Universidad de Navarra
Facultad de Ciencias

Effects of sedimentary humic acids on endophytic microbiomes: implications and agronomical potential

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Efectos de los ácidos húmicos sedimentarios en los microbiomas endófitos: implicaciones y potencial agronómico

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Effects of sedimentary humic acids on endophytic microbiomes: implications and agronomical potential

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A dissertation submitted by Mr David de Hita Mejía in partial fulfillment of the requirements of the Degree of Doctor at the University of Navarra, Spain

Memoria presentada por Don David de Hita Mejía para aspirar al grado de Doctor por la Universidad de Navarra

We have directed the research leading to this Dissertation at the Department of Environmental Biology, and hereby authorize its submission before the designated Tribunal.

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Biología Ambiental y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

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"Agriculture, for an honorable and high-spirited man, is the best of all occupations and arts through which a man can procure sustenance."

Xenophon of Athens

Como todo lo importante en la vida, esta tesis no es el fruto del esfuerzo exclusivo de una sola persona. Pese a ello, el ser yo quien la ha de defender me pone en la necesidad de reconocer y agradecer a todos aquellos que de una manera u otra me han ayudado en la realización de esta tesis doctoral.

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Esta tesis doctoral incluye una colección de manuscritos en diferentes estados de publicación, cada uno de los cuales constituye un capítulo. Los manuscritos se reproducen íntegros y en el idioma en el que fueron publicados o enviados para su publicación, incluyendo siempre un resumen en castellano.

En cumplimiento de la normativa para la presentación de tesis doctorales en la Facultad de Ciencias de la Universidad de Navarra se incluyen los siguientes apartados: (1) un Resumen integrador del contenido de la tesis doctoral; (2) una Introducción general que sitúa el trabajo realizado en su contexto teórico, planteando los Objetivos de la tesis doctoral; (3) una Discusión general, y (4) un apartado de Conclusiones generales.

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List of abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AHL	N-acyl homoserine
BIOM	Biological Observation Matrix
BLASTn	Basic Local Alignment Search Tool nucleotide
BNF	Biological Nitrogen Fixation
CKs	Cytokinins
DOM	Dissolved Organic Matter
DW	Dry Weight
ET	Ethylene
FA	Fulvic Acids
FAO	Food and Agriculture Organization
FW	Fresh Weight
GAs	Gibberellines
HA	Humic Acids
HGT	Horizontal Gene Transference
HS	Humic Substances
IAA	Indol-3-acetic Acid
IPS	Inorganic Phosphate Solubilization
ISR	Immune System Resistance
JA	Jasmonic acid
JAIle	Jamonyl-L-Isoleucine
LEfSe	Linear discriminant analysis Effect Size
MCI	Metal Chelation Index
NGS	Next Generation Sequencing

NMDS Non-metric Multidimensional Scaling

NO Nitric Oxide

NOM Natural Organic Matter

OTUs Operational Taxonomic Units

PGP Plant Growth Promotion

PGPB Plant Growth Promoting Bacteria

PGPR Plant Growth Promoting Rhizobacteria

PM H^{+}-ATPase Plasma Membrane H^{+}-ATPase

PNA Peptide Nucleic Acid

RDP Ribosomal Data Project

RH Relative Humidity

ROS Reactive Oxygen Species

SA Salicylic acid

SEM Scanning Electronic Microscopy

SHA Sedimentary Humic Acids

SOD Superoxide Dismutase

SOM Soil Organic Matter

TEM Transmission Electronic Microscopy

VOCs Volatile Organic Compounds

GENERAL ABSTRACT

RESUMEN GENERAL

The current world situation, a growing population in a climate change scenario, requires new solutions from agriculture. New practices for sustainable agriculture are demanded by society. In this context, it is proposed the use of humic substances, specifically sedimentary humic acids (SHA), for plant growth promotion. These substances are widely utilized in agriculture for increasing the crop yields and soil fertility; in the field, SHA usually is applied as a foliar spray, but most of the studies focused on disentangling their mechanism of action have studied the direct SHA application to the roots. In addition, little is known about how the plant microbiome is affected in response to the treatments with SHA. Microbiome studies have increased in the last years as a consequence of the arising of new generation sequencing techniques and the cheaper prices associated with these techniques. Most of the have been centered on soil and rhizosphere microbiomes, while, the plant endosphere microbiome, due to its intrinsic challenges, is still poorly understood. However, this microbiome has enormous importance because it can be inherited for the offspring. For all these reasons, this dissertation has evaluated how the treatment with SHA, applied either as a foliar spray or supplied to the root zone, influences on the plant endophytic microbiome. To achieve this goal, firstly, we have characterized in Chapter 1 the hormonal, phenological and biochemical changes provoked by SHA foliar application in cucumber plants grown in hydroponics. Then, in Chapter 2, we have assessed the effect of SHA on endophytic microbiome composition from cucumber plants also grown in hydroponics and if the application strategy promotes or decreases certain microbial populations. Simultaneously, in Chapter 3, we have isolated bacterial endophytes from cucumber plants previously treated with a root application of SHA and grown in the same conditions as in the previous chapters. These bacterial isolates have been characterized as plant growth promoters depending on the presence of plant growth promoter traits (nitrogen, iron and phosphorus acquisition and phytohormone production). Finally, we have tested in Chapter 4, the SHA plant growth effect in barley plants grown under greenhouse conditions and different N-fertilization regimes, with the aim to evaluate the endophytic microbiome response in a realistic scenario.

The main results of this work showed, in Chapter 1, that the SHA application increases plant growth regardless of the application strategy, but the mechanisms of action exerted in the plant were different depending on how the SHA was applied. In contrast with root SHA application, the foliar application did not induce the PM H^+-ATPase activity, did not increase the ABA concentration in plant roots and did not

increase the IAA-mediated lateral root promotion. However, foliar application increase the length of principal and secondary roots, modified the leaf surface and the starch content in chloroplasts and increased the concentration of JA, JA-Ile and SA concentrations, either in leaves and roots. In Chapters 2 and 4, we have observed that the SHA application can modify the endophytic plant microbiome significantly but did not affect equally to fungal and bacterial communities nor the different plant compartments. SHA application had a negative impact on fungal Operational Taxonomic Units (OTUs) richness, whereas the bacterial richness increased or remained neutral. In Chapter 2, we found that both types of SHA applications (foliar or root) differently modified the composition of the cucumber microbiome. In general, SHA increased the presence of *Ascomycota* fungi, as well as of bacteria from *Actinobacteria* phylum and *Pseudomonadales* order, but the effect was highly dependent on plant genotype, in contrast with the results obtained in Chapter 4 in barely plants. These enriched bacterial groups in the cucumber microbiome were also found between the isolated endophytes in Chapter 3. In fact, some of the more promising Plant Growth Promotion (PGP) candidates belonged to these groups: *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas* and *Arthrobacter* genera. Most of the isolates were able to grow in an N-free medium and to produce phytohormones; both traits are essential to prevail in endosphere habitats. Further studies should be carried out for assessing the performance of the best candidates *in vivo* assays. Finally, we found that N-fertilization only modified the endophyte microbiome slightly in barley plants. We observed that the combination of N-fertilization and SHA application allowed greater Biological Nitrogen Fixation (BNF) microbes recruitment, especially *Rhizobium* bacteria.

La actual situación mundial, una población en aumento a la par que un escenario de cambio climático, requiere de nuevas soluciones para la agricultura. Además, la sociedad demanda nuevas prácticas para una agricultura más sostenible. En este contexto, se propone el uso de las sustancias húmicas, más específicamente los ácidos húmicos de origen sedimentario (AHS), para promover el crecimiento vegetal. Estas sustancias son ampliamente utilizadas en agricultura para aumentar el rendimiento de los cultivos, así como de la fertilidad del suelo; en el campo los AHS son aplicados normalmente como sprays foliares, sin embargo la mayoría de los estudios científicos que se han centrado en desentrañar su mecanismo de acción, lo han estudiado mediante su aplicación directa a las raíces. Por otro lado, poco se sabe sobre la respuesta del microbioma vegetal ante este uso de los AHS. Los estudios sobre el microbioma han aumentado en los últimos años como consecuencia del advenimiento de la nueva generación de secuenciadores y la bajada de precios asociada a estas nuevas tecnologías. La mayoría de los esfuerzos en este campo se han centrado en el estudio de los microbiomas del suelo y de la rizosfera; consecuentemente, el microbioma endófito sigue siendo pobremente entendido, debido principalmente a los desafíos intrínsecos a su estudio. Sin embargo, este microbioma es de una enorme importancia, debido a que puede ser heredado por la descendencia. Por todas estas razones, en esta tesis se ha evaluado cómo los AHS, según su estrategia de aplicación, impactan en el microbioma endófito. Para conseguir este objetivo, en primer lugar hemos caracterizado en el Capítulo 1, los cambios hormonales, fenológicos y bioquímicos provocados por la aplicación foliar de los AHS en plantas de pepino crecidas en hidroponía. Después, en el Capítulo 2, hemos evaluado el efecto de los AHS en la composición del microbioma endófito de plantas de pepino crecidas en hidroponía, así como si el tipo de aplicación de los AHS promovía o inhibía ciertas poblaciones microbianas. Al mismo tiempo, en el Capítulo 3, aislamos endófitos bacterianos de plantas de pepino previamente tratadas con AHS y crecidas en las mismas condiciones que en los anteriores capítulos. Estos aislados bacterianos fueron caracterizados y clasificados en función de la presencia de características típicamente promotoras del crecimiento vegetal (adquisición de nitrógeno, hierro y fósforo, y la producción de fitohormonas). Finalmente, comprobamos en el Capítulo 4 el efecto promotor de crecimiento vegetal de los AHS en plantas de cebada crecidas en invernadero y con diferentes regímenes de fertilización nitrogenada, con el objetivo de evaluar la respuesta del microbioma endófito en un escenario más realista.

Los principales resultados de este trabajo en el Capítulo 1 mostraron que aplicar AHS sin importar la estrategia de aplicación incrementa el crecimiento vegetal, pero el mecanismo de acción para cada tipo de aplicación es distinto. Al contrario que la aplicación radicular de AHS, la aplicación foliar no indujo la actividad de la H^+-ATPasa de membrana plasmática, tampoco incrementó la concentración en raíz de ABA y no incrementó el crecimiento de raíces laterales mediado por IAA. Sin embargo, este tipo de aplicación incrementó la longitud de las raíces principal y secundarias, modificó la superficie foliar y el contenido de almidón cloroplástico, así como incrementó la concentración de JA, JA-Ile y SA tanto en la raíz como en la hoja. Además, en los Capítulos 2 y 4, observamos que la aplicación de AHS modificaba significativamente la composición del microbioma endófito vegetal; aunque el efecto no era el mismo en hongos o bacterias, ni en los diferentes compartimentos vegetales. La aplicación de AHS afectó negativamente a la riqueza en OTUs (*Operational Taxonomic Units*) de los hongos, mientras que la riqueza bacteriana era incrementada o se mantenía neutral. Además, en el Capítulo 2, encontramos que el tipo de estrategia de aplicación también modificaba de manera significativa la composición del microbioma. Generalmente, la aplicación de AHS aumentó la presencia de los hongos *Ascomycota* y de las bacterias pertenecientes al filo *Actinobacteria* y al orden *Pseudomonales*; pero este efecto es muy dependiente del genotipo vegetal, tal y como comprobamos en el Capítulo 4. Estos grupos bacterianos promovidos por el uso de AHS también se encontraron entre los aislados del Capítulo 3. De hecho, los candidatos más prometedores pertenecían a estos dos grupos bacterianos: géneros *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas* y *Arthrobacter*. La mayoría de los aislados encontrados fueron capaces de crecer en medio libre de nitrógeno y de producir fitohormonas, siendo ambas características esenciales para la vida en la endosfera. Futuros ensayos *in vivo* se deberán realizar para evaluar el potencial uso de inóculos de estos aislados como promotores del crecimiento vegetal. Finalmente, hemos encontrado que la fertilización nitrogenada sólo modificó levemente la composición del microbioma endófito en plantas de cebada. Hemos observado, además, que la combinación de fertilización y la aplicación de AHS permite un mayor reclutamiento por parte de la planta de microorganismos fijadores de nitrógeno atmosférico, especialmente bacterias del género *Rhizobium*.

INTRODUCTION

1. Contextualization

1.1. Agriculture historical importance.

Plant domestication was a crucial step in human civilization, enabling the development of complete, reliable, and productive agriculture (Puruggaman et al., 2009; Berg and Raaijmakers, 2018). Agriculture has been a driving force for mankind since its beginning more than ten thousand years ago in the ancient Middle East. The food production allowed our ancestors to abandon the hunter-gathering way of life and to create settlements. Agriculture was the first step to increase human societies; these societies would evolve in political complexity, and as a consequence handwriting, religions, weapons or monumental structures appear. Such enormous modifications were possible because agriculture (and the main plant species selected for domestication) elevated the production efficiency by hectare in front of previous conditions. Hence, agriculture allowed to feed more humans than the hunter-gathering lifestyle. When and how a human civilization discovered the agriculture profoundly influenced their technological advances and, thus, their fate.

The rise and fall of many human civilizations have been deeply linked to changes in agriculture management or the discovery of new crops. For instance, Ancient Egypt was extremely dependent on the Nile floods for maintaining the agricultural system that held up their population. When the Nile floods regime changed, the ancient Egyptian economy decayed, and consequently the pharaohs lost their power against Greek and Romans. Also widely known is the fate of Greenland vikings. After several centuries of colonization, this culture never could adapt their European agriculture to arctic environments. Finally, another example happened in the European age of empires after the America discovery. During that age many new crops were adapted to Europe and the standard of life was increased. As a main consequence, Europe dominated the world for more than three centuries, and still, nowadays, Europe and European-descendants countries as U.S. or Canada lead economically, culturally, and technologically the humankind.

As history has continuously shown us, only significant improvements in agriculture have allowed population growth. Concomitantly with this growth, cultural, political and technological changes have appeared. New strategies for enhancing agriculture must be carried on with the aim to face global climate change, to help

developing countries, to reduce famines, and to decrease the economic differences between human beings.

1.2. Modern agriculture as a source of environmental problems.

No other agricultural improvement has supported the global population growth as the Green Revolution did during the '60s of the past century. This revolution supposed an increase of the population never seen before, going from 3000 million to more than 7600 million of people nowadays[1]. But this revolution has also been accompanied by some drawbacks due to the excessive use of chemical fertilizers and pesticides, changes in land use, ecologically aggressive agricultural practices, or loss of crop diversity, among others (Sergaki et al., 2018, and references therein). These drawbacks have provoked detrimental effects on soil physicochemical and biological properties.

Thus, soils have become gradually and increasingly degraded and unprofitable for crop production. This situation has been exacerbated by human and animal food consumption for an increasing human population in a climate change scenario (Pukalchik et al., 2019). In these intensively managed agroecosystems, the application of fertilizers has been used to compensate for the soil fertility loss, while the tillage has disrupted the microbial soil communities (Johnson et al., 1997). At the same time, we must face massive challenges associated with the N, C and P cycling disturbances.

Also, during the last century, plant genotype selection was carried out, prioritizing only the crop yield under fertilizing conditions and pathogens absence (Philippot et al., 2013). Therefore, nowadays, plant cultivars could be harboring dysfunctional microbial communities that are not used to face climate change conditions like droughts, salinity, recurrent pests, or fertilization shortage.

1.3. Proposed solutions and perspectives.

There is a growing interest in the development and implementation of sustainable land management practices (Sanz et al., 2017; Gazzola et al., 2019; Pukalchik et al., 2019). It is predicted by 2050 that the crop demand would have been doubled the actual demand (Tilman et al., 2011; Toju et al., 2018), and this prevision requires fast and immediate solutions.

[1] www.fao.org/faostat/

New practices such as crop diversification, use of local cultivars, or intercropping, have been proposed. These practices will allow soil fertility maintenance, C sequestration, nutrient cycling and will also prevent soil erosion (Sanz et al., 2017). Besides, these actions would help to enhance the suppressive disease soils (Bonilla et al., 2015), which are the living proof that plant microbiomes not only promote the plant growth but also protect the plant against pathogens (Shiomi et al., 1999; Chaparro et al., 2012; Sergaki et al., 2018). Similar to the animal guts and immune responses, there are two types of suppression: general suppression (common to all soils), and specific suppression that is susceptible to be loss by soil pasteurization but also can be transfer to other soils (Sergaki et al., 2018). Finally, "smart farming" has been proposed. It consists in the use of novel sensing technologies that allow the real-time diagnostic, forecasting plant physiological conditions based on soil and climate physicochemical data (Schlaeppi and Bulgarelli, 2015; King, 2017). Inside smart farming practices, it is englobed the use of microbiome data.

In the last decade, microbiome research has modified our perspective of the complexity and structure of microbial communities (Sergaki et al., 2018). The microbiome concept not only considers the plant microbiota but also the genetic information of each individual serving to the plant fitness. That is the main reason why the use of customizing microbiomes for improving crop yields has been suggested; a strategy that would be specially benefited by the current genetic uniformity of crops (Sergaki et al., 2018). The importance of maintaining a diverse and well-balanced microbiome is crucial for impelling a new Green Revolution. Maximizing the microbiome functionality would face the apparition of new pathogen strains, climate change, and the fertilizer shortage and the environmental costs of their use (Toju et al., 2018). Therefore, promoting microbial diversity and biotic interactions will be a valuable tool to develop more sustainable agricultural systems, less dependent on chemical inputs (Lemanceau et al., 2017). One limitation of this approach is that microbial recruitment by plant roots is highly dependent on the soil and microbial environment, root exudates, and plant genotypes. To overcome this limitation, the use of organic amendments can increase microbial diversity and, at the same time, maintain a certain grade of stability. This organic matter would act as a food resource for the microbiota and would decrease the physical perturbations by increasing the soil aggregation (Siegrist et al., 1998; Wang et al., 2017). Moreover, humic substances, a part of the soil organic matter, have already been proven to promote plant development and microbial activity (Olaetxea et al., 2018).

The prospection of microorganisms that exhibit either new or improved functionalities has been widely investigated. Furthermore, these microbes could be used in future investigations to identify the molecular mechanisms behind their plant growth promotion (Sergaki et al., 2018). The microbial isolates application to plants sometimes failed in their purpose to improve plant growth due to the competence with soil or plant microbiomes; or because they do not persist against a wide range of climatic, biotic, and abiotic conditions (Sergaki et al., 2018). Microbial consortia or the use of natural carriers as humic substances would allow a better performing of this strategy (Finkel et al., 2017; Olivares et al., 2017).

The promotion of plant growth through the use of endophytic microorganisms has been proposed as a useful solution (Sessitsch et al., 2019). Endophytes are the microorganisms that inhabit the inner plant tissues without harm them (Hallmann et al., 1997). Endophytic isolates are specially appropriated to be used as biofertilizers and biopesticides in sustainable practices, especially in those crops phylogenetically related to the original host (Long et al., 2008; Afzal et al., 2019). However, the current challenge is to successfully transfer the lab-generated knowledge to the field (Sessitsch et al., 2019). In this context, an interesting strategy that is currently the subject of different investigations (Canellas et al., 2015; Olivares et al., 2017) is the combined used of endophytic plant growth promoters and humic substances. This approach comprises the benefits of increasing crop yields and maintaining soil fertility in the framework of sustainable agricultural practices.

2. Humic substances

2.1. Origin, Classification and Chemical characteristics

Humic substances (HS) are the result of the gradual transformation-degradation process of fresh organic matter originated from plant and animal decomposition. The group of processes, either abiotic (oxidation, reduction, polymerization) as biotic (by microorganisms), is called humification (Stevenson, 1994). Humification is a long process that can last for years, even thousands of them, and is conditioned by physico-chemical environmental conditions such as temperature, oxygen availability, water content, pH, or soil texture, among others (Stevenson, 1994; Hayes, 2009; Huang and Hardie, 2009). The organomineral soil layers may contain a myriad of organic molecules in different transformation degrees such as sugars, amino acids, phytoregulators, organic acids,

proteins or organic chemical and biologically complex fractions, commonly called humus (Piccolo, 2002, Magdoff and Weil, 2004b, Olaetxea et al., 2018). There are different classifications of HS, but the most common criterion has been their water solubility as a pH function. Those HS soluble at basic and acidic pH are the fulvic acids (FA), those that only are soluble in water at basic pH are the commonly called humic acids (HA), and finally, the HS that are insoluble at all pH are known as humins (Stevenson, 1994). Nowadays the biological and chemical sense of this classification is subjected of intense scientific debate (Lehmann and Kleber, 2015; Kleber and Lehmann, 2019; Olk et al., 2019). Nevertheless, recent works have pointed out that the singular structural features of HS obtained by this methodology are not present in natural biomolecules such as proteins, polysaccharides, lignin, or cellulose (Cao and Rohr, 2018; Perminova et al., 2018). For instance, despite the high polydispersity, HS has singular features mainly associated with the fraction and not with the origin. Hence, this classification remains valid for agronomical purposes.

Inside the HS structure there are multiple functional groups free to form metallic bonds with polyvalent elements, for example carboxylic and phenolic groups, amines and amides, or reduced sulphur. Other common binding sites are the salicylic acid-like, catechol-like, phthalic acid-like and hydroquinolones-like molecules (Senesi, 1992; Van Trump et al., 2011; Olaetxea et al., 2018). Besides, depending on the type of HS, the pH, the metal concentration, the ionic strength, the soil solution composition, or the temperature, the metal-HS bonds could be intra- or intermoleculars (Senesi, 1992). Through the metal-HS conformation the bioavailability of these elements increases, besides their solubility and stability, according to the pH and ionic strength of the soil solution (García-Mina et al., 2004).

This mechanism has been studied in deep for Fe-HS complexes. Fe is an essential nutrient to plants thus; the high stability of these complexes due to the bonds with carboxylic and phenolic groups has increased the availability of this element in alkaline and calcareous soils (Senesi, 1992; Fuentes et al., 2013). However, Cu, Zn, and Mn complexes with HS seem to create bonds with the N-functional groups. These differences in chemical bonds may affect the biological effect of HS in plants. The work of Scaglia et al. (2016) linked the lateral root proliferation in plants with a higher content in labile O-functional groups, mainly carboxylic groups in aromatic or aliphatic structures. But also this work linked the increase in root dry weight with HS enriched in recalcitrant O-groups and N-alquil structures (Scaglia et al., 2016).

Finally, it has been recently demonstrated that not only the functional groups may modify the biological activity of HS, but the molecular conformation of these substances in solution (Aranaz et al., 2018). In this work, using a fungal enzyme (laccase), the HS conformation was modified, so the plant growth promotion was reduced in *Arabidopsis* and cucumber seedlings. It is this structural and chemical composition diversity that allows the long-time persistence of HS in nature, impeding the fast microbial degradation but enabling their biological function in soils and plants.

2.2. Uses of humic substances

HS have been part of agriculture since ancient times, but it has been at the beginning of the modern agriculture when soil organic matter (SOM) has attracted the attention of industry and science (Chen et al., 2004).There is a strong positive correlation between soil fertility and the content of humified natural organic matter (NOM) (Magdoff and Weil, 2004a)

The humic-based soil amendments are habitually used by the food industry due to their efficiency and environmentally friendly behavior. These reasons have become HS application an important biostimulant in the last decades, and it is estimated that the worldwide market size will reach 1 billion US$ by 2024 (Pukalchik et al., 2019, and references therein). The plant biostimulants are defined as "a formulated product of biological origin that improves plant productivity as a consequence of the novel or emergent properties of the constituents, and not as a sole consequence of the presence of known essential plant nutrients, such as plant growth regulators, or plant protective compounds" (du Jardin, 2015; Yakhin et al., 2017; Pukalchik et al., 2019). In fact, HS are commercially sold accompanied (or not) with plant nutrients, both mineral and/or organic. HS for commercial purposes are usually extracted by the alkaline method from lignite, peat, composts and organic waste materials and contains between 15-85% of HS (Perminova et al., 2005; Yakimenko and Terekhova, 2011; Lamar et al., 2014). Therefore, the main economic interest and use of HS is plant growth promotion. This plant growth promotion, as it will be explained later, is the result of direct interaction with plant tissues and physiology but also due to indirect effects as pH buffering, water retention, or nutrient mobilization (Pukalchik et al., 2019).

Another promising use of HS is as remediation agents in degraded and polluted lands due to their competence improving the physical, chemical, and biological properties of soil (Pukalchik et al., 2017; Tregubova et al., 2017). HS are especially useful in heavy

metal and contaminated organic soils (Hui et al., 2019). The complexation of these soil pollutants reduces, for example, the toxicity of Aluminium or Cr(VI) from aqueous solutions (Janos et al., 2009; Trevisan et al., 2010). Furthermore, it has been proved the capability of HS avoiding arsenic and pesticide soil contamination (Trevisan et al., 2010; Bezuglova et al., 2019).

In agriculture, there have been, traditionally, two different ways to HS application, a direct application to roots (through fertirrigation systems) or by foliar spray (Olaetxea et al., 2018; De Hita et al., 2019; Justi et al.; 2019). Most of the studies concerning the use of HS have been mainly focused in the application to the roots, where the mechanisms of action in plants have been described in multiple experimental conditions, although some of them remain partially unknown (Chen et al., 2004; Rose et al., 2014; Olaetxea et al., 2016, 2018). In contrast, the foliar application mechanism of action has been usually overlooked in scientific reports in spite of being a common practice in extensive agriculture (Olaetxea et al., 2018). It seems clear that a non-identical plant response must be triggered by foliar application due to the fact of the different nature of the plant organs where the HS are applied. For instance it has been proposed that in foliar application, the role of metal-complexing ability of HS would be minimal, while interaction with plant microbial communities or great accessibility to HS colloids should be taken into account (Olaetxea et al., 2018; Justi et al.; 2019). The use of HS foliar applications has been proved especially efficient in plant growth under stress conditions (Canellas et al., 2015; Van Oosten et al., 2017), but also in normal conditions, though in this situations the effects are less consistent than those occurring when HS are applied to the roots (Olk et al., 2013; De Hita et al., 2019). Disentangling the plant response induce by foliar application will improve the HS-based biostimulants' use and formulation to increase crop yields.

2.3. Humic substances as plant promoters and effects in plant physiology.

The biological action of HS in plant growth and fitness has been proved in diverse experimental conditions and numerous studies (Chen et al., 2004; Rose et al., 2014). Habitually the effect of HS in plants has been classified in indirect effects and direct effects.

Indirect effects:

HS indirect effects are those effects produced by HS in soil application that modify the environment where the plants are. Some of these effects involve changes in

soil porosity, aggregation, or gas exchange. But also changes in the activity of soil microorganisms or, as mentioned above, changes in nutrients bioavailability (Stevenson, 1994). The latter indirect effect has been extensively studied, probably due to its importance in alkaline, calcareous, and agricultural soils, where the nutrients are retained, forming oxides, hydroxides, or carbonates, and the increment in bioavailability is economically interesting (Olaetxea et al., 2018). As proposed in Olaetxea et al. (2018), there are two different pathways through the HS indirectly improving the plant nutrition: the complexity pathway and the biochemical pathway.

The complexity pathway is related to the ability of HS to create natural chelates mainly with metallic nutrients (Fe, Mn, Cu, and Zn), increasing their availability, solubility, and stability in soil solution (Senesi, 1992, Chen et al., 2004). The metal-HS complexes enhanced the availability of plants when they are stable and soluble in soil solution. Besides, the bioavailability of other nutrients like P or S, through metallic bridge creation, may be increased by HS application in soils (Baigorri et al., 2013; Urrutia et al., 2014).

The biochemical pathway is linked to an enhancement of mineral nutrition thanks to the activation of main physiological players in nutrient root uptake and transport and metabolism of these nutrients inside the plant (Quaggiotti et al., 2004; Jannin et al., 2012).

i. Macronutrients:
    a. Nitrogen: HS increases N root uptake and assimilation (Rose et al., 2014) through the activation of root plasma membrane H^+-ATPase (PM H^{+-}-ATPase) (Pinton, 1997). Also, HS are able to up-regulate nitrate transporters codifying genes (Jannin et al., 2012) and enhance cytokinins root-shoot translocation (Mora et al., 2010).
    b. Sulfur: HS may enhance S root uptake, also mediated by of root PM H^{+-}-ATPase. In addition, an increase in the expression of S-transporters genes has been observed (Jannin et al., 2012).
    c. Phosphorus: Besides increasing P-bioavailability in soil, HS can modify the gene expression of the phosphate transporters in plants under P-sufficiency conditions but not under P-deficiency (Urrutia et al., 2014; Jindo et al., 2016).
    d. Potassium, Calcium, and Magnesium: HS action is associated with an increase in K, Ca, and Mg concentrations in leaf tissues, probably

involving the root-shoot translocation of these elements (Mora et al., 2010; Rose et al., 2014).

ii. Micronutrients: the root uptake of these essential nutrients usually is related to the HS complexation mentioned above. Nevertheless, various studies have shown that SHA from leonardite may activate root transcriptional and post-transcriptional pathways to face Fe deficiency (chelate reductase or $Fe_{(II)}$ transporter) (Aguirre et al., 2009; Billard et al., 2014). As a result, a rise in Fe concentration in leaf tissues and an increment in chloroplast efficiency have been observed (Billard et al., 2014; Abros´kin et al., 2016). Similar responses were observed in Cu, Mn, and Zn transport codifying genes (Billard et al., 2014).

Direct effects:

These effects would imply a direct interaction with plant cells, and would cause changes in root/shoot cells, or in the whole plant, that would promote plant growth. Hence, the effect of the HS application can vary according to the plant organ:

i. In roots:

The main observable effect of HS application is the change in root architecture (lateral and adventitious root promotion) and their development (dry weight increase) (Trevisan et al., 2010, Rose et al., 2014; Canellas et al., 2015b; Olaetxea et al., 2018). The direct application of HA to the plant root enhance the levels of indole-3-acetic acid (IAA) and nitric oxide (NO), increasing the lateral root density (Mora et al., 2012). There is also an up-regulation in root cell auxin-related genes (Trevisan et al., 2010). This increase in phytohormones level concomitantly activates the root PM H^{+}-ATPase (Zandonadi et al., 2007; 2010; Olaetxea et al., 2019). Furthermore, the use of HS increases the gene expression of various ATPase isoforms (Olaetxea et al., 2019). Finally, the increase of abscisic acid (ABA) and ethylene, regulated by the IAA and NO response, also favor the lateral root promotion.

Otherwise, the increase in root dry weight is independent of this hormonal cascade (Mora et al., 2012). This effect possibly is due to the involvement of reactive oxygen species (ROS) that regulates finely many physiological processes like root development or adaptive stress response (Kaur and Pati, 2017). It is known that HS modify the cell content in ROS as well as the

homeostasis enzyme expression like superoxide dismutase (SOD), catalase, or ascorbate oxidase (García et al., 2012, 2016; Berbara and García, 2014). Moreover, ROS regulation has been proved to be located in cell elongation and proliferation root regions (García et al., 2016).

ii. In shoots:

The application of HS also increases shoot growth. It has been observed that in plant shoot, there is a rise in nitrate concentration coupled with a root-shoot translocation of active cytokinins (Rahayu et al., 2005; Sakakibara et al., 2006; Olaetxea et al., 2018). Also, this cytokinin translocation is related to the increase in root PM H^{+}-ATPase; hence the shoot growth is linked with the rise in IAA and NO root concentration (Mora et al., 2014a,b). A logical consequence of this link between shoot and root effects is the increase in ABA concentration. The ABA increasing in root is the main responsible for hydraulic conductivity regulation and aquaporin activity. Both actions allow the HS mediated shoot development (Olaetxea et al., 2015; De Hita et al., 2019). Thus, all these signaling pathways would be partially responsible for HS-plant growth promotion.

Finally, HS are also known because they improve plant growth, especially when plants have to face abiotic and/or biotic stresses (Canellas et al., 2015; Aguiar et al., 2016; García et al., 2016; Pukalchik et al., 2019). Furthermore, the functional complementarity of HS and stressed plant necessities is noteworthy. Several studies have demonstrated that HS, besides ROS regulation, are able to modify the enzymatic and plant stress-related transporters' expression (Trevisan et al., 2011; Jannin et al., 2012; García et al., 2016). Even though HS promoted the secondary metabolite biosynthesis such as proline or phytoalexins, both of them related to stress alleviation (Berbara and García, 2014; Aguiar et al., 2016; García et al., 2016). It is for that reason that HS application allows management versus saline stress, increasing the plant growth (Van Oosten et al.; 2017; De Hita et al., 2019). Additionally, biotic stress response might be related to changes in jasmonic acid (JA), jasmonyl isoleucine (JAIle), and salicylic acid (SA), but not relevant works have explored this pathway.

In resume, a reliable hypothesis about HS action in plants is that the HS interacts physically and chemically with superficial root cell walls. That provokes mild transient stress, also called eustress, which would trigger biochemical and genetic events cascade that results in effective plant growth (Olaetxea et al., 2016, 2018).

2.4. Effect in microbial communities

The NOM is known to be highly influenced by rhizospheric micro- and macro-organisms (Pinton et al., 2007, Hinsinger et al., 2009; Olaetxea et al., 2018, Pukalchik et al., 2019). The molecular complexity of NOM has a biological effect in soil food chain and, thus, in microbial and invertebrates communities (Pukalchik et al., 2019). It is well-known the relationship between NOM and earthworms, though the earthworms reduced the NOM particle size. In fact, some authors have explored the HS use as decontaminant protecting the earthworms in polluted soils (Shen et al., 2015; He et al., 2017). In contrast, other studies have pointed out the HS potential as biological control agents against nematodes (Kesba and Al-Shalaby, 2008; Seenivasan and Senthilnathan, 2017).

Humic substances act as nutrient providers for microorganisms (slow-release biostimulants), mainly organic matter decomposers (heterotrophic microbes). This decomposition is essential in C and N soil cycles because both elements are retained in microbial biomass while microorganisms release CH_4, CO_2 or NO_2 (greenhouse gases) (Murphy et al., 2007; Zhu et al., 2013). Gorlenko et al. (2012) observed that low HS doses increased soil microbial diversity, microbial activity, and community stability. However, in the same study, higher doses of HS reduced these parameters. Another study carried out by Maji et al. (2017), concluded that an HS from vermicomposting increased bacteria and fungi population in plant rhizosphere; besides, the nodule bacteria and mycorrhizal fungi colonization was also promoted. According to Wallenstein and Burns (2011), the HS can interact directly with cytoplasm and extern cell surface of microorganisms, modifying their ability to form protozoic cysts or bacterial and fungi spores (Stevenson et al., 2004).

On the one side, several studies have been focused in the direct effect of HS on microbial diversity (Dong et al., 2009; Puglisi et al., 2009; Schoebitz et a., 2016; Pukalchik et al., 2019 and references therein). Most of them concluded that HS habitually affect more bacterial than fungal communities. The bacterial phyla usually promoted by HS application in soils are *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes,* and *Proteobacteria* (Van Trump et al., 2011; Yuan et al., 2017). However, other authors have found opposite results, being the fungi promoted when HS were applied; besides, the HS doses effect and fungal abundance (Sellamuthu and Govindaswany, 2003). Undoubtedly, more research is needed to conclude a general rule between HS use and microbial diversity.

But on the other hand, most of the research about HS-microbes relationship has been carried out in artificial liquid and solid medium. This strategy has been used for

assessing the direct interaction between HS and microbial growth and metabolism (Crowther et al., 2018; Pukalchik et al., 2019). Either beneficial or inhibitory effects have been observed in bacteria and fungi when HS has been added to the growth mediums.

i. Beneficial effects: HS have promoted the biomass growth of some bacterial species as *Acinetobacter*, *Agromyces*, *Arthrobacter*, *Bacillus*, *Microbacterium*, *Nocardiodes*, *Pseudomonas*, *Rhodococcus*, *Sphingopyxis*, or *Streptomyces* (Tikhonov et al., 2010). Another beneficial effect is the plant root colonization HS-mediated by bacteria and mycorrhizal fungi (Gryndler et al., 2005; Olivares et al., 2017).
ii. Inhibitory effects: most of the works carried out in artificial medium agreed in the growth inhibitory effect of HS in soil fungi (Pukalchik et al., 2019 and references therein). Generally, saprophytic fungi soil populations are reduced by antagonistic fungi growth stimulation due to the HS application. Some of the plant pathogens inhibited by HS are *Botrytis cinerea*, *Rhizoctonia cereal*, *Fusarium graminearum,* or *Phytophthora infestans* (Wu et al., 2016). Thus, HS should be considered as an alternative treatment to traditional fungicides.

Probably the type of effect over microorganisms depends on the HS structure and functional groups: quinones, phenols, carboxylic acids, and hydrophilic groups (de Melo et al., 2016; Scaglia et al., 2016). For instance, the HA significantly affect bacterial transport and deposition due to changes in their physicochemical properties (Yang et al., 2016). Quinones are responsible for ROS formation, so a high abundance of them will increase the level of ROS, and it would increase the antimicrobial effect of HS by breaking the lipidic membranes (de Melo et al., 2016). Otherwise, the HS enriched in phenolic groups would protect the microorganisms due to the antioxidant activity of these groups (Kulikova et al., 2018).

At the end, another role of HS is as microorganism protectors. Several studies have found that the application of HS at the same time with herbicides may help the development of natural bacterial communities (Taspinar et al., 2017; Bezuglova et al., 2019). This protector effect is different according to the life-strategy of bacteria: fast-growing bacteria are more susceptible to herbicides than slow growers (Bezuglova et al., 2019). As a consequence of this protection, HS have been explored as potential carriers for plant growth promoting bacteria (PGPB). The use of PGPB able to fix nitrogen or solubilize P in foliar sprays with HA has been explored in low fertility soils with

promising results (Canellas et al., 2015a, b; Olivares et al., 2017; de Azevedo et al., 2019). Olivares et al. (2017) have hypothesized that the HS action in root development: lateral root development, increment in number of hair roots, increase in the PM H^{+}-ATPase and the increase in water transporters gene e expression (de Azevedo et al., 2019), and the sorption of HS in roots, would be the responsible for an increase in endophytic plant colonization. As a result of this colonization, the microbes would complement the HS action in terms of increasing nutrient acquisition and assimilation, promoting the plant growth through phytohormones production, and enhancing the plant defense system. Furthermore, these effects will be essential under hydric stress conditions, or in low N fertilized soils (de Azevedo et al., 2019).

3. Microbiome

3.1. Concept, implications and generalities.

Land colonization by plants was only possible thanks to the help of soil microorganisms. Microbes can stimulate the plant hormonal balance, increase the nutrient root uptake, promote the plant growth, or suppress the soil-borne diseases; in exchange, plants sustain microbes by rhizodeposition (Bais et al., 2006; Lemanceau et al., 2017). These rhizodeposites, mostly organic acids, are a source of available organic C for microbes. Indeed, plants liberate them to attract and select from the soil microbial communities the best partners for increasing their fitness.

Recently, the advances in Next Generation Sequencing (NGS) technologies have boosted the plant-microbe interactions field, mainly in the microbial ecology area (Lemanceau et al., 2017). As a consequence, the new term microbiome has been coined. The microbiome is the set of typical microbial communities from an area/environment/host; it is an adaptation of the term *biome* to the field of microbiology. The microbiome plays a pivotal role in plant biology, participating in processes such as germination, growth, health, protection against stress and phytochemistry (Mendes and Raaijmaakers, 2015). The main difference between microbiome and microbiota is that microbiome considers the genome of the microbiota as a part of the holobiont (Rosenberg and Zilber-Rosenberg, 2016; Lemanceau et al., 2017).

The holobiontic theory defends that the unique biologic unit is the holobiont (multicellular host + microbiome), even as evolutionary unity of selection. The major arguments supporting this theory are: (i) All the multicellular organisms harbor a

microbiota, (ii) this microbiota might be inherited by the offspring, and (iii) thus, not only the host genome is transmitted between generation so also it is the microbiota genomes. Hence, both the host and the associated microorganisms would act like a superorganism in order to benefit the holobiont fitness (Rosenberg and Zilber-Rosenberg, 2016).

As a consequence of this theory, we could find a subgroup of microbial taxa that would be shared by all the individuals of one species: the core microbiome. In theory, the core range of microbiota species/OTUs systematically associated with a certain plant genotype would represent the taxonomic core microbiome (Lemanceau et al., 2017; Durán et al., 2018; Toju et al., 2018). But due to the fact that the environmental reservoirs of microbes may change depending on the site where the plant/holobiont is, different individuals from the same plant genotype may harbour highly divergent taxonomic core microbiomes. For that reason, the concept of functional core microbiome has been proposed, as it has been observed that any given plant genotype recruits similar functional microbiomes independently to the site of sampling (Lemanceau et al., 2017). The functions of the microbiome as a whole favour the holobiont fitness (health and nutrition).

Depending on the phenological and developmental state of a plant, the associated microbiota also varies across time (Mougel et al., 2006; Shi et al., 2016). Other factors affecting plant core microbiomes are the host genotype, soil type, plant compartment, plant immune response, biogeographical distribution, or season. These factors may have low impact on taxonomical core microbiomes, but a high impact on functional core microbiomes (Ofek-Lalzar et al., 2014; Bulgarelli et al., 2015). A characteristic of holobionts is the heritability of their microbiomes, underscoring the importance of natural and human selection (domestication) (Blouin et al., 2015; Panke-Buisse et al., 2015; Berg and Raaijmakers, 2018). Furthermore, inside the holobiont the horizontal gene transference (HGT) between different microbial taxa is a common phenomenon. HGT is important to increase the PGP traits abundancy and redundancy inside the holobiont (Polz et al., 2013; Bruto et al., 2014).

3.2. Relationships inside the plant-microbiome system.

Soil is known to be one of the most diverse habitats on Earth, containing billions of bacteria and millions of fungi. This enormous diversity is driven by the capability of microbes to perform HGT between phylogenetically unrelated groups (McDonald and Currie, 2017; Sergaki et al., 2018). Soil microorganisms are essential in nutrient cycling

like N, C, or P (Bender et al., 2016; Lladó et al., 2017), and it is in the interphase between soil and plant roots where these communities become crucial for agriculture. Similarly to the vertebrates gut, plant roots are inhabited by microbial communities, taxonomically structured to provide fitness to their host (Hacquard et al., 2015). These communities are attracted from the soil to the rhizosphere, which is highly populated (10^7-10^9 bacterial cfu/g soil), but the most specialized microbes are found in the rhizoplane. Here the bacteria reach populations between 10^5-10^7 cfu/g (Benizri et al., 2001). Only a little part of plant-related microbes are able to inhabit the inner plant tissues; hence, the endosphere populations are lower and rarely exceed 10^5 cfu/g fresh weight, mainly in shoot endosphere (Hallman et al., 2001; Compant et al., 2010). Furthermore, the alpha diversity decreases according to the soil-rhizosphere-rhizoplane-root endosphere-above ground tissues endosphere gradient (Reinhold-Hurek et al., 2015; Durán et al., 2018).

3.2.1. Type of communities in the microbiome:

The microbiome can be formed not only by bacteria, but also by archaea, fungi, oomycetes, and viruses. From all of them, the most relevant to plant fitness are the bacteria, the fungi and the oomycetes (Durán et al., 2018; Hassani et al., 2018). Bacterial and fungal communities are clearly different among soil, rhizosphere, and endosphere (Durán et al., 2018; MacPherson et al., 2018).

Bacteria (and archaea) community:

Beneficial bacteria are a class of bacteria that helps their host to tolerate biotic and abiotic stresses threatening its development (Militute et al., 2015). These bacteria can live outside the plant (soil bacteria, rhizobacteria, and epiphytes) or internally as endophytes (Hardoim et al., 2008; Compant et al., 2010).

The composition of these communities is not random; bacteria and archaea are strongly selected by the plant host genotype, and, therefore, are taxonomically and functionally highly conserved in plant microbiomes (Durán et al., 2018; Hassani et al., 2018). Other factors influencing the community composition are surrounding soil, plant compartment, plant immune response, developmental stage, UV light, or season, among others (Müller et al., 2016; Hassani et al., 2018, and references therein).

Bacterial communities have been extensively studied. Commonly, the bacterial plant-related communities are conformed by *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Planctomycetes,* and *Verrucomicrobiales*. Although the root-associated

communities are different from those inhabiting the phyllosphere (niche specialization), there is a certain grade of overlapping (Hassani et al., 2018).

On the other side, archaeal communities have been rarely assessed, but at the moment, no pathogenic isolates have been found. The main archaeal groups found in plant microbiomes are *Thaumarchaeota*, *Crenarchaeota,* and *Euryarchaeota* (Müller et al., 2016).

Fungi and oomycetes communities:

There is a great fungal diversity colonizing above- and belowground plant organs. The main groups found in plants are the so-called true fungi, *Ascomycota,* and *Basidiomycota* phyla (Jumpponen et al., 2009; Toju et al., 2013; Hardoim et al., 2015). Also, in roots *Glomeromycota* and other ectomycorrhizal fungi are commonly found. *Pythium* is the most abundant genus in *Oomycota* (Durán et al., 2018).

Fungal microbiome composition also depends on the soil, plant compartment, host genotype and season (Hassani et al., 2018, and references therein). But, comparing to bacterial microbiome, the fungal communities from soil and plant are more influenced by stochastic variations and respond differently to the factors (Lekberg et al., 2012; Wang et al., 2013; Powell et al., 2015). For instance, fungi and oomycetes are mainly distributed according to biogeographic factors (Talbot et al., 2014; Coleman-Derr et al., 2016; Durán et al., 2018). Consequently, there is a limited dispersion and climatic selection of these microbial kingdoms, so they show a high grade of endemism (Peay et al., 2010; Talbot et al., 2014; Tedersoo et al., 2014).

3.2.2. Microbial interactions:

These microbes may create positive interactions with the plants (mutualists), neutral interactions (commensals), or negative interactions (pathogens). Microbe-microbe interactions have emerged recently as an important feature in the plant episphere (phyllosphere and rhizosphere) (Agler et al., 2016; Hassani et al., 2018). These interactions are essential in the microbiome functioning; for instance, the defense against pathogens, the plant growth and the mycorrhizal symbiosis promotion (Durán et al., 2018).

The competition for organic matter between microbial kingdoms explains the strong positive correlation among the microorganisms from the same kingdom and the negative correlation between bacteria and filamentous fungi (interkingdom competition) (Durán et al., 2018; Zhalnina et al., 2018). This interkingdom competence mainly occurs in soil-root interphase during the microbiota (rhizospheric and endophytic) colonization.

In that situation, biocontrol traits are essential, and for that reason they are recurrent among the plant-related microbes (Durán et al., 2018).

Positive interactions

i. Nutritional interdependency: it is the reciprocal metabolite exchange among dependent microbes to compensate for the metabolic deficiencies, habitually in poor nutrient environments (Sckink, 2002; Morris et al., 2013; Mee et al., 2014). This sort of interaction promotes the community connection, and in final stages it can suppose the adaptive loss of genes in the members of the relationship (Mas et al., 2016). This loss can be irreversible.

ii. Biofilm formation: biofilms are micro-architectural constructions where microbial communities are embedded. The coordinate production of polymeric extracellular substances allows its formation (Stoodley et al., 2002). Biofilms provide selective advantages for the microorganisms as antimicrobial molecules and protection. The formation process is dependent on high cellular density and HGT gene acquisition (Van Acken et al., 2014; Zhang et al., 2014). Biofilms are essential to prevent the entrance of the pathogen in plants, and they can be formed exclusively by bacteria or by a mixture of fungi and bacteria (Hassani et al., 2018).

iii. Quorum sensing: Gram – bacteria can monitor their population density by means of the N-acyl-l-homoserine lactone (AHL) production (Eberl, 1999). AHL are widely spread in bacterial phylogeny, which allows them to cooperate or interfere (quorum quenching). Other communication molecules are the volatile organic compounds (VOCs), oxalic acid, trehalose, glucose, or thiamine (Hassani et al., 2018).

iv. Dispersion improvement: it has been demonstrated that certain bacteria use the hyphae of filamentous eukaryotes as a dispersal vector, there are the so-called "fungal highways" (Kohlmeier et al., 2005; Worrich et al., 2016). These mycelial networks enable the HGT between bacteria separated in the space and the endosphere entrance (Larousse et al., 2017).

v. Bacterial endosymbiosis in fungi: plant-associated fungi recruit bacteria from the environment as endosymbionts; these bacteria can be inherited vertically through the fungal spores (Bianciotto et al., 2000; Partida-Martínez et al., 2007). This type of association has been reported between *Burkholderiaceae* and *Bacillaceae* bacterial families and plant-associated fungi from genera like

Rhizophagus, *Gigaspora*, *Laccaria*, *Morteriella*, *Ustilago,* or *Rhizopus* (Hassani et al., 2018).

Negative interactions

i. Resources competition: some microbes can increase their speed and efficiency in the uptake of limiting resources, preventing the growth of the other microorganisms. For instance, the liberation of bacterial siderophores for iron complexation impedes, at the same time the growth of some Fe-dependent plant pathogens (Ahmed and Holmström, 2014).
ii. Contact dependent competitive strategies: some bacteria, mostly from *Proteobacteria* phyla, use the type VI secretion system as a molecular weapon to liberate toxins in eukaryotic cells or other prokaryotes (Records, 2011). These mechanisms improve the bacterial fitness inside the plant environment (increasing the niche colonization). Also, the type III secretion system is essential in colonization interactions from bacteria to fungi or bacteria to oomycetes (Hassani et al., 2018).
iii. Antimicrobial compounds secretion: bacteria and fungi secrete chemical compounds with the aim to suppress the growth of competitor microorganisms (Raaijmakers and Mazzola, 2012). Occasionally, this production only occurs in community (biofilm) or co-cultivation contexts.
iv. VOCs emission: bacteria as *Pseudomonas*, *Serratia*, *Stenotrophomonas,* or *Streptomyces* are known by their ability to produce VOCs with inhibitory effect in fungal and oomycete growth (Tyc et al., 2017).
v. Predation: it is a well-known ability of microbes inhabiting the soil-root interphase. There are both mycophages bacteria or mycoparasitic fungi, which actively ingest fungal hyphae and lead those fungi to death (Hassani et al., 2018).

3.3. Endophytes behavior and plant growth promotion. Seed microbiome.

Endophytes are described as those microorganisms isolated from surface-sterilized plant tissues without noticeable harm signs (Hardoim et al., 2008; Santoyo et al., 2016). Besides, most of the endophytes are considered as a subgroup of rhizobacteria with the ability to invade their host (Reinhold-Hurek et al., 2015). The majority of the endophytes have a biphasic life cycle, alternating environment, and plant. Inside the host the beneficial effects of these bacteria are greater than those of rhizobacteria (Hardoim et

al., 2008; Afzal et al., 2019). The endosphere is the ecological niche of endophytes. Endosphere is a unique protective ecological niche that provides a safe and consistent environment unperturbed by the fluctuating environmental conditions affecting epiphytes (Shentilkumar et al., 2011). In return, endophytes promote the host plant development, alleviate stress conditions and produce allelopathic substances against other plant competitors (Rosenblueth and Martínez-Romero, 2006; Cipollini et al., 2012; Afzal et al., 2019).

The endophytes have been isolated and characterized from several plant hosts; for instance, agronomical valuable crops, prairie plants, extreme environment plants, wild plants, and perennial plants (Afzal et al., 2019 and references therein). These microbes have been isolated in all plant tissues: roots, stems, leaves, seeds, fruits, tubers, rhizomes, ovules, and nodules (Rosenblueth and Martínez-Romero, 2006; Chebotar et al., 2015; Liu et al., 2019).

As a part of the plant microbiome, the endophytes pertain to the different microbial kingdoms previously described? (bacteria, archaea, fungi and oomycetes), being bacteria the group more extensively studied. For that reason, the endosphere colonization process, the endophytic diversity and the PGP mechanisms below described will be focused on bacterial endophytic microbiome, including information about the other kingdoms if available.

3.3.1. Plant colonization by endophytes:

The potential endophytes need a battery of colonization traits to come into the plants. The colonization process is complex and based on plant-microbe cross-talk. Usually, this process is initiated in the roots, where the root exudates are recognized specifically by each bacterial taxa (Rosenblueth and Martínez-Romero, 2006; Hardoim et al., 2008; Afzal et al., 2019). The whole process is developed in different phases (Figure 1):

i. Rhizosphere colonization: this is a highly competitive process for endophytes because they must occupy a space and take nutrients in a populated environment (Raaijmakers and Mazzola, 2012). Motility and polysaccharide production are two of the most important traits to success in such an environment (Hardoim et al., 2008; Santoyo et al., 2016). Those bacteria attracted by the root exudates have to attach themselves to the rhizoplane. This attachment can occur in root cracks from lateral root growth regions or

root hairs (Hardoim et al., 2008; Olivares et al., 2017). After that, bacteria form micro-colonies and biofilms to diminish the competition with rhizobacteria and to facilitate their entrance in the endosphere (Afzal et al., 2019). Hence, the whole process is dependent on the plant root exudation patterns, the bacterial attachment and motility, bacterial quorum sensing and growth rate, and the bacterial nutrient efficiency and antimicrobials production (Compant et al., 2010).

ii. Endosphere colonization: The endosphere penetration process can be active or passive. Passive penetration occurs through root emergency cracks, root tips, and pathogenic wounds (Hardoim et al., 2008). However, the active colonization depends of the bacterial proliferation and the attachment by lipopolysaccharides, flagella, type IV pili, adhesins, twitching motility and quorum sensing (Böhm et al., 2007; Hardoim et al., 2008; Hori and Matsumoto, 2010; Afzal et al., 2019); although each taxon has their colonization region and pattern (Zachow et al., 2010). Plant-cell degrading enzymes segregation (mainly pectinases and cellulases) is another important colonization trait. In fact, the main difference between endophytes and plant pathogens is the production level of these enzymes: low levels in endophytes, and high in pathogens (Elbetagy et al., 2000). By maintaining low levels of these degrading enzymes, endophytes can avoid the plant innate defense systems, promoting at the same time the ISR (Induced System of Resistance). Endophytes also avoid the plant immune system by lowering its population density in comparison with pathogenic microbes (Zinniel et al., 2002). In this colonization step the plant genotype plays an important role as well, selecting their endophytic partners (by rhizodeposition) and by a selective defensive response (Rosenblueth and Martínez-Romero, 2006; Reinhold-Hurek et al., 2015).

iii. Systemic colonization: it is still not clear if the endophyte colonization in aboveground tissues is as important as it is in roots. Although, the inability to colonize these plant compartments probably is related to vertical heritability of endophytes through seeds (Truyens et al., 2015; Berg and Raaijmakers, 2018; Nelson, 2018). It is widely known that only a few bacterial species are able to spread themselves systematically in the plants; thus, those endophytes are highly niche adapted. Once the endophytes reach the root endosphere,

only few of them can move inside the plants using their flagella, the plant-cell degrading enzymes, or by the plant transpiration stream (Afzal et al., 2019). Besides, some of these endophytes may enter the plant through the stomata (Senthilkumar et al., 2011).

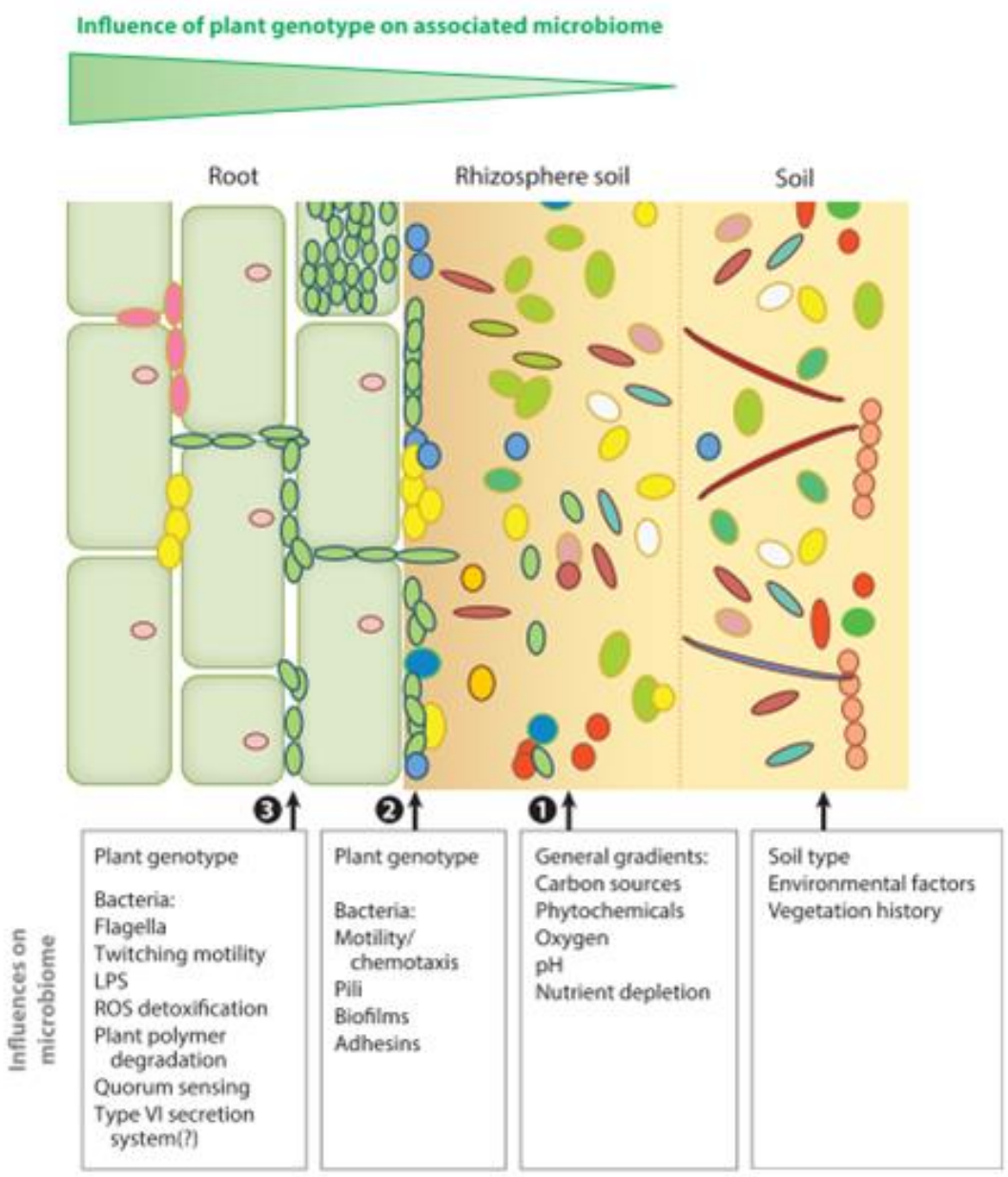

Figure 1. Enrichment model for microorganisms colonizing different root microhabitats (Reinhold-Hurek et al., 2015). Endosphere is inhabited lower complexity communities. Specific bacterial traits are required for colonize this compartment, but host genotype has the strongest influence on community structure compare with the other compartments. LPS,=lipopolysaccharide; ROS=reactive oxygen species

3.3.2. Endophyte diversity:

Despite the fact that endophytes are a subpopulation of rhizobacteria and soil bacteria, the community composition of endophytic communities is different from them. Also, the methods utilized to study endophytes are different and may influence the result. Finally, the factors affecting this community are completely different from those affecting epiphytes.

i. Factors driving the endophytic community composition: the most important factors are those related to the plant host: host age, genotype, geographical localization, development stage, and plant compartment. Secondarily, climate conditions such as temperature, UV light, soil pH, soil type, nutrient soil concentrations, environmental pollutants or pathogens presence would also affect the endophyte community (Müller et al., 2016; Afzal et al., 2019).
ii. Methods in endophyte community studies:
    a. **Culture-based methods:** these methods only evaluate the cultivable bacteria. Plant tissues must be sterilized superficially with sodium hypochlorite, ethanol, and/or hydrogen peroxide as sterilizing agents. This process can influence the observed endophytic diversity (Eevers et al., 2015; Richter-Heitmann et al., 2016). Another factor affecting the cultivable endophytic community is the choice of the culture medium (Eevers et al., 2015). Unfortunately, cultivable bacteria usually represent a low percentage of the real community (Sergaki et al., 2018; Afzal et al., 2019).
    b. **Culture-independent methods:** after the tissue sterilization (same bias as culture base methods), the DNA is extracted from plant tissues. The main drawback of this step is controlling the quantity of plant DNA extracted. There are multiple methods to analyze the endophytic community like ARDRA (Amplified rDNA Restriction Analysis), DGGE (Denaturing Gradient Gel Electrophoresis), TGGE (Temperature Gradient Gel Electrophoresis), T-RFLP (Terminal Restriction Fragment Length Polymorphism) or ARISA (Automated Ribosomal Intergenic Spacer Analysis). But, due to the lower costs in massive sequencing achieve in the last decade, metagenomics studied have prevailed. Metagenomics studies usually carried out to assess the endophytic community are: amplicon metagenomics (only one gene

or phylogenetic marker), shotgun metagenomics (the whole genomes of the community are sequenced) and RNA-seq metagenomics (assessment of the metabolically active populations). Furthermore, other –omics are also used, like metaproteomics (to evaluate the microbiome protein expression) or metabolomics (to evaluate the microbiome metabolite production) (Afzal et al., 2019 and references therein).

c. ***In situ* studies:** endophytic communities could be assessed in the plant using the FISH (Fluorescent In Situ Hybridization) technique (Lo Piccolo et al., 2010).

iii. Endophyte diversity in different plants: *Proteobacteria* is the prominent bacterial phylum in plant endospheres, especially *a-*, *β-* and *γ-proteobacteria*, being the latter the most diverse (Militute et al., 2015; Santoyo et al., 2016). The other common phyla are, as in the rhizosphere, *Actinobacteria*, *Bacteroidetes,* and *Firmicutes*. *Acidobacteria*, *Planctomycetes,* and *Verrucomicrobia* are also present in endospheres, but to a lesser extent (Hardoim et al., 2008; Santoyo et al., 2016; Afzal et al., 2019). Common genera in the endosphere are *Azospirillum*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Microbacterium*, *Micrococcus*, *Pantoea*, *Pseudomonas* and *Stenotrophomonas* (Hallman et al., 1997; Hardoim et al., 2015).

3.3.3. Plant growth-promoting traits:

Endophytes are known for their beneficial effects on plants (Figure 2). These effects can be direct (enhancing/modulating phytohormonal levels or increasing the availability of nutrients) or indirect (decreasing pathogens, alleviating stress, or promoting the plant defense).

i. Nutrient acquisition:

a. **Nitrogen (N):** diazotrophic endophytes are those microbes that harboring the nif gene (codifies the nitrogenase), are able to fix biologically the atmospheric N_2, which increases the N bioavailability for the plants (Carvalho et al., 2014). Nitrogenase is a highly conserved enzyme, and it can be transmitted between phylogenetically distant bacteria by HGT (Gaby and Buckley, 2012). Some diazotrophic endophytes are some strains of genera *Azoarcus*, *Azospirillum*, *Burkholderia*, *Gluconoacetobacter,* and *Herbaspirillum*. Diazotrophic endophytes perform better than rhizospheric

diazotrophs improving the nutritional status in plants under N limiting conditions (Hurek and Reinhold-Hurek, 2003). Moreover, these endophytes increase the N fixation rate and the plant accumulation of this nutrient (Gupta et al., 2013).

b. **Phosphorus (P):** most of the soil P is found as insoluble forms. Some endophytes are able to solubilize the precipitated phosphates by means of acidification, chelation, ionic exchange, or organic acid production (Nautiyal et al., 1999). Furthermore, some of them can produce acid phosphatases to mineralize organic phosphates (Van der Heijden et al., 2008). Finally, these microbes prevent P adsorption and fixation under limiting conditions by assimilating solubilized P; acting as a P sink, that could be provided to the plant as needed (Khan and Joergensen, 2009).

c. **Iron (Fe):** Fe is an important nutrient to plants; this element is part of many proteins involved in respiration and transpiration processes (Afzal et al., 2019). Usually, Fe in the soil is forming insoluble compounds as carbonates, hydroxides, oxides or phosphates unavailable to the plants. Endophytes produce chelating agents called siderophores. These siderophores complex the Fe increasing the availability of this nutrient. Also, siderophores discourage the growth of the pathogens by Fe depletion in the environment (Ahmed and Holmström, 2014).

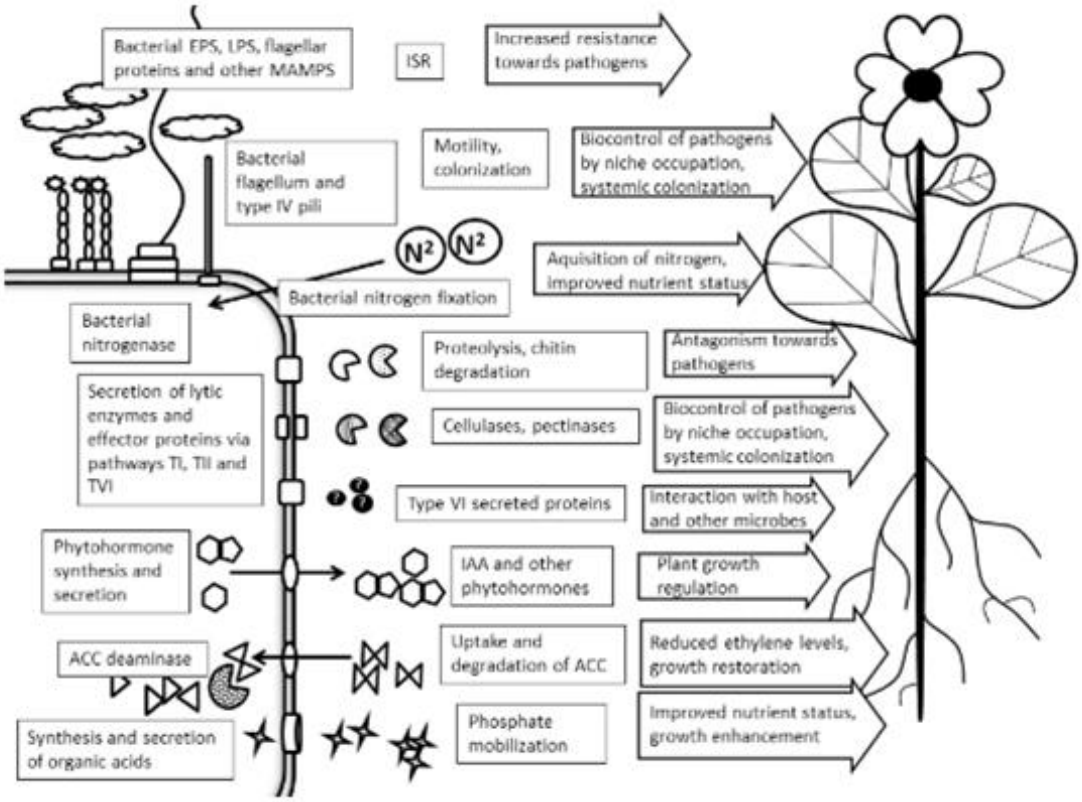

Figure 2. Plant Growth Promoting (PGP) traits from endophytes and their effects on plant development (adapted from Hardoim, 2011). EPS=extracellular polysaccharide; LPS=lipopolysaccharides; MAMP=microbe associates molecular pattern; IRS=induced systemic resistance; TI=type one protein secretion system; TII=type two protein secretion system; TVI=type six protein secretion system; IAA=indole-3-acetic acid; ACC=1-aminocyclopropane-1-carboxylate.

ii. Phytohormone production and modulation:

a. **Indol-3-acetic acid (IAA):** is the most common auxin. This auxin is involved in plant physiologic processes like cell-cell signaling, plant development regulation, plant defense system induction, lateral and adventitious root formation, photosynthesis, secondary metabolites biosynthesis, and stress conditions resistance (Spaepen et al., 2007; Glick et al., 2012). Therefore, the endophyte production of IAA promotes the plant biomass, the root volume, and the lateral root number (Afzal et al., 2019). Nevertheless, high concentrations of this hormone might produce stunted growth. Phytopathogen usually produced greater quantities of IAA than endophytes, which increase the ethylene concentration and promotes the plant defense (Rashid et al., 2012). Thus, the ability to degrade IAA for maintaining stable

levels in plant is also an important endophyte trait (Leveau and Lindow, 2005).

b. **Ethylene (ET):** This hormone controls the response to biotic/abiotic stress. Besides, it controls the root initiation, leaf senescence, root nodulation, abscission, cell elongation, fruit ripening and auxins transport (Sun et al., 2016). When a stress causes an increase in ET concentration, the root growth is reduced. Endophytes can modulate this ET concentration producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The endophytes use the enzyme to hydrolyze the ACC, an ET precursor, as a N source, and so alleviating the stress (Hardoim et al., 2008; Santoyo et al., 2016; Sun et al., 2016).

c. **Cytokinins (CKs) and Gibberellins (GAs):** only a few works have addressed the role of these two phytohormones families in endophyte-mediated plant growth. Cohen et al. (2009) concluded that microbial GAs are important to plant stress alleviation, while Bhore et al. (2010) identified some CKs in broth extracts from two bacterial endophytes.

iii. Pathogen suppression by endophytes:

Endophytes produce antipathogenic substances like antibiotics, toxins, siderophores, hydrolytic enzymes, and antimicrobial VOCs (Sheoran et al., 2015). Most of the producers belong to *Bacillus*, *Enterobacter*, *Paenibacillus*, *Pseudomonas*, *Serratia* and *Actinobacteria* classes (Afzal et al., 2019). Besides suppressing the bacterial pathogen's growth, some endophytes also prevent fungal pathogen growth. The antifungal activity of these endophytes may be caused by the production of fungal cell-wall degrading enzymes, like chitinases, proteases or glucanases (Zhang et al., 2012).

Besides, endophytes act as inductors of the ISR, protecting the holobiont from fungi, bacteria, and viruses (Alvin et al., 2014). ISR primes the defense mechanisms to protect the plant from pathogens' future attacks. This system can be activated by the endophytes through the JA/ET mediated pathway or the SA mediated pathway (Pieterse et al., 2012). In fact, the same endophyte can induce both signaling pathways to protect the plant from two different pathogens (Conn et al., 2008).

3.3.4. Seed microbiome, an endophyte subgroup:

Traditionally seeds were considered sterile, but sometimes can be a pathogen transmission vector. For avoiding this, phytosanitary measures such as mechanical, thermic, physical, chemical, and biological treatments have been proposed (Berg and Raaijmakers, 2018). However, the growth of omics has shown that seeds are not sterile and that they contain beneficial microbes inside. Hence, despite the fact that part of the microbiome could be inherited horizontally (ecological heritance) by a soil reservoir shared by parents and offspring; it is only through the seeds how a subgroup of microbial endophytes may vertically trespass to the next plant generation (Lemanceau et al., 2017, Berg and Raaijmakers, 2018; Nelson, 2018).

These living microorganisms colonize seeds through the carbohydrates transport pathway (leaf-coat seed), via pollen grains, via necarthodes o by the flower stigma. Inside the seed, endophytes can inhabit the seed coat, the cotyledon, or the root hypocotyl (Berg and Raaijmakers, 2018). Seed microbiome composition is plant genotype-specific and it is correlated with the common pathogens affecting that plant genotype (Rybakova et al., 2017).

Future plant breeding programs should take into account the seed microbiome for new cultivars selection (microbiome engineering) (Adam et al., 2018). Aside from seed microbiome selection, Pérez-Jaramillo et al. (2016) has proposed the "back to the roots" strategy combining this new breeding strategy with the use of local biostimulants, the so-called SynBiotic (prebiotic + probiotic), consisting in the use of microbes previously present in crop ancestors. This strategy would reverse the domestication process that is believed to have affected plants' capability to create beneficial associations with endophytes (Pérez-Jaramillo et al., 2016; Berg and Raaijmakers, 2018; Nelson, 2018). For instance, the domestication footprint is noticeable in the increase in domesticated crops of *Proteobacteria* endophytes (fast-growth bacteria), while in the crop wild ancestors, the phylum *Bacteroidetes* (slow-growth bacteria) was more common. This change was also observed in human guts, and it has been called "domestication syndrome".

4. Aim of this thesis

Currently, the demanding for more producing agriculture to feed a growing population, but also the social interest in sustainable agriculture, requires a rapid and efficient response from science. In this context, the use of humic substances as a valuable tool to reach these objectives. Despite the fact that humic substances have been used since ancient times, their mechanism of action is still under debate. Traditionally, SHA mechanism of action has been studied in root applications (Aguirre et al., 2009; Zandonadi et al., 2010; Jannin et al., 2012; Mora et al., 2014a,b; Olaetxea et al., 2019; Zanin et al., 2019; have been focused in the direct application to the roots, but, however, in agriculture, the foliar application is an extended practice (Olk et al., 2013; Canellas et al., 2015a).

Furthermore, little is known about the effect of these substances on plant-associated microorganisms. Most of the studies only have evaluated their effect in soil microbes or in microorganisms grown in artificial mediums. We proposed the study of their effect in relationship with the plant fitness. In the last decade, the development of NGS has increased the knowledge of plant microbiomes, highlighting their importance as plant fitness regulators, although mainly rhizosphere and soil microbiomes have been explored (Hacquard et al., 2015; Müller et al., 2016). Nevertheless, still little is known about the endophytic microbiome due to the working difficulties.

Hence, in this dissertation, our aim is to assess how the different SHA application strategies can affect plant development and the endophytic microbiome composition. We have explored the possible links between SHA action in plants and the microbiomes changes under different conditions. For achieving our goal, we will:

i. Define the mechanism of action of SHA foliar application in cucumber plants grown in hydroponics. We will compare the plant growth induced by foliar and root applications, but also the hormonal, phenological and biochemical changes occurring inside the plant (Chapter 1).
ii. Evaluate the effect on cucumber plant endophytic microbiome of the different SHA application strategies. We will carry out the experiments under the same conditions as Chapter 1. We will analyze the bacterial and fungal endophytic communities through the use of amplicon massive sequencing techniques. Besides, if there are some differences, we will identify those

microbial taxa especially enriched or depleted by the SHA application (Chapter 2).

iii. Identify the microbiome response to N-fertilization in barley plants grown under greenhouse conditions and its combination with SHA application. Chapter 4, also, will allow us to describe the SHA application effect on the endophytic microbiome in monocots plants, its long-term effect and in a more realistic scenario.

iv. Isolate bacterial endophytes from cucumber plants previously treated with a root application of SHA. Then we will characterize their potential as plant growth promoters in their ability to increase the bioavailability of the nutrients and to induce plant growth.

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CHAPTER I

Discriminating the short-term action of root and foliar application of humic acids on plant growth: emerging role of jasmonic and salicylic acids

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Frontiers in Plant Science (Under review)

Abstract

Humic substances (HS, fulvic and humic acids) are widely used as fertilizers or plant growth stimulants since ancient times, although their mechanism of action still remains partially unknown. Humic substances may be applied either directly to the soil or as foliar sprays. Despite both kind of applications are commonly used in agricultural practices, the major part of the studies regarding the elicited response in plants as a consequence of the treatment with HS are based on the root-application of these substances. The present work aimed at discriminating between the mechanisms of action of foliar application versus root application of a sedimentary humic acid (SHA) on plant development. For this purpose, six markers related to plant phenotype, plant morphology, hormonal balance and root-plasma membrane H+-ATPase (PM H^+-ATPase) were selected.

Both application strategies improved the shoot and root growth. Foliar applied- and root applied-SHA shared the capacity to increase the concentration of indole-3-acetic acid in roots and cytokinins in leaves. However, foliar application did not lead to short-term increases in either abscisic acid root-concentration or root-PM H+-ATPase activity which are, however, two crucial effects triggered by SHA root-application. Both application modes increased the root concentrations of jasmonic acid and jasmonoyl-isoleucine. Foliar-applied SHA led to a medium-term increase in the concentration of salicylic acid in roots and leaves. These hormonal changes caused by foliar application could be explained by the loss of leaves trichromes, the decrease in cuticle soluble waxes and the diminution of chloroplasts size seen by microscopy. These results support the hypothesis that the beneficial effects of SHA applied to roots or leaves may result from plant adaptation to a mild transient stress caused by SHA application.

Keywords

Humic substances, humic acids, foliar application, root application, shoot growth, root growth, jasmonic acid, salycilic acid.

1. Introduction

The beneficial influence of specific fractions of soil organic matter (SOM) on the fertility of soils is well stablished (Magdoff and Weil, 2004a, 2004b; Chen et al., 2004). One of such fractions is the organic matter present in the soil solution, which is also known as dissolved organic matter (DOM) (Olaetxea et al., 2018). This fraction includes various families of organic molecules that range from simple compounds like amino acids, sugars or polycarboxylic acids to complex molecules resulting from biotic and/or abiotic transformation of fresh organic matter in the soil (Stevenson, 1994; Olaetxea et al., 2018). The process involved in the transformation of fresh organic matter is normally known as humification, and the groups of organic molecules resulting from it are called humic substances (HS). Humic substances are fractionated according to their solubility as a function of pH (Stevenson, 1994): humic acids (HA) are soluble at basic pH but insoluble at acidic pH, fulvic acids (FA) are soluble at both basic and acidic pH values, whereas humin is insoluble at all pH values (Stevenson, 1994). Despite both the chemical nature and structural features of HS are still a matter of scientific debate (Lehmann and Kleber, 2015; Kleber and Lehmann, 2019; Olk et al., 2019), recent studies indicate that HS have singular structural domains that are not present in other biomolecules such as proteins, polysaccharides, lignin or cellulose (Cao and Rohr, 2018; Perminova et al., 2018).

Many studies showed that HS from different origins applied to plant roots can improve plant growth and mineral nutrition (Chen et al., 2004; Rose et al., 2014; Olaetxea et al., 2018). These positive effects involve various mechanisms, including the action of HS on soil and rhizosphere properties (indirect effects), and their interactions with plant roots (direct effects) (Chen et al., 2004; Olaetxea et al., 2018). A highly relevant indirect effect is the capacity of HS to increase the pool of plant available nutrients in the rhizosphere (Chen et al., 2004; Olaetxea et al., 2018). Direct effects involve a coordinated action of HS on several signalling pathways regulated by relevant plant hormones (Nardi et al., 2002; Canellas et al., 2015; Olaetxea et al., 2018). Both direct and indirect effects are influenced by HS structural features (Canellas et al., 2012; García et al., 2016).

The capacity of HS to enhance plant growth promoted the development of commercial products based on HA and FA as biostimulants for plant production (Rose et al., 2014; Canellas et al., 2015). In general, marketed HS-based products can be applied not only on the soil (root area) but also as foliar sprays (Rose et al., 2014; Canellas et al., 2015). While

the mechanisms of action involved in the plant growth promoting effect of soil applied HS have been characterised in various studies (Chen et al., 2004; Canellas et al., 2015; Olaetxea et al., 2018), the beneficial action of foliar-applied HS remain unexplored to date. Indeed, it is assumed that foliar-applied HS promote plant growth by mechanisms similar to those involved in HS root application (Chen et al., 2004). However, there are many differences regarding the mechanisms of absorption, transport and interaction of root- versus foliar-applied HS. For example, the range of concentration of HS that is needed to improve plant growth via foliar application is much lower compared to root HS application (Chen and Aviad, 1990). Likewise, HS applied to the leaves do not interact with the soil and rhizosphere. It is therefore plausible that the mechanisms underlying the response of plants to foliar compared to root applied-HS may involve nutritional, metabolic and physiological differences which have not been assessed yet.

Hence, the aim of this study is to evaluate some of the mechanisms triggered after foliar application of a well-characterized HA previously found to improve plant growth (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). Our hypothesis is that the interaction of HS with plant leaves may induce some kind of mild stress signals that may activate hormonal and molecular pathways involved in the regulation of plant stress responses. As the nature of HS-leaf interactions in the phyllosphere may be quite different from that of HS-root rhizosphere interactions, we hypothesise the occurrence of potentially different mechanisms responsible for the beneficial effects of both HS supply modes on plant growth.

For a proper comparison of the effects of foliar- versus root-applied HA, the same HA (a sedimentary HA extracted from leonardite referred to as SHA from now on), plant species (cucumber) and experimental settings were implemented as in previous root-applied SHA trials (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). For comparison between the mechanisms associated with root-applied SHA and foliar-applied SHA and as previously shown (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019) the following parameters were used as markers of the beneficial effects:

i. The short-term increase in both root and shoot dry matter. Several studies have shown the capacity of root-applied SHA to increase dry matter production of the root and shoot of cucumber plants (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019).

ii. The short-term increase in lateral root production. Previous studies have shown that root-applied SHA increased root dry weight (D.W.) as well as the whole number of adventitious-lateral roots in cucumber plants (Mora et al., 2012).
iii. The short-term increase in root indole-3-acetic acid (IAA) and abscisic acid (ABA) concentrations. Previous studies have shown the capacity of root-applied SHA to increase the concentration of root IAA and ABA in cucumber plants (Mora et al., 2010, 2012). Further studies demonstrated that these effects play an essential role in the mechanism underlying the promoting action of root-applied SHA on shoot growth (Mora et al., 2012; Olaetxea et al., 2015).
iv. The short-term increase in cytokinin leaf-concentrations. Previous studies reported the capacity of root-applied SHA to increase the shoot (stem and leaves) concentration of several cytokinins in cucumber plants (Mora et al., 2010). Further studies indicated that these effects of SHA play an essential role in the shoot growth promoting action of root-applied SHA (Olaetxea et al., 2019).
v. The short-term increase in root plasma membrane (PM) H+-ATPase activity. Previous studies reported the capacity of root-applied SHA to increase the activity of root PM H+- ATPase in cucumber (Mora et al., 2010). This effect was accompanied by a significant up-regulation of the genes codifying some root PM H+-ATPase isoforms (Aguirre et al. 2009). Further studies have shown that these effects play a relevant role in the mechanism underlying the increases in root and shoot growths caused by root-applied SHA (Olaetxea et al., 2019).
vi. The short-term increase in shoot nutrient concentrations. Previous studies showed an effect of root-applied SHA in increasing the concentration of several essential elements in the leaves of cucumber plants (Mora et al., 2010).

These six markers reflecting short-term effects of root-applied SHA on the growth of cucumber plants cultivated in hydroponics were analysed after SHA foliar treatment of cucumber plants. The study is complemented with the determination of the variation of other hormones that may also play a role in the mechanisms responsible for the beneficial effects of foliar-applied SHA in cucumber plants.

Other mechanisms expressed as long-term plant responses to SHA-foliar application might also be of great relevance in the whole action of SHA on plant development when applied on the leaves. However, we hypothesize that the short-term responses of plants to

foliar-applied SHA play a crucial role in the triggering of the evolvement of the plant response to SHA during its phenological life cycle.

2. Material and methods

2.1. Extraction and Purification of a Leonardite HA (SHA)

Sedimentary humic acids (SHA) were extracted from a leonardite originated in the Danube basin (Czech-Republic). The extraction and purification of SHA were performed according to the International Humic Substances Society methodology with some modifications as described in Aguirre et al. (2009) (Supplementary Information). The main physico-chemical features of SHA are described in Supplementary information (Table S1, Figures S1 and S2).

2.2. Plant Material

Cucumber (*Cucumis sativus* L. var. Ashley) seeds were germinated in the dark, on perlite and filter paper moistened with a 1mM $CaSO_4$ solution. The germination chamber conditions were 25ºC and 75% relative humidity (RH). One week after, seedlings were transferred to a hydroponic system with vessels filled with 7 L of nutrient solution. This solution contained: 0.63 mM K_2SO_4, 0.5 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.30 mM $MgSO_4$, 0.25 mM KNO_3, 0.05 mM KCl, 0.87 mM $Mg(NO_3)_2$, 40 µM H_3BO_3, 27.3 µM $MnSO_4$, 2 µM $CuSO_4$, 2 µM $ZnSO_4$, and 1.4 µM Na_2MoO_4. The solution was supplemented with 80 µM iron as Fe-ethylenediamine-N,N_9-bis(2-hydroxyphenylacetic acid) chelate (80% [w/w] ortho-ortho-isomer). The average value of the pH of the nutrient solution during the experiment was 6.7. The different experiments were performed in a growth chamber (Aralab, S.A.) where the experimental conditions were set up to 25ºC/21ºC and 70%/75% RH in a day-night cycle and the photoperiod was 15h/9h (PAR of 250 µmol m^{-2} s^{-1}).

Evaluation of the effects caused by SHA when applied on the leaves: The foliar SHA treatment consisted of a SHA aqueous solution (pH 6) plus 0.1% Tween20 (vol/vol, Sigma-Aldrich, MO, USA), sprayed on the leaves of cucumber plants after 10 days upon plant germination. The spray was applied both on the abaxial and adaxial side of leaves. Control plants were treated with water plus 0.1% Tween20 (vol/vol, Sigma-Aldrich, MO, USA). Each treatment consisted of five replications with one plant per replicate, for the different harvest times.

Two different set of experiments were carried out:

i. A set of short-term experiments based on the results obtained in previous studies on the short-term effect of SHA applied on the roots of cucumber plants (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). In these experiments the concentration of SHA applied on the leaves were 20, 30 and 40 mg L^{-1} of organic C. Plants were harvested after 4h, 24 h, 48 h and 72 h.
ii. In order to complement the results obtained in the short-term experiment, a second set of experiments using a higher concentration of SHA (100 mg organic $C{\cdot}L^{-1}$) and extending the harvest period to 8 days was also performed. We named these experiments as medium-term experiments throughout the manuscript.

Foliar treatments were always applied 2 hours after the start of the diurnal period.

2.3. Measurement of root and shoot dry matter

In short-term experiments, cucumber plants were harvested 4h, 24h, 48h, and 72h upon SHA application, whereas in medium-term experiments plants were harvested upon 72h (3 days) and 192h (8 days). Five plants were harvested for each treatment and each harvest time.

Control and foliar-SHA treated plants were harvested at the same time of the day for avoiding diurnal variations: 6 h after the start of the light period. Shoots and roots were sectioned with a scalpel before fresh weight (FW) measurement. Cotyledons were removed after weighing. Plant tissue samples were then dried at 50 ºC for 3 days in a lab stove, and their dry weight (DW) was subsequently measured.

2.4. Root morphology studies

Root morphology images were acquired with the software WINRHIZO (REGENT INSTRUMENTS INC, Canada) implemented in a scanner (EPSON Perfection V700 Photo). Images were taken from short term experiments at different times for control and SHA foliar-applied treatment corresponding to 40 mg L^{-1}. Three plants per treatment and harvest time were analysed.

2.5. Leaf morphology studies

Morphological features of leaves of both control plants and plants treated with 100 mg L-1 of SHA on the leaves, were analysed by transmission (TEM) and scanning (SEM) electron microscopy. Second true leaves (fully expanded) were harvested after seven days from the onset of the treatments. For both SEM and TEM, 4 mm^2 pieces were cut and subsequently fixed in 2.5% glutaraldehyde-4% paraformaldehyde (Electron Microscopy Sciences (EMS),

Hatfield, PA, USA) for 6 hours at 4ºC. Then they were rinsed in ice-cold phosphate buffer, pH 7.2, 4 times within a period of 6 hours and left overnight.

For SEM, fixed leaf tissues were dehydrated in a series of absolute ethanol (Merck, Germany) (i.e., 30%, 50%, 70%, 80%, 90% and 100%; x3 times each concentration). They were subsequently subjected to critical point drying (Leica EM CPD300, Leica Microsystems, Germany). Before observation, samples were gold-sputter and examined with a JEOL 6400 SEM (Tokyo, Japan).

For TEM, fixed and phosphate buffer rinsed cucumber leaf samples were post-fixed for 1.5 h in 1:1 water: 2% aqueous osmium tetroxide (TAAB Laboratories, Berkshire, UK) solution containing 3% potassium ferrocyanide (Sigma-Aldrich). Tissue were consequently washed with distilled water (x3), dehydrated in a series of 30, 50, 70, 80, 90, 95 and 100% acetone (x2, 15 min each concentration) and embedded in acetone-Spurr's resin (TAAB Laboratories) mixtures (3:1, 2h; 1:1, 2h; 1:3, 3h) and kept in pure resin overnight (kept at 25ºC). Pure resin sample embedding was carried out in blocks which were incubated at 70°C for 3 days. Semi-thin leaf sections were cut, mounted on nickel grids and post-stained with Reynolds lead citrate (EMS) for 5 min, prior to TEM observation (Jeol 1010, Tokyo, Japan equipped with a CCD megaview camera) at 80 kV.

2.6. Mineral nutrition analysis

The concentration of the mineral nutrients in leaves was determined in the plants corresponding to control and SHA-foliar applied treatment for medium-term experiments (five replications with one plant per replicate, for the different treatments and harvest times). Leaf-samples (0.15 g dry sample) were subjected to acid digestion (8 mL of concentrated HNO_3 and 2 ml of H_2O_2) in a microwave (Thermo Scientific) at a controlled temperature of 200 ºC. Digested samples were then diluted with deionized water (dH2O) in 25 mL volumetric flasks, and the nutrient concentrations were measured by ICP-OES (iCAP 7400 DUO, Thermo Scientific).

2.7. Determination of IAA, ABA, cytokinins, SA, JA and JA-Ile in roots and leaves

SHA foliar treated cucumber plants (five replicates per treatment and harvest times, with one plant per replicate) were harvested in the same way that previously was described for DW measurement. Samples were frozen in liquid nitrogen at harvest and stored at -80ºC prior to analyses.

IAA, ABA, SA, JA and JAIle concentration analysis in plants tissues: The content of IAA, ABA, SA, JA, and JA-Ile in plant tissues was analysed by high-performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). These hormones were extracted and purified as described in Silva-Navas et al. (2019) from 0.25 g of ground frozen plant tissue (root or leaves), homogenized with 2.5 mL of precooled (-20ºC) methanol:water:HCOOH (90:9:1, v/v/v, with 2.5 mM Na-diethyldithiocarbamate) and 25 µL of a stock solution of 1000 ng ml^{-1} of deuterium-labelled internal standards in methanol. Samples were shaked in a Multi Reax shaker at room temperature for 60 min at 2000 rpm. Immediately afterwards, solids were separated by centrifugation at 20,000 g for 10 min using a Sigma 4-16K Centrifuge, and re-extracted with 1.25 mL of fresh extraction mixture by shaking for 20 min and subsequent centrifugation. Aliquots of 2 mL of the pooled supernatants were separated and evaporated in a RapidVap Evaporator operating at 40 ºC. The residue was re-dissolved in 500 µL of methanol/0.133% acetic acid (40:60, v/v) and centrifuged at 20,000 g for 10 min before the injection in the HPLC-ESI-HRMS system. Detail description of the quantification is reported in Silva-Navas et al. (2019).

Cytokinins concentration analysis in plant tissues: The endogenous content of the following cytokinins was analysed: trans- and cis-zeatin (tZ and cZ), dihydrozeatin (DHZ), trans- and cis-zeatin riboside (tZR and cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenosine (BAR), meta-topolin (mT), meta-topolin riboside (mTR), ortho-topolin (oT) and ortho-topolin riboside (oTR). Extraction process was carried out following the method described in Silva-Navas et al. (2019), using 0.25 g of frozen plant material (root or leaves) previously ground with liquid nitrogen. Sample homogenization was made with 4 mL of precooled (-20ºC) methanol-water-formic acid (15:4:1, v/v/v), and with 25 µL of a stock solution of 100 ng/mL of each deuterium-labelled standard (in methanol). An overnight extraction at -20ºC was carried out, after which solids were separated (20,000 g, 10 min, 4ºC) in a Sigma 4-16K Centrifuge. Then, they were re-extracted with 2 mL of extraction mixture and centrifuged again. Supernatants were passed through a Sep-Pak C18 cartridge (ref. WAT054945, Waters Co., Milford, MA) preconditioned with 2 mL of methanol and 2 mL of extraction medium. Afterwards, the eluted was evaporated near to dryness with a RapidVap Evaporator and the residue was re-dissolved in 2 mL of 1M formic acid. This solution was applied to an Oasis MCX column (ref. 186000254, Waters Co., Milford, MA) preconditioned with 2 mL of methanol and 2 mL of 1M formic acid. Column was washed with 2 mL of 1M formic acid, 2 mL of methanol and

2 mL of 0.35M NH4OH, applied in succession. Finally, cytokinins bases and ribosides were eluted with 2 mL of 0.35M NH4OH in 60% methanol (v/v). The eluted was evaporated to dryness in the RapidVap Evaporator and re-dissolved with 250 µL of methanol and 250 µL of 0.04% formic acid and centrifuged (20,000 g and 10 min) before injection in HPLC-ESI-HRMS system. Description of quantification and data processing was detailed in Silva-Navas et al. (2019).

Furthermore, the effects caused by root-applied SHA on the concentration of jasmonic acid (JA), jasmonoyl-isoleucine (JAIle) and salicylic acid (SA) were also evaluated from cucumber leaves and roots. The 10 days old cucumber plants were treated with 100 mg L^{-1} of SHA added to the nutrient solution. Plant growth conditions were similar to those described above for the experiments with SHA applied on the leaves. Each treatment consisted of five replications with one plant per replicate, for the different harvesting times.

2.8. Root PM H+-ATPase activity

The root PM H+-ATPase activity was analysed in cucumber plants treated with 40 and 100 mg L^{-1} of SHA applied on the leaves. Harvest times were 24, 48 and 72 h from the onset of the treatments. Each treatment for the different harvest times consisted in five replicates with one plant per replicate.

Plasma membrane vesicles were extracted from apical roots (2 g FW from 2 plants for each sample) using a sucrose-gradient technique previously described in Mora et al. 2010. Briefly, apical roots corresponding to different treatments were cut and ground in a mortar with a pestle in an ice cold extraction buffer containing: 250 mM sucrose, 10% (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EDTA, 2 mM DTT (dithiothreitol), 2 mM EGTA, 2 mM ATP, 1 mM PMFS, 20 mg mL-1 chymostatin, 5.7% (w/v) choline-iodine, and 25 mM BTP (1,3-bis [TRIS (hydroxymethyl) methylamino] propane) buffered to pH 6.7 with MES. The homogenate mix was filtered through four layers of sterile gauze and then centrifuged 3 min at 13,000 g and 4ºC (4K15 Sigma Laboratories centrifuges, Sartorius). The supernatant was conserved and centrifuged 25 min again under the same conditions. The pellets were recovered and resuspended in extraction buffer; this solution was loaded onto 1.5 mL tubes with the sucrose density gradient which consisted in 700 mL of 1.17 g/cm^3 sucrose over 300 mL 1.13 g/cm^3.

Sucrose solutions were prepared in 5 mM BTP-MES (pH 7.4) with all the protectants present in the extraction buffer. The gradients were centrifuged (Beckman Coulter Microfuge 22 R Centrifuge) for 1 h at 13,000 g, and the vesicles banding at the interface were collected, resuspended again in extraction buffer for cleaning the residuals of sucrose, and centrifuged for 30 min at 13.000g. The resulting pellets were resuspended in 0.5 mL of conservation buffer (20% glycerol; 5mM DTT; 0.5mM ATP; 50μg/ml chymostatin; 2mM EDTA; 2mM EGTA; 2mM BTP buffered with MES; pH 7.0). Finally, the PM vesicles were frozen with liquid N_2 and stored at -80ºC for enzyme activity measurements.

Enzyme activity was measured following the guidelines of ATPase /GTPase Assay Kit (DATG-200 kit, BioAssay Systems ATPase/GTPase – QuantiChromTM). The total protein measure was based on the Bradford assay. The measurements were acquired with a GENios spectrophotometer (TECAN).

2.9. Gene expression studies

The whole roots of harvested plants were immediately frozen in liquid N_2. Prior to RNA extraction, roots were ground to a fine powder using a 6870 Freezer/Mill (SPEX SamplePrep). Five independent RNA biological replicates were used per treatment and harvest time (corresponding to five roots per treatment, belonging to five different plants). Total RNA was extracted from 80 mg of powdered root tissue using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Diirefn, Germany) following the manufacturer's instructions. Genomic DNA contamination was avoided by including a digestion step with DNase. The Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA, United States) was used to assess the integrity and the concentration of the RNA in the extracts, using RNA StdSens Chips. First strand cDNA synthesis was performed with the iScript cDNA systnehsis kit (Bio-Rad, Hercules, CA, United States) in aliquots containing 1 μg RNA.

Real time-PCR was performed on 50 ng cDNA aliquots, using iQ SYBR Green supermix containing hotstart iTaq DNA polymerase, in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). Gene expression of 6 different plasma membrane H+-ATPAses expressed in roots was analysed: CsHA2, CsHA3, CsHA4, CsHA8, CsHA9, CsHA10 23, using the same primer sequences as in Olaetxea et al. 2019 (Supplementary information; Table S2). Relative normalized gene expression was calculated assigning roots from non-SHA treated plants as a control and using two different reference genes: CsTUA (α-tubulin) and CsCYCLO (cyclophilin).

2.10. Statistical analysis

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

3. Results

3.1. Foliar-applied SHA led to a significant shoot and root growth increases but did not induce changes in leaf nutrient concentrations.

In the short-term experiment, the leaves of cucumber plants were treated with three doses of SHA: 20, 30 and 40 mg of organic C L^{-1}. The production of root and shoot dry matter was measured after 72 h from the onset of treatments. The results obtained show that the concentration of 40 mgL^{-1} caused a significant increase in both shoot and root dry matter production, although the effect on the shoot was slight (Figure 1). In order to evaluate if these results might be more relevant with higher doses of SHA and for longer time responses, a medium-term experiment involving a higher dose of SHA (100 mg organic C L^{-1}) was performed. We selected this concentration of SHA because is the concentration that presented higher shoot- and root- DW production in experiments with SHA applied to the roots (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). In this case, plants were harvested after 3 days and 8 days from the onset of the foliar treatment. The results showed that foliar-applied SHA (100 mg L^{-1}) caused a significant increase in shoot dry matter production after 3 and 8 days from the onset of the treatment (Figure 2). However, in this case the effects on root dry matter were significant only after 8 days (Figure 2).

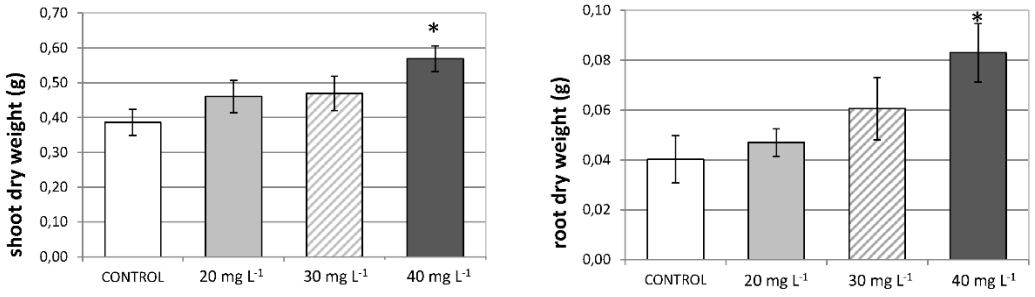

Figure 1. Effects of foliar application of different SHA doses (20, 30 and 40 mg organic C L-1) on the shoot- and root- dry weight (DW) of cucumber plants after 72 h from the onset of treatments. Significant differences (Anova test; $p \leq 0.05$) between treatments and control plants are indicated by an asterisk.

On the other hand, foliar-applied SHA (100 mg L^{-1}) did not affect the concentration of mineral nutrients in plant leaves after 3 or 8 days from the onset of the foliar treatment (Supplementary information, Figure S3).

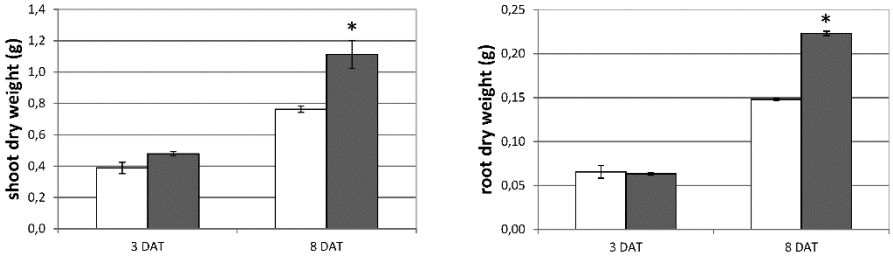

Figure 2. Effects of the foliar application of 100 mg organic C L^{-1} of SHA on the shoot- and root-dry weight (DW) of cucumber plants after 3 and 8 days from the onset of treatments. Significant differences (Anova test; $p \leq 0.05$) between treatments and the control are indicated by an asterisk. White bars: control; dark grey bars: SHA.

3.2. Foliar-applied SHA did lead to noticeable changes in root architecture

Images of the roots of cucumber plants corresponding to the control and foliar-applied SHA (40 mg L^{-1}) harvested 72h from the onset of treatments are presented in Figures 3 and 4. Clear effects on root architecture were observed upon SHA foliar-treatment. The qualitative analyses of the results indicated that the roots of control plants presented shorter principal roots but higher proportion of adventitious-lateral roots than plants treated with SHA, which had longer principal and secondary roots, higher volume and more dry matter production.

Figure 3. Whole root of cucumber control plants after 72 h from the onset of the treatments.

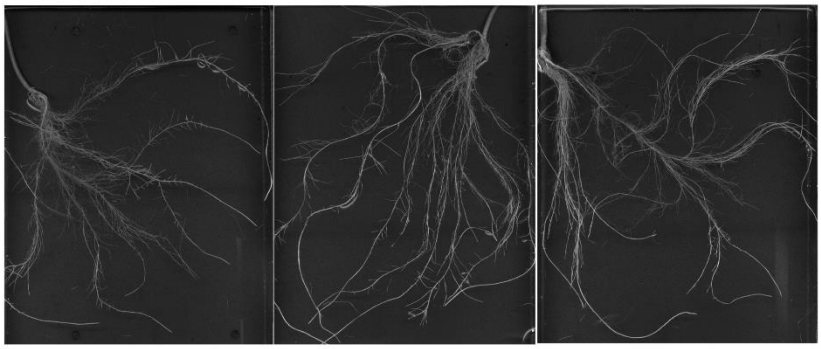

Figure 4. Whole root of 40 mg L^{-1} SHA-foliar treated cucumber plants after 72 h from the onset of the treatments.

3.3. Foliar-applied SHA increased IAA but not ABA root concentrations

Foliar-applied SHA at 40 mg L^{-1} caused a significant increase in IAA root concentration after 48 h from the onset of treatments (Figure 5A). This effect was accompanied by a concomitant increase in IAA concentration in the leaves (Figure 5A).

Foliar-applied SHA at 100 mg L^{-1} did not cause any significant increase in IAA concentration in both roots and leaves for the different sampling times between 3 and 8 days from the onset of the treatment (Figure 5B).

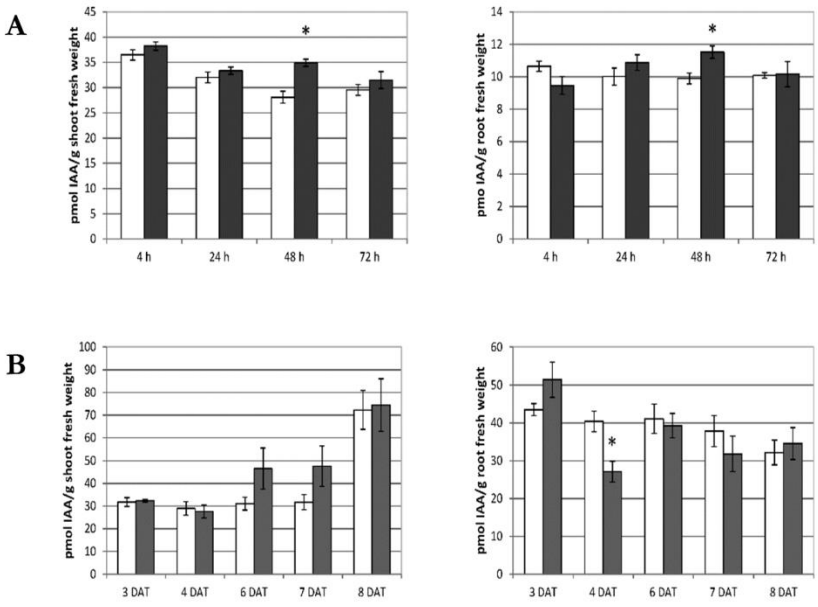

Figure 5. IAA concentration in shoots and roots of control and SHA foliar-treated plants. (A) 40 mg L^{-1} foliar-treated plants (white bars: control; dark grey bars: SHA); (B) 100 mg L^{-1} foliar-treated plants (white bars: control; light grey bars: SHA).

However, 40 mg L^{-1} foliar-applied SHA did not cause any significant increase in ABA concentration in both roots and leaves, but a slight decrease (Figure 6A). However, 100 mg L^{-1} foliar-applied SHA caused an increase after 6 days in roots that was accompanied with concomitant increases in the leaves after 7 and 8 days (Figure 6B).

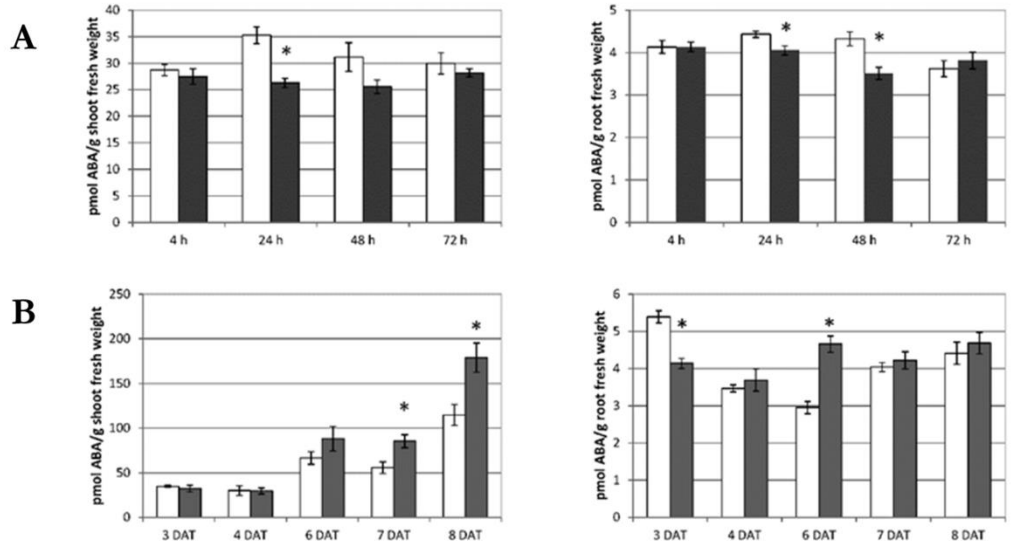

Figure 6. ABA concentration in shoots and roots of control and SHA foliar-treated plants. (A) 40 mg L-1 foliar-treated plants (white bars: control; dark grey bars: SHA); (B) 100 mg L-1 foliar-treated plants (white bars: control; light grey bars: SHA).

3.4. Foliar-applied SHA increased several cytokinins concentrations both in roots and leaves

40 mg L^{-1} of foliar-applied SHA caused an increase in the leaf concentrations of tZR after 72 h, cZ after 24 and 48 h, and iPR after 72 h (Figure 7A). In the case of tZ a slight increase was observed after 72 h that was not significant ($p = 0.13$) (Figure 7A). In the roots, foliar-applied SHA caused a significant increase in the concentration of iP after 4 h, iPR after 4 and 24 h, and cZ after 72h (Figure 7A). A slight increase in tZ after 4 h was also observed ($p = 0.09$) that was accompanied by a significant decrease after 72h.

The foliar application of 100 mg L^{-1} SHA caused significant increases in leaves of DHZR after 3 and 6 days, and iPR after 8 days (Figure 7B). Slight increases in cZR after 7 days ($p = 0.06$) and iP after 6 days ($p = 0.06$) were also observed (Figure 7B). No significant results were obtained in the roots (data not shown).

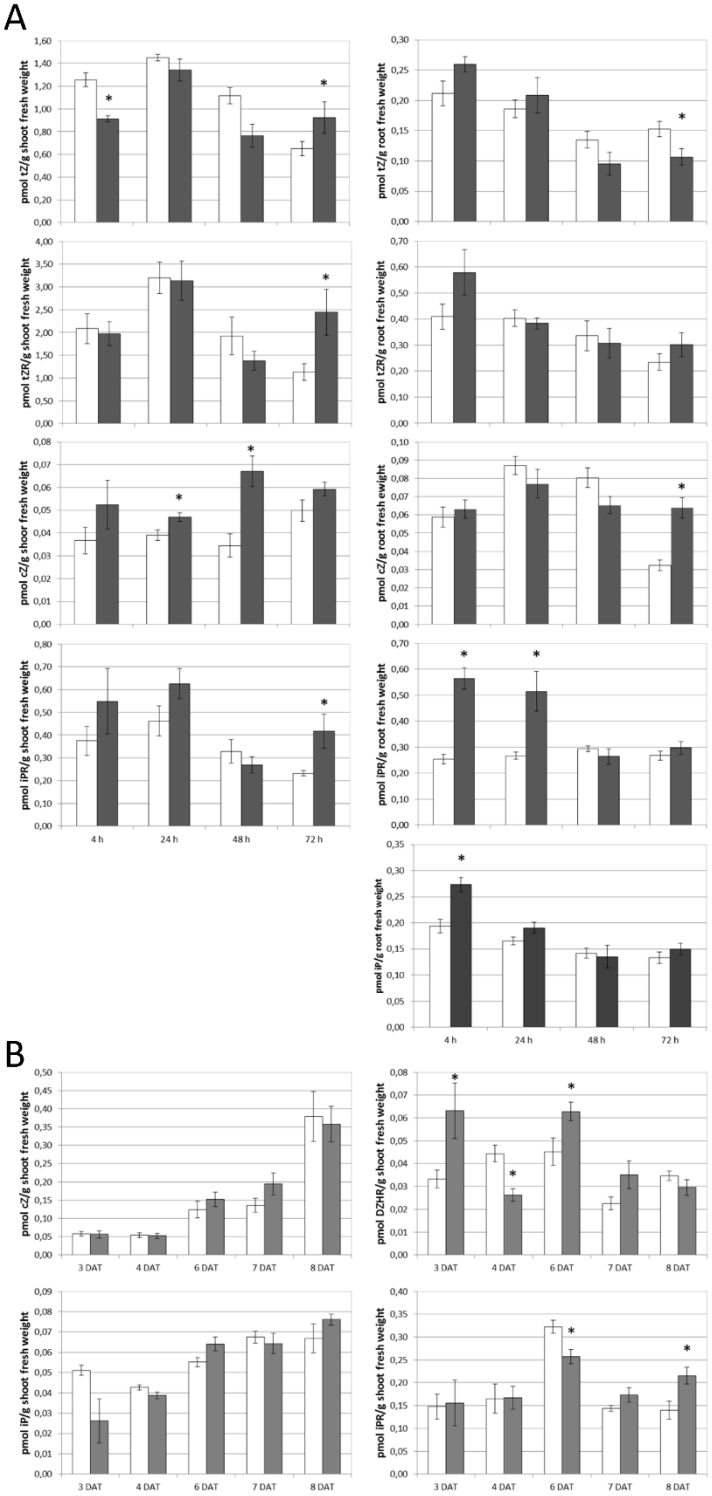

Figure 7. Cytokinin concentration in shoots and roots of control and SHA foliar-treated plants. (A) 40 mg L^{-1} foliar-treated plants (white bars: control; dark grey bars: SHA); (B) 100 mg L^{-1} foliar-treated plants (white bars: control; light grey bars: SHA).

3.5. Foliar-applied SHA did not induce short-term increases in either PM H^+-ATPase- activity or the expression of genes codifying for different isoforms of this enzyme in plant roots

The capacity of foliar-applied SHA at the two doses employed in the experiments (40 and 100 mg L^{-1}) to increase the activity of root PM H^+-ATPase activity was also studied. Neither foliar-applied SHA treatments induced a short-term increase in root PM H^+-ATPase activity (Figure 8). Likewise, 100 mg L^{-1} foliar-applied SHA did not affect the short-term expression of genes codifying PM H^+-ATPase in the root (Supplementary information, Figure S4).

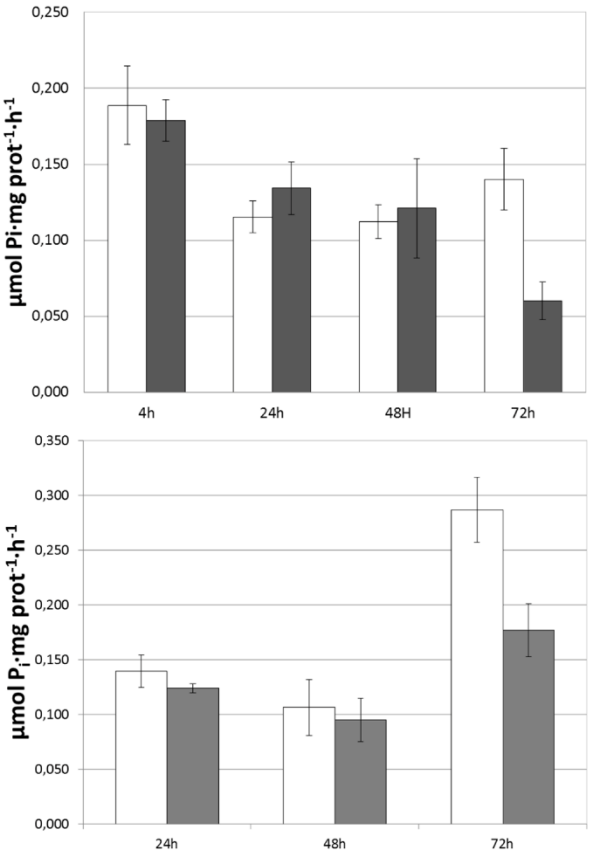

Figure 8. Root PM-ATPase activity of control and SHA foliar-treated plants. White bars: control; dark grey bars: 40 mg L^{-1} SHA, light grey bars: 100 mg L^{-1} SHA.

3.6. Foliar-applied SHA led to significant increases in the leaf- and root- SA and JA/JAIle concentrations

Considering that the deposition of SHA onto the leaves does not occur in nature and may present certain analogies with aggressions caused by external agents, the main plant hormones that are involved in the plant responses to this type of affection were also analysed in roots and leaves: SA, JA, and JA-Ile.

The results obtained show that 40 mg L^{-1} of foliar-applied SHA caused a significant increase in the root concentration of JA and JA-Ile after 72 h from the onset of treatments, whereas SA concentration was not affected (Figure 9A). In leaves, however, 40 mg L^{-1} of foliar-applied SHA caused an increase in JA after 72h and tended to increase SA concentration after 24 h ($p = 0.081$) and JA-Ile concentration after 4h ($p = 0.065$) (Figure 9A).

On the other hand, 100 mg L^{-1} of foliar-applied SHA increased the concentration of SA in leaves after 6 and 7 days and in roots after 7 days (Figure 9B). SHA foliar treatment also caused an increase in the root concentration of JA after 3, 6, 7 and 8 days from the onset of the treatment, and after 6 and 8 days in the leaves (Figure 9B). These effects were accompanied by concomitant increases in JA-Ile in the roots after 7 and 8 days, and a tendency to increase in the shoot after 6,7 and 8 days (Figure 9B).

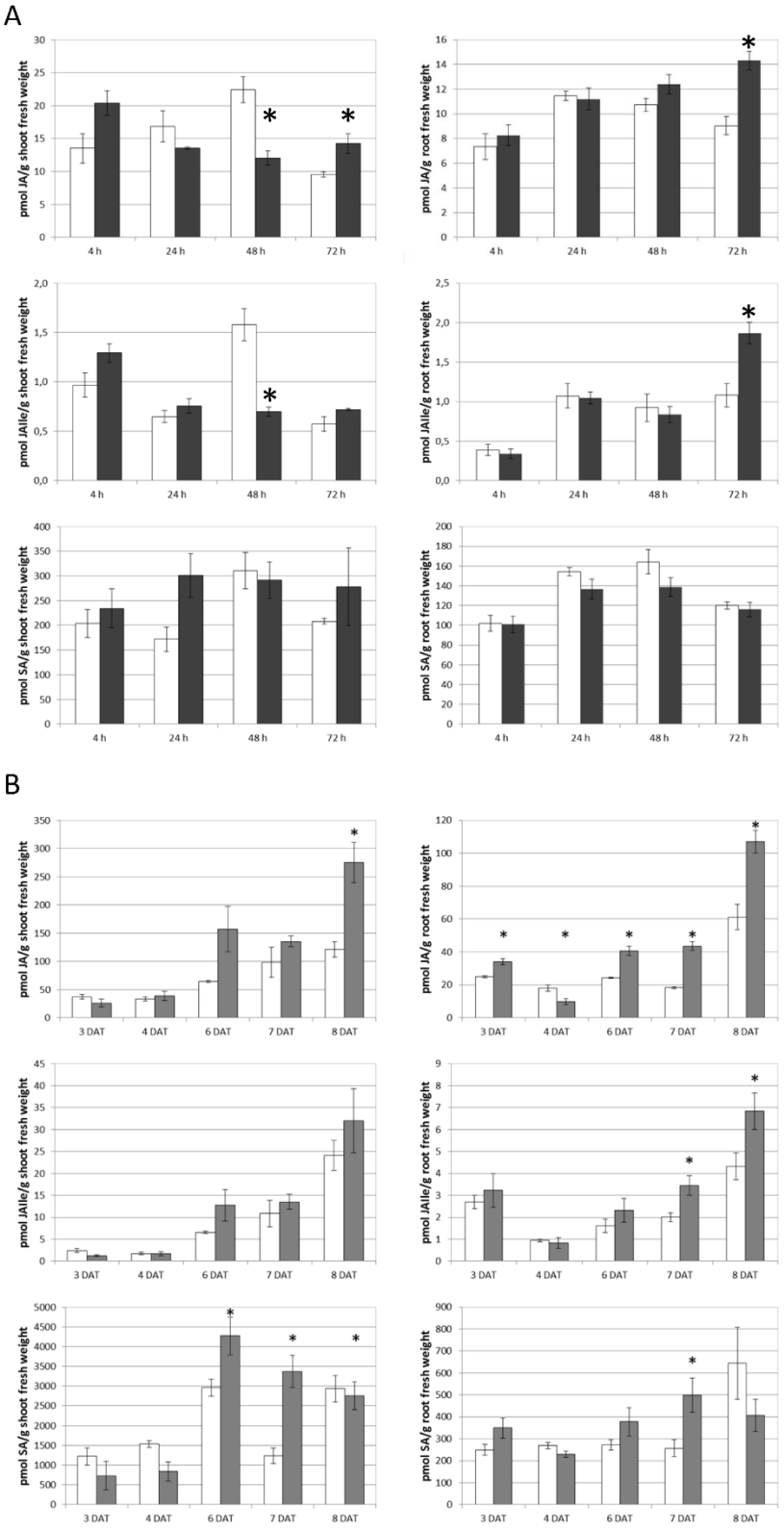

Figure 9. JA, JAIle, and SA concentration in shoots and roots of control and SHA foliar-treated plants. (A) 40 mg L^{-1} foliar-treated plants (white bars: control; dark grey bars: SHA); (B) 100 m g L^{-1} foliar-treated plants (white bars: control; light grey bars: SHA).

In order to compare these results with those corresponding to SHA-root application and considering that there are not experimental results regarding the effects of root-applied SHA on the concentration of SA, JA, and JA-Ile, the action of 100 mg L^{-1} root-applied SHA on the concentration of these plant hormones were also investigated in cucumber. The results obtained show that root-applied SHA (100 mg L^{-1}) did not have a significant effect on the leaf-concentration of SA and JA/JA-Ile for the considered sampling times (data not shown). However, a significant increase in both JA and JA-Ile was observed in the roots (Figure 10).

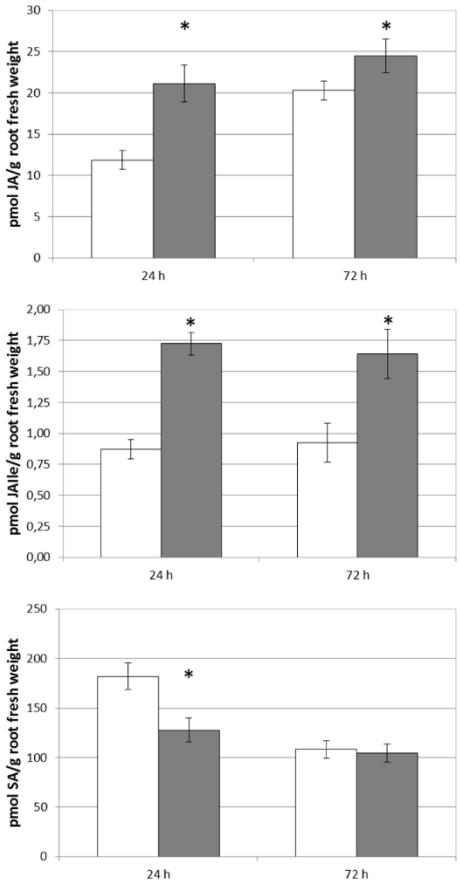

Figure 10. JA, JAIle, and SA concentration in the root of cucumber plants treated with 100 mg L^{-1} SHA applied to the roots (white bars: control; grey bars: SHA).

3.7. Foliar-applied SHA affected leaf surface structure, wax amounts and mesophyll cell starch

Images from both scanning (SEM) and transmission electron microscopy (TEM) revealed that the foliar application of 100 mgL^{-1} SHA affected some leaf structures, such as trichomes, cuticles, and starch granules. Images from SEM showed that the leaves of plants treated with 100 mg L^{-1} foliar-applied SHA have undergone a loss of trichomes in both adaxial and abaxial leaf sides, compared to control plants (Figure 11A-11D), whereas there were no differences in the number of stomata or in the proportion of open/closed stomata

(Figure 12A-12D. This result is in line with the values of stomatal conductance, which showed that there were no statistical differences between the stomatal conductance of control plant and 100 mgL^{-1} foliar-applied SHA treated plants (data not shown).

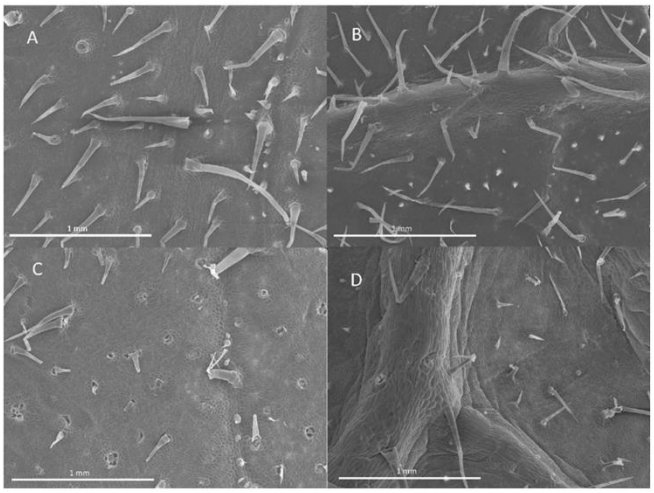

Figure 11. Scanning electron micrographs of cucumber leaf surfaces 7 days after foliar application: adaxial (A) and abaxial (C) leaf side of control leaves, adaxial (B) and abaxial (D) leaf side of 100 mg L^{-1} SHA-sprayed leaves.

The cuticles of leaves from plants treated with 100 mgL^{-1} foliar-applied SHA were thinner than those from control plants, as shown in TEM images (Figure 13 A,E,D,H), with a diminution in the concentration of soluble cuticular waxes (data not shown).

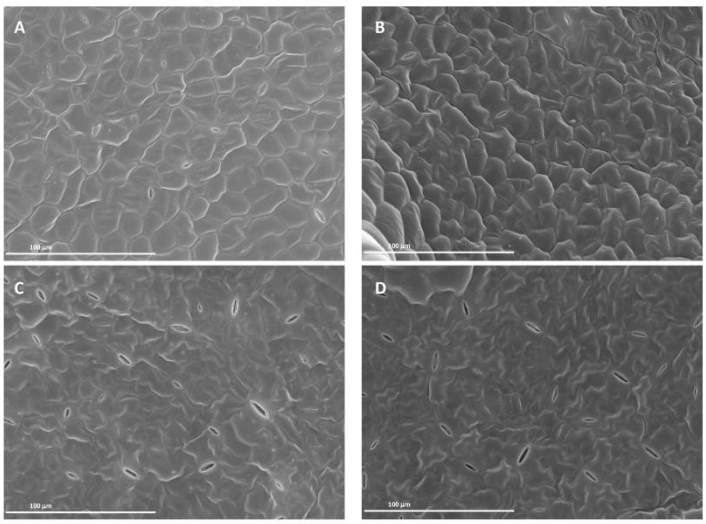

Figure 12. Scanning electron micrographs of cucumber leaf surfaces 7 days after foliar application: adaxial (A) and abaxial (C) leaf side of control leaves, adaxial (B) and abaxial (D) leaf side of 100 mg L^{-1} SHA-sprayed leaves.

The last noteworthy change caused by foliar SHA treatment was a diminution of the size of starch granules present in the chloroplasts, in comparison with non-treated leaves from control plants (Figure 13 C,G).

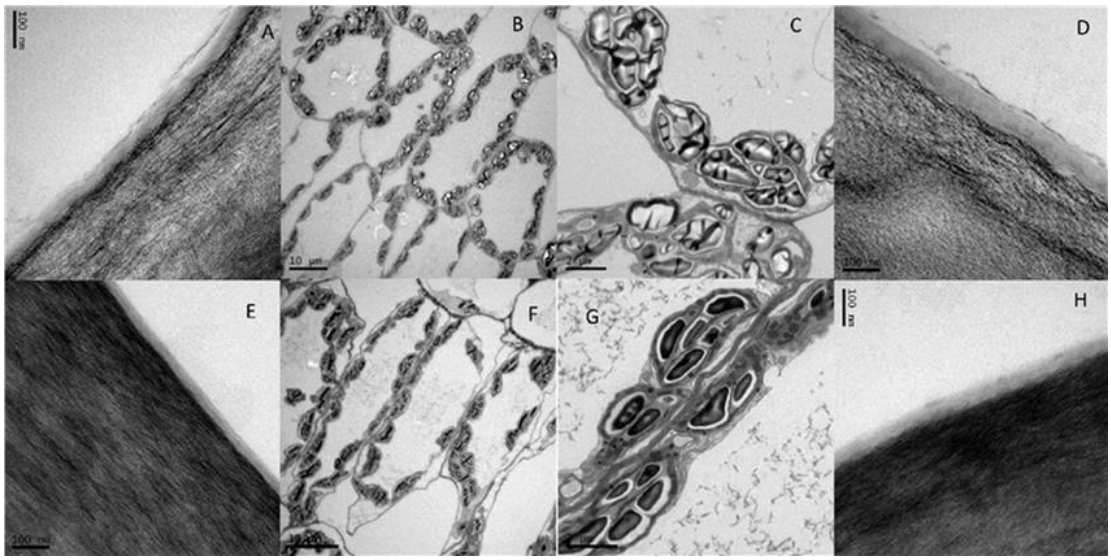

Figure 13. Transmission electron micrographs of control (A, B, C, D) and 100 mg L^{-1} SHA-sprayed (E, F, G, H) cucumber leaves, 7 days after foliar treatment. Images correspond to: (A, E) Adaxial cuticle, (B, F) palisade parenchyma cells, (C, G) detail of chloroplast containing starch in mesophyll cells, and (D, H) abaxial leaf cuticle.

4. Discussion

4.1. Different mechanisms underlay the plant growth promoting action of foliar- versus root-applied HA

In agreement with previous results on the application of HS to plant leaves (Rose et al., 2014; Canellas et al., 2015), foliar-applied SHA was found to promote significant increases in both shoot and root dry matter for the different SHA concentrations employed (40 and 100 mgL^{-1}) (Figures 1-2). These effects were observed at short term for both doses (72 h) and at medium term for the higher dose (8 days) (Figures 1-2). These results are in line with the results obtained with root-applied SHA (100 mg L^{-1}) using the same experimental model and experimental conditions (Aguirre et al., 2009; Mora et al., 2010, 2012, 2014; Olaetxea et al., 2015, 2019). In principle, these results indicate that SHA is able to promote plant growth regardless the mode of application. However, this fact does not mean that the mechanisms of action underlying these effects are similar to each other.

In fact, some differences were observed regarding the effects on root morphology and architecture. Many studies have reported the capacity of root-applied HA to promote the proliferation of adventitious-lateral roots (Nardi et al., 2002; Canellas et al., 2012; García et al., 2016; Olaetxea et al., 2018). In the case of our experimental model, Mora et al. (2012) reported that root-applied SHA promoted the number of lateral roots as well as root growth in short-term experiments. However, the short-term response to foliar-applied SHA showed that SHA tended to reduce the presence of adventitious-lateral roots with respect to control plants (Figures 3-4). However, SHA foliar treatment increased principal and secondary root length and root dry weight with respect to the control (Figures 3-4). This fact might be explained by the different effect of foliar-applied SHA and root-applied SHA on the concentration in roots of two phytoregulators related to the regulation of root growth and architecture: IAA and ABA. Several studies have shown that the capacity of root-applied HA to enhance lateral root proliferation appears to be mediated by auxin signalling pathways (Nardi et al., 2002; Canellas et al., 2015; Olaetxea et al., 2018). Studies in our experimental model showed that SHA applied to the roots increased the root concentration of IAA and ABA (Mora et al., 2012; Olaetxea et al., 2015). However, whereas inhibitors of IAA biosynthesis and action did not prevent the SHA-mediated increase in root growth (Mora et al., 2012), the inhibition of ABA biosynthesis prevented this SHA effect on root growth (Olaetxea et al., 2019). These results suggest that, at least in our experimental model, the role of ABA in the mechanisms underlying the action of root-applied SHA on root development appears to be more relevant than that of IAA. This hypothesis is supported by the lack of effects of foliar-applied SHA on lateral root proliferation, because this treatment increased IAA but not ABA root concentrations (Figures 5-6).

Therefore, the short-term effects of SHA applied to the leaves and those for SHA applied to the roots show different patterns regarding root development and architecture.

Previous studies using the same experimental model reported that the improvement in shoot growth associated with root-applied SHA was linked to the increase in IAA caused by SHA in the roots (Mora et al., 2014). SHA applied to the leaves also caused an increase in the concentration of IAA in roots (Figure 5), thus suggesting that IAA could play a relevant role in the shoot growth promotion resulting from foliar-applied SHA.

Further studies also involving a similar experimental model, showed that root PM H^+-ATPase activity played a crucial role in the shoot growth-promoting action of root-applied SHA (Olaetxea et al., 2019). In fact, the use of inhibitors of the activity of this enzyme

prevented the increase in shoot growth mediated by SHA applied to roots (Olaetxea et al., 2019). It is therefore plausible that this enzyme may also be involved in the increase in shoot growth caused by foliar-applied SHA. However, the results obtained in experiments including the two doses of foliar-applied SHA (40 and 100 mg L^{-1}) associated with short-term increases in shoot growth, did not show any noticeable short-term effect on root PM-H^+-ATPase activity (Figure 8). Therefore, although a medium- and/or long- term stimulation of root PM H^+-ATPase activity resulting from foliar-applied SHA cannot be ruled out, this action would not explain the short-term enhancement of shoot growth promoted by foliar SHA application (Figures 1-2). In addition, the lack of effects of foliar-applied SHA on the root PM H^+-ATPase activity may explain why foliar applied-SHA (100 mg L^{-1}) did not change the leaf concentration of the nutrients analysed (Supplementary information, Figure S3) since this enzyme is directly involved in root nutrient uptake (Olaetxea et al., 2018).

Another event that played a relevant role in the mechanism underlying the shoot-growth promoting action of root-applied SHA in our experimental model was a short-term increase in the concentration of some cytokinins in the leaves and roots (Mora et al., 2010). In the case of foliar-applied SHA we also observed an increase in the root and shoot concentration of several cytokinins (Figure 7). This fact is in line with the enhancement of shoot growth in foliar SHA treated plants. In the case of root-applied SHA the effect of cytokinin concentration in leaves was mediated by the stimulation in root-PM H^+-ATPase activity (Olaetxea et al., 2019). Nevertheless, for foliar-applied SHA this mechanism does not appear to be involved in the regulation of this process since this treatment did not have any short-term effect on root-PM H^+-ATPase activity.

In our experimental model, root ABA also played an important role in the promotion of shoot growth after root SHA application (Olaetxea et al., 2015, 2019). However, foliar-applied SHA did not modify ABA concentrations in roots (Figure 6), thus suggesting that this event is not involved in its effect on shoot growth. Therefore, in addition to IAA, other signaling pathways different from root PM- H^+-ATPase and root ABA must be involved in the shoot growth promoting action of foliar-applied SHA and the increase in cytokinin leaf concentration resulting from this treatment.

4.2. HA applied on the leaves, but also to the roots, affects SA and JA signalling pathways

As described in the introduction, the interaction of HA with leaf surfaces does not occur in nature and can be sensed by plants as an external aggression. In such case, plants normally activate SA and JA/JA-Ile signalling pathways as a defensive and adaptive response

(Wasternack and Hause, 2013; Nazar et al., 2017). It is therefore plausible that foliar-applied SHA may activate these signalling pathways. In this framework, the results obtained regarding the root- and leaf- concentration of SA and JA/JA-Ile are very relevant. Our results confirm this hypothesis since SHA applied to leaves clearly affected the concentration in roots and leaves of JA, and JA-Ile that is the active form of the hormone (Figure 9). At short-term, the results in leaves showed that SHA (40 mg L-1) tended to increase SA JA and JA-Ile, whereas in roots both JA and JA-Ile increased after 72 h (Figure 9A). These results were clearer in medium-term experiments with a higher dose of SHA (100 mg L^{-1}), which showed increases in SA and JA/JA-Ile in both leaves and roots for specific sampling times (Figure 9B). Therefore, our findings are compatible with a role of SA and/or JA signalling pathways in the mechanisms underlying the plant growth promoting effect of foliar-applied SHA.

In principle, these results suggest that foliar-applied SHA may cause some damage at a leaf surface level. Analysis of foliar-treated SHA leaves by SEM and TEM some anatomical changes associated with SHA supply. On the one hand, SHA treatment decreased trichome densities (Figure 11). Some studies carried out with *Arabidopsis* showed that whereas wounding and JA increased the number of trichomes, SA decreased it (Traw and Bergelson, 2003). In our case, both JA and SA increased upon SHA foliar treatment. However, regarding trichome development the role of SA appears to be predominant.

Other studies showed that ABA likely modified cuticle composition and structure, increasing its firmness and decreasing its permeability (Ziv et al., 2018). In our experiments, we observed and increased in ABA concentrations in shoots treated with foliar-applied SHA (100 mg L^{-1}) (Figure 6B). Transmission electron micrographs of leaves treated with SHA showed changes in the cuticle that appeared thinner but denser than in control leaves (Figure 12). Significant decreases in the concentration of soluble cuticular waxes were also determined after SHA foliar application. These structural and chemical changes may be associated with a reaction between SHA and at least cuticular waxes forming a stable polymer, with these lipids being incorporated into the supramolecular conformation of SHA. However, further studies are needed in order to elucidate the potential effect of foliar-applied SHA on cuticle structure, chemical composition and barrier properties.

A further interesting finding was the decrease in leaf mesophyll starch accumulation in the chloroplasts upon SHA foliar application (Figure 12). This effect was unexpected since the application of HA to plant roots is associated with an increase in chloroplast starch accumulation (Jannin et al., 2012). This effect may be potentially linked to a mobilization of

carbohydrates associated with higher metabolic activity and regulated by cytokinin activity. However, the effect o foliar SHA supply on leaf starch concentrations should be studied in future investigations.

In order to compare the effects of SHA foliar application on JA, JA-Ile and SA compared to root-applied SHA, we carried out a new experiment exploring the action of SHA applied to the roots on the concentration of these hormones. This experiment was performed using the same experimental model and under similar experimental conditions to those used in foliar SHA application and preliminary root SHA supply trials (Olaetxea et al., 2019). Surprisingly, SHA root application led to significant short-term increases in the root concentration of both JA and JA-Ile, whereas no clear effects were observed in leaves (Figure 10). No effects on SA concentration in roots and leaves were observed (Figure 10). As in the case of foliar-applied SHA, these results are consistent with some potential involvement of JA signalling pathway in the whole mechanism of action of root-applied SHA on plant growth.

Regarding the potential roles that could play SA and JA in the mechanisms responsible for the plant growth-promoting action of SHA applied on either roots or leaves, several studies reported negative cross-talk between the SA and JA in the regulation of several processes related to plant development, such as plant defense mechanisms and root development (Traw and Bergelson, 2003). It is therefore complicated to discuss the simultaneous role of both SA and JA in the regulation of the same process. It is well known that SA is generally involved in the regulation of plant responses to biotrophic and hemi-biotrophic pathogens, whereas JA is involved in plant responses to necrotrophic pathogens and herbivorous (Wasternack and Hause, 2013; Nazar et al., 2017).

Some studies described that the application of low concentrations of SA increased root growth and root dry matter production (Deef et al. 2007). Conversely, several studies reported that JA inhibited plant growth but promoted lateral root formation (Wasternack and Hause, 2013). In our experiments with foliar-applied SHA, we observed short-term increases in JA and JA-Ile root concentrations that were not accompanied by a reduction in root growth or increases in lateral root formation (Figures. 9A and 3,4 respectively). On the contrary, we observed an increase in root dry matter production and a reduction in adventitious-lateral root formation (Figures. 3, 4). This fact suggests that JA signalling pathways do not play a dominant role in short-term effects of foliar-applied SHA on root development. As discussed above, the increase in root IAA concentration upon SHA foliar application suggests a dominant role of IAA in the regulation of root development in this

specific case. Likewise, these results also suggest that these processes might be regulated by the ratios, the relative proportion, between specific hormones involved in root development regulation such as IAA, ABA, cytokinins, SA and JA. Regarding root-applied SHA, the results obtained are compatible with a relevant role of JA in the SHA mediated effects on lateral root production but not regarding the increase in root growth, which appears to be principally linked to root ABA (Olaetxea et al 2015, 2019).

Finally, the effects of foliar-applied SHA on SA and JA signalling pathways are compatible with the induction of higher resistance of treated plants against eventual pathogen attacks. In any case, it is concluded that more research is required to elucidate the role of JA and SA in the whole mechanism underlying the beneficial action of HA on plant development and, eventually, plant defense against pathogens.

Furthermore, it is highly likely that additional biochemical and molecular processes may also be involved in the long-term response of plants sprayed with HS. However, in light our findings, the short-term reaction of plants to HS application has great influence in the whole action of HS during the entire growing cycle (Olaetxea et al., 2018).

5. Conclusion

In summary, the results obtained are compatible with the hypothesis that the beneficial action of foliar-applied SHA or root-applied SHA on plant growth may result from molecular and biochemical events triggered by a transient mild stress associated with SHA application (Figure 14).

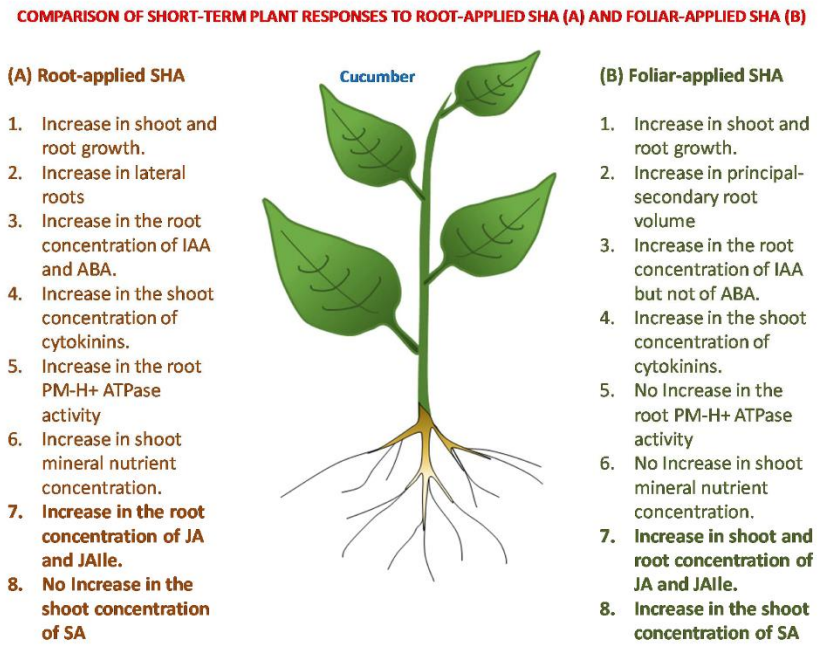

Figure 14. Comparison of some short-term responses on cucumber plants to root-applied SHA and foliar-applied SHA. In bold the new markers added in this study to the humic acids plant growth promotion mechanism.

6. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

The study was conceptualized and designed by DDH, MF, VF, and JMGM. Experimental work was performed by DDH, MF, and MO. Hormone detection was assessed by AZ. Microscopy studies were performed by VF. Data analysis was carried out by DDH, MF, and JMGM. Manuscript draft was prepared by JMGM, DDH, MF, VF, and AZM.

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9. Acknowledgements

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10. Data Availability Statement

All datasets generated in this study are included in the manuscript and the supplementary files.

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CHAPTER II

Sedimentary humic substances application strategies modify differentially the cucumber plants endophytic microbiome

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In preparation

CHAPTER III

Culturable bacterial endophytes from sedimentary humic acid-treated plants: Potential use in plant growth promotion

De Hita, D; Fuentes, M; Zamarreño, AM; Ruiz, Y & García-Mina, JM

Frontiers in Plant Science (Under review)

Abstract

The global decrease in soil fertility leads to a new agricultural scenario where eco-friendly solutions play an important role. The plant growth promotion through the use of microbes, especially endophytes and rhizosphere microbiota, has been proposed as a useful solution. The main plant growth promoting features of these microorganisms suggest an effect in plant fitness similar to the effect produced by humic substances. In this work, a sedimentary humic acid from leonardite has been used to promote the growth of cucumber plants grown in a hydroponic system, and culturable endophytic bacteria have been isolated from these plants. The pre-treatment with this sort of humic substances has been suggested to modify certain endophytic taxa; because of that, it is believed that it may exist a complementarity between endophytes and humic substances actions.

The culturable endophytes that have been isolated from humic acid-treated cucumber plants have been identified as members of four main phyla: *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. Isolates were characterized according to the following plant growth promoting traits: nitrogen fixation/scavenging, phosphate solubilization, siderophore production and plant hormone production. Most of the isolates were able to fix/scavenge nitrogen and to produce plant hormones (indole-3-acetic acid and several cytokinins), whereas only few isolates were able to solubilize phosphate and/or produce siderophores. The most promising endophyte isolates for future use as plant growth promoting bacterial inocula were *Pseudomonas sp.* strains (that showed all traits); *Sphingomonas sp.*, *Stenotrophomonas sp.* strains, or some *Arthrobacter sp.* and *Microbacterium sp.* isolates could potentially be good plant growth promoters. These results suggest that a combined use of humic substances and endophytes could improve plant growth due to the increase in mineral nutrient acquisition and the stimulation of plant growth with hormonal regulators.

Keywords

Endophyte, plant growth promotion, microbiota, humic, phosphate solubilizing bacteria, cucumber

1. Introduction

In the last decades the human population has grown exponentially, reaching 7,600 millions of people in 2018, and as the Food and Agriculture Organization (FAO) has predicted, in 2050 the world population will be near to 10,000 million[3]. This fact involves an increasing pressure over global food production and the land surface dedicated to that purpose. However, approximately 35% of world surface is already dedicated to crop production, according to FAO database[4], and increasing the crop land surface is not an ecologically valid solution, being in fact very controversial in most of developing countries where population demands new eco-friendly politics. Only increasing crop yields appears as a possible solution to prevent food shortage in this future scenario, although the excessive use of NPK chemical fertilizers is already negatively affecting soil fertility, soil microbial activity, and may cause the pollution or/and eutrophication of water reservoirs (Torrent et al., 2007; Ulén et al., 2007; Youssef and Eissa, 2014).

Therefore, more rational, environmentally-friendly, and efficient agricultural practices are needed. One approach is the use of biofertilizers containing living microorganisms (Dastager et al., 2010; Bhardwaj et al., 2014; Canellas et al., 2015; Suhag, 2016; Pérez et al., 2016; Dias et al., 2017). This strategy has recently gained relevance with the development of a new generation of gene sequencing techniques which have allowed the assessment of microbe-plant relationships and the development of a new evolutionary model, the holobiontic theory (Rosenberg and Zilber-Rosenberg, 2016). This model proposes that microbiota would evolve over time to improve the fitness of the plant under changing environmental conditions such as drought, salinity, nutrient deficiency or soil contamination (Murphy et al., 2015; Fidalgo et al., 2016; Soussi et al., 2016; Kumar et al., 2017).

Among the different kinds of biofertilizers, those including plant growth promoting rhizobacteria (PGPR) are frequent (Mahaffee and Kloepper, 1997; Bhattacharyya and Jha, 2012; Sarathambal et al., 2015; Kumar et al., 2017; Gouda et al., 2018). The main effect of PGPR in plants is the improvement of both nutrient availability in the rhizosphere and the plant resistance to biotic/abiotic stresses (Gouda et al., 2018). However, the application of these microorganisms has several efficiency limitations when applied to the soil under field conditions due to the competition with

[3] http://www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf
[4] http://www.fao.org/faostat/en/3data

native soil microbiota and their low survival rate. These facts cause the poor reproducibility of the agronomical results of PGPR-based treatments in field crops (de Oliveira et al., 2006). Recent studies have shown that a promising approach to overcome all these limitations in efficiency might be the application of PGPR directly on the leaves (Canellas et al., 2013; 2015; Olivares et al., 2017).

In contrast with PGPR, the endophytic microbiota has been only recently explored as a potential source of beneficial microorganisms for improving plant growth (Brader et al., 2013; Sessitsch et al., 2019). Endophytes are those microorganisms inhabiting inner plant tissues (Hallmann et al., 1997). Their main origin is the rhizosphere so that they share the same advantages as those of PGPR but showing special characteristics that may overcome some of the limitations associated with the use of PGPR even when applied to the leaves. Endophytes are well adapted to living within the plant, thus favoring in some way their efficacy as plant growth promoters (Reiter and Sessitsch, 2006; Hardoim et al., 2008; Compant et al., 2010). Indeed, endophytes have evolved to transmit themselves to the next plant generation through seed colonization (Johnston-Monje and Raizada, 2011; Truyens et al., 2015; Nelson, 2018). In fact, this trait would justify the use of PGP endophytes instead of PGPR inoculums: the evolutionary selection of endophytes and the capability of being inherited through plant generations provide them with high biocompatibility with plant tissues thus increasing their possibilities to help plants to growth under normal conditions or under stress conditions (López et al., 2018).

On the other hand, several studies have shown the compatibility and synergetic beneficial action on plant growth of PGPR and humic substances (HS) when applied together (Olivares et al., 2017 and references therein). HS are a specific fraction of soil organic matter that can be extracted using alkaline solutions (Stevenson, 1994) and have been proven to promote the plant development by increasing nutrient availability in soils and activating plant metabolism (Mora et al., 2010; 2013; Zandonadi et al., 2013; García et al., 2014; Olaetxea et al., 2018; de Hita et al., 2019; Zanin et al., 2019).

Recently, a study carried out in our laboratory showed that the application of a sedimentary humic acid (SHA) extracted from leonardite was able to modify the population and distribution of a number of endophyte microorganism families in cucumber (de Hita et al., 2018). This study showed that although SHA treatment was not particularly accompanied by the presence of new families of microorganisms, the relative

proportion between them varied. However, taking into account that many of the endophytes found in both control and SHA-treated plants are potentially able to synthetize or promote the synthesis of phytoregulators that play relevant roles in the mechanism of action of SHA on plant growth such as indole-3-acetic acid (IAA) or cytokinins (CKs) (Olaetxea et al. 2018), it was proposed that endophytic microbiome might be involved in some way in the plant growth promoting action of SHA (de Hita et al., 2018). Besides that, although endophytes and SHA may act separately within the plant promoting its growth, the two types of action show potential complementarity to each other. In fact, the increase in plant growth observed upon SHA application could also include the possible positive action of endophytes.

In order to explore this hypothesis, we have studied here the main plant growth enhancing traits of culturable bacteria endophytes isolated from cucumber plants treated with SHA. We compare these traits with the main key effects involved in the SHA promoting action of the growth of cucumber plants and discuss the possibility of certain interrelation between the biostimulant effect of SHA and the plant growth promoting effects of culturable endophytes. This work will allow us to isolate and identify promising candidates for future applications as enhancers of plant growth alone or in combination with HS.

2. Material and methods

2.1. Plant material and growth conditions

Cucumber seeds (*Cucumis sativus* L. var. Ashley) were sown in bed of sterile perlite and wet filter paper, and placed in a germination chamber in darkness, at 25ºC, and 75% relative humidity. One week later the seedlings were transferred to a hydroponic system in a growth chamber whose day/night conditions were: 16h/9h (irradiance of 250 μmol m^{-2} s^{-1}), 25ºC/21ºC and 70%/75% relative humidity. The nutrient solution utilized was previously described in Mora et al. (2010) and Olaetxea et al. (2015), with minimum changes in the final concentration of Fe-EDDHA and $MnSO_4$ (80 μM and 27.3 μM, respectively). After ten days, plants were treated with a 100 mg L^{-1} C of a sedimentary humic acid (SHA) obtained from leonardite as described in Mora et al. (2010) and characterized in Aguirre et al. (2009). The treatment was applied directly to the nutrient solution two hours after the start of the diurnal period. Plants were harvested seven days from the onset of the treatments.

2.2. Plant surface sterilization and bacterial endophyte isolation

SHA-treated cucumber plants were surface-sterilized prior to the isolation of bacterial endophytes. Firstly, three different cucumber plants were rinsed, separately, with autoclaved deionized water (dH_2O) to wash the nutrient solution from the roots. Then, cucumber plants were rinsed with commercial bleach (<5% sodium hypochlorite) containing 0.1% Tween 20 for 3 min. The next steps consisted of three consecutive washes with autoclaved dH_2O for 5 min each one and stirring in an orbital shaker. Finally, roots, stem, and leaves were separated with a sterile scalpel and frozen with liquid N_2 for later use. To verify the surface sterilization, 1 mL of the last wash was plated and cultured on R2A agar medium. Plates were incubated at 27ºC for three days. No growth was detected for any plant. All sterilization steps were carried out in a laminar flow cabinet in sterile conditions.

The plant organs were ground with sterile mortar and pestle using autoclaved peptone water (0.9 mL per tissue gram) to recover the microorganisms. The liquid was filtered through sterile gauze to eliminate plant debris. This filtrate was used (100 µL) for microbial culturing ten-fold serial dilutions in autoclaved peptone water (10^0-10^{-4}). One milliliter was plated, by the pouring plate method, in R2A agar. Plates were incubated for seven days at 27ºC. Morphologically single colonies from each plate were selected, picked, streaked, and re-streaked on new R2A agar plates to obtain axenic cultures of each isolate. Finally, each pure culture was inoculated in LB broth and incubated for 20 h-72 h, at 27ºC and 160 rpm in a microbiological incubator; then bacterial stocks in 25% glycerol were prepared and conserved at -80ºC. Only those isolates reaching a minimum optical density (OD) of 0.6 at 600 nm were considered viable for further analysis. A total of 72 isolates were successfully grown and conserved in glycerol stocks.

2.3. Isolate identification

Partial PCR amplification of the 16S rRNA gene was performed directly from the glycerol stocks, using the universal primers F799 (5'-AACMGGATTAGATACCCKG-3'), and 1492R (5'-AAGGAGGTGATCCANCCRCA-3') (Chelius and Triplett, 2001; Hogg and Lehane, 1999). The PCR mix contained: 1 µL of 10 µM F799 primer, 1 µL of 10 µM 1492R primer, 2.5 µL of bacterial glycerol stock, and 10.5 µL Premix Ex Taq RR003A. The PCR was performed in a iCycler iQ thermocycler, with the following protocol: an initial denaturation step at 98ºC for 1 min; 30 PCR cycles at 98ºC for 10 s, 57ºC for 30 s, 72ºC for 1 min; and a final extension at 72ºC for 5 min. PCR products

were purified with the NucleoSpin Gel and PCR Clean-up kit from Machery-Nagel, following the manufacturer guidelines.

The DNA concentration in each purified PCR product was measured in a Nanodrop ND-1000 spectrophotometer. Capillary sequencing was carried out by CIMA Lab Diagnostics. Sequencing reads were searched against RDP SeqMatch (Cole et al. 2014) and BLASTn databases using default parameters. Only those taxonomical assignments identical in both databases were considered. If this was not possible, BLASTn[5] taxonomical assignment prevailed. The reference sequences selected belonged to GenBank 16S partial sequences and were used for building the phylogenetic tree. The reference sequences and the sequences of the isolates were aligned by Clustal Omega web service[6] (Madeira et al. 2019) with default parameters except for the number of combined iterations, max guide tree iterations, and max HMM iterations, that were shifted from default to 5 in all of them. The alignment tree distances resulting from Clustal Omega were used as basic data to create the circular cladogram tree in iTOL[7] (Letunic and Bork 2019).

2.4. Bacterial endophytes characterization

2.4.1 Growth on nitrogen-free medium

The endophyte isolates were tested for their capability to fix or scavenge nitrogen using NFC medium (10 g/L mannitol, 0.2 g/L $MgSO_4.7H_2O$, 0.2 g/L KH_2PO_4, 0.2 g/L NaCl, 0.2 g/L $CaSO_4.2H_2O$, 5 g/L $CaCO_3$, 15 g/L European bacteriological agar; pH 7.2), based in Ashby's mannitol agar (Liu et al., 2016; Li et al., 2018). Microorganisms were picked from the glycerol stocks (10 μL), streaked on NFC medium, and incubated at 30ºC for seven days. Those plates with positive growth were re-streaked over fresh NFC plates twice (each seven days). Only the plates with consistent bacterial growth at the end of the process (after 21 days) were considered positive isolates for nitrogen-free medium growth trait. An *Azotobacter vinelandii* DSMZ 85 strain was used as a positive control microorganism.

2.4.2. Inorganic phosphate solubilization

For the detection of mineral phosphate solubilizer microorganisms, NBRIP agar was used as the culture medium: 10 g/L glucose, 5 g/L $Ca_3(PO_4)_2$, 5 g/L $MgCl_2.6H_2O$, 0.25 g/L $MgSO_4.7H_2O$, 0.2 g/L KCl, 0.1 g/L $(NH_4)_2SO_4$, 15 g/L European

[5] https://blast.ncbi.nih.gov
[6] EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/clattalo
[7] Interactive Tree of Life, https://itol.embl.de

bacteriological agar, pH 7-7.2 (Nautiyal, 1999; Truyens et al., 2013). Glucose was dissolved in a small volume of sterilized dH_2O, filter-sterilized (0.45 μm), and then added to the sterilized medium.

Each isolate was tested in two different plates, placing in each of them five 10 μL-drops from the corresponding bacterial glycerol stock. After seven days at 27ºC in darkness, clear halos around positive isolates were measured. These isolates were classified as fast solubilizers. Seven days later, solubilization halos were measured again, and those isolates with new clear halos were classified as slow solubilizers. The IPS ratio (Inorganic Phosphate Solubilization ratio) between the halo diameter and the colony diameter was also used as a classification parameter (Batista et al., 2018). A *Bacillus megaterium* var. *phosphaticum* DSMZ 3228 strain was used as a positive control for inorganic phosphate solubilization.

2.4.3. Siderophore production

Isolates were tested as siderophore producers with the CAS-agar protocol developed by Schwyn and Neilands (1987) and modified by Cordero et al. (2012). Firstly, all the PIREX glasswares were deferrated, rinsing with 10% HCl (vol/vol) overnight and five consecutive washes with dH_2O. Then, the CAS-Fe-HDTMA dye was prepared (1 L): 10 mL $FeCl_3$ 10mM dissolved previously in 100 mM HCl, 590 mL 1 mM Chrome azurol sulfonate, and 400 mL 2 mM HDTMA. The solution was autoclaved (25 min, 121ºC) in an opaque PIREX bottle and stored at room temperature. After that, the CAS-agar was prepared, containing 30.24 g PIPES, 1 g/L NH_4Cl, 3 g/L KH_2PO_4, 20 g/L NaCl, adjusting at a final pH of 6.8, and finally adding 9 g/L agar noble. After autoclaving (20 min, 121ºC), 30 mL of filter-sterilized (0.45 μm) 10% (w/vol) casamino acids, 10 mL of filter sterilized (0.45 μm) 20% glucose (w/vol) and 100 mL of previously prepared CAS-Fe-HDTMA solution were added to the medium and dispensed in plates. Each isolate was tested in the same way as in the phosphate solubilization assay. After seven days at 27ºC in darkness, yellow-orange halos around positive isolates were measured. These isolates were classified as siderophore producers. The MCI ratio (Metal Chelation Index ratio) between the halo diameter and the colony diameter was also used as a parameter to evaluate the siderophore production (Batista et al., 2018). A *Pseudomonas sp.* DSMZ 25842 strain was used as a positive control for siderophore production.

2.4.4 Plant hormones production

The production of plant hormones by bacterial isolates was tested by growing each isolate in 5 mL of LB broth supplemented with filter-sterilized (0.45 μm) 5 mM L-Tryptophan, for IAA production (Lin et al., 2015; Gilbert et al., 2018). Isolates were grown in triplicates, for 20h at 28ºC with 250 rpm shaking in 50 mL sterile centrifuge tubes. OD at 600 nm of all isolates was measured, but those that did not reach a minimum OD_{600} value of 0.6 were not considered for hormone concentration measurements. In resume, only 55 isolates were considered for hormone production analyses. The cultures were centrifuged at 5200 rpm for 10 min, and supernatants were transferred to clean 12 mL tubes and stored at -80ºC until hormone quantification. A *Pseudomonas sp.* DSMZ 25842 strain was used as a previously known IAA producer. Final concentration for each replicate was calculated after subtracting the control (LB medium) hormonal concentration and dividing by the OD_{600} value.

The content of acidic hormones (IAA; jasmonic acid, JA; jasmonoyl isoleucine, JA-Ile; abscisic acid, ABA; and salicylic acid, SA) and CKs (isopentenyladenine, iP; isopentenyladenosine, iPR; trans- and cis-zeatin, tZ and cZ; trans- and cis-zeatin riboside, tZR and cZR; dihydrozeatin, DZ; dihydrozeatin riboside, DZ) were analysed by high performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS).

The procedures for the determination of acidic hormones and CKs are different and were performed separately using two different aliquots from the same sample/culture. The quantification was carried out in a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific), equipped with an HESI(II) source, a quadrupole mass filter, a C-Trap, a HCD collision cell and an Orbitrap mass analyzer, following the methodology elaborately described in Silva-Navas et al. (2019).

The content of IAA, JA, JA-Ile, ABA, and SA was analyzed as follows: for each triplicate of every bacterial culture, and for three replicates of the pure culture medium (without bacteria, as a blank), aliquots of 90 μL of culture broth were added to 10 μL of internal standard (1000 ng ml^{-1} of deuterium-labelled internal standards in metanol), 150 μL of MeOH, and 150 μL of acetic acid 0.133%, and centrifuged at 20,000 g (Sigma 4-16K Centrifuge) for 10 min before the injection in the HPLC-ESI-HRMS system, using

exactly the same conditions detailed in Silva-Navas et al. (2019), and the same identification and quantification procedure.

For the analysis of the production of CKs by endophytic bacterial isolates, 100 μL of the culture medium for each bacteria and replicate were mixed with 25 μL of internal standard (100 ng/mL of each standard in metanol), 225 μL of MeOH and 150 μL of formic acid 0.04% and centrifuged at 20,000 g for 10 min before the injection of the sample. Three aliquots of 100 μL of the pure culture medium without bacteria were subjected to the same procedure, with the purpose of serving as a blank for the determination of the concentration of hormones produced by the bacteria. The measurement conditions, detection and quantification have already been described in Silva-Navas et al. (2019).

2.5 Statistical analysis

For comparison between hormonal productions by genus, ANOVA signification tests were carried out followed by HSD Tukey post-hoc tests. The statistical tests were performed with the stats package in R (RStudio, 2016). The $p \leq 0.05$ was used as statistically significant threshold.

3. Results

3.1. Taxonomic diversity of SHA-treated cucumber associated endophytic microbiota

As mentioned in the introduction, the treatment with SHA did not induce the appearance of new microbial phyla, but modified the distribution and relative proportion of some microbial taxa (de Hita et al., 2018). Therefore, it is assumed that the endophytes isolated from SHA-treated plants would be also present in control plants. The number of endophytic bacterial isolates obtained from three different plants of cucumber previously grown in the presence of 100 ppm C SHA in the nutrient solution was 80, but only 72 of them were able to easily grow in LB broth and considered for further analyses. For the taxonomic identification, BLASTn and RDP SeqMatch databases were utilized. Both identifications were similar, with only a misleading identification in one isolate (CR329.a, Table S1). Most of the microorganisms identified (97% of the isolates) showed ≥97% of similarity with reference sequences in BLASTn database; whereas those isolates with less than 97% similarity could be new species (genus *Paracoccus* and genus *Dyadobacter*) or sequencing chimeras.

The cladogram tree represents the closest classification of isolates to reference sequences in BLAST (Figure 1). The phylum *Proteobacteria* was the most abundant (44%) followed by *Actinobacteria* (24%), *Firmicutes* (31%), and *Bacteroidetes* (1%) (class, order, and family distribution of isolates are shown in Figure S1). The most diverse group of bacteria belonged to *Proteobacteria* with five different families (*Xanthomonadaceae*, *Pseudomonadaceae*, *Sphingomonadaceae*, *Rhodobacteraceae*, and *Methilobacteraceae*) representing two different classes (*a-* and *γ-proteobacteria*). Both *Actinobacteria* and *Firmicutes* were represented by only one phylogenetic class: *Actinobacteriia* and *Bacilli* respectively. In each class there were two (*Micrococcaceae* and *Microbacteriaceae*) and three (*Staphylococcaceae*, *Paenibacillaceae*, and *Bacillaceae*) different families. *Bacteroidetes* phylum had only one isolate belonging to *Cytophagaceae* family. The most significant diversity at genus level was found in *Bacillus* and *Microbacterium*, comprising seven and six different species respectively. The most abundant species was *Stenetrophomonas maltophilia* with 15 isolates, followed by *Arthrobacter* aurescens (seven strains) and *Pseudomonas oryzihabitans* (seven strains).

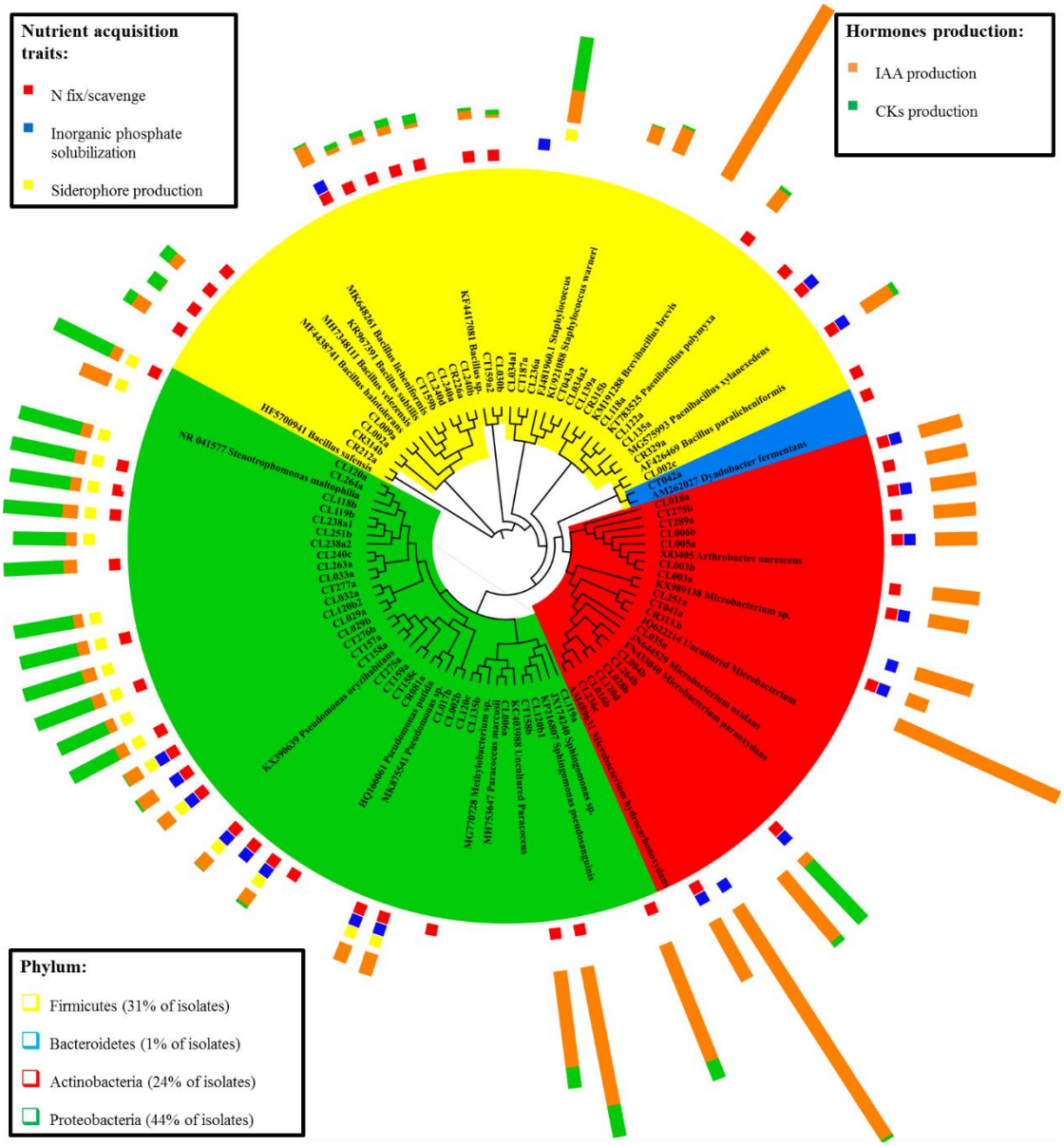

Figure 1. Cladogram representing the identification of the endophytic bacterial isolates and the corresponding plant growth promotion traits. Isolates are grouped by their sequence similarity to the BLASTn reference sequence. Phylum taxonomic level is showed by color: Firmicutes (yellow), Bacteroidetes (blue), Actinobacteria (red) and Proteobacteria (green). Plant growth promotion traits are separated by nutrient acquisition traits (presence or absence) and hormone production.

3.2. *General framework of the plant growth promotion traits characterization in endophyte isolates*

Endophytic isolates were screened for their *in vitro* PGP traits. The traits selected were those related to the mineral nutrient acquisition (nitrogen fixation/scavenging, inorganic phosphate solubilization and iron siderophore production) and those associated with plant growth regulators (IAA and CKs plant hormones) production. After clustering the sequences and representing the presence/absence of the studied PGP traits in the

cladogram (Figure 1), *Actinobacteria*, *Firmicutes*, and *Proteobacteria* isolates were clustered in three diferenciated clades, whereas the *Bacteroidetes* isolate appears as an intermediate group between *Firmicutes* and *Actinobacteria* phyla. This representation also showed that the isolates clustering together showed similar PGP performance according to the traits that have been studied; although the functional strain diversity was highlighted. The cladogram confirmed the identification of isolates by BLASTn and RDP SeqMatch.

The biological nitrogen fixation/scavenging was the most prevalent PGP trait within the nutrient acquisition features studied, with 68 % of isolates being able to grow in an N free medium (Figure 2). This trait is showed by diverse phylogenetic groups (Figure 1) and found in isolates from leaves, stems and roots.

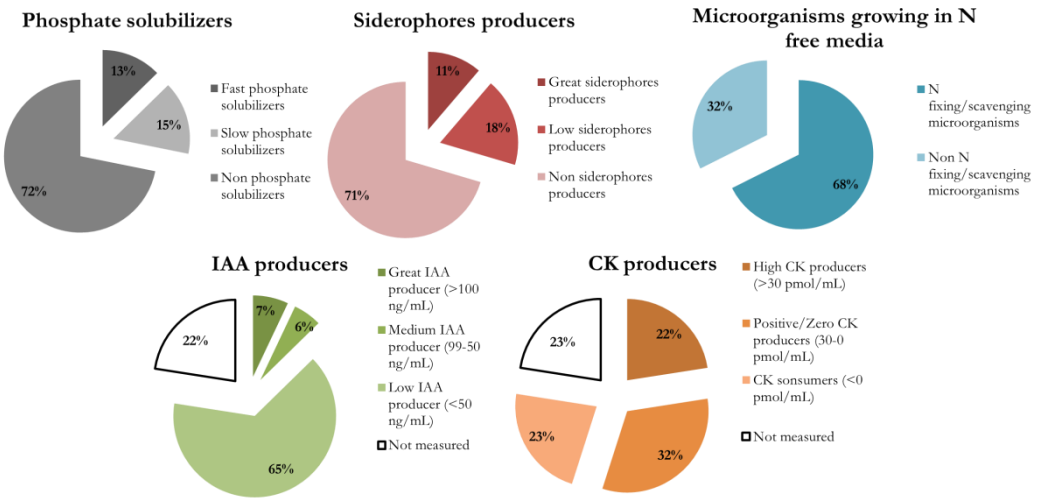

Figure 2. Prevalence of each plant growth promotion trait in the endophytes isolated from cucumber plants pre-treated with a sedimentary humic acid. The traits evaluated were: inorganic phosphate solubilization at 14 days, siderophore production at 7 days, capability of growing in a N-free medium, and phytohormones production (IAA and CKs).

Regarding inorganic phosphate solubilization, it was performed by only 28% of isolates. They were classified as: fast solubilizers, which solubilized phosphate after 7 days and had the greatest IPS ratios after 14 days; or slow solubilizers, whose halos of solubilization were visible not at 7 days but after 14 days, or showed small IPS ratios. The fast solubilizers (9 isolates) were identified as *Pseudomonas* genus. The slow phosphate solubilizers were more diverse, and there were 11 isolates from six different genera: *Arthrobacter*, *Paenibacillus*, *Microbacterium*, *Stenotrophomonas*, and *Staphylococcus* (Table S2).

Most of the isolates producing siderophores able to chelate iron (29% of isolates) belong to to *Pseudomonas* and *Stenetrophomonas* genera (Figure 2, Table S3). Those isolates

producing a MCI ratio higher than 1.5 were classified as great siderophore producers, all *Pseudomonas* strains pertained to that group.

We have also screened for the production of a wide group of plant hormones (listed in Materials and Methods section), but only IAA, cZ, cZR, iP, and iPR were detected in our cultured endophytic isolates.

There were 16 isolates that did not grow appropriately in LB broth supplemented with 5 mM Trp, so the hormonal production was not measured for those bacteria (Table S1). The rest of the isolates were able to produce IAA and CKs. IAA production ranged between 1-245 ng/mL (Figure 3), and the isolates were classified according to their IAA production in three levels: low producers (<50 ng/mL), medium producers (50-99 ng/mL) and great producers (>100 ng/mL). Most of the isolates were low producers (65% of all isolates), but 7% of isolates (5 strains) were great IAA producers and identified by BLASTn as *Microbacterium paraoxydans*, *Sphingomonas pseudosanguinis*, *Sphingomonas sp.*, an uncultured *Microbacterium*, and *Brevibacillus brevis*. IAA production appears as a phylogenetically diverse trait involving different bacterial taxa.

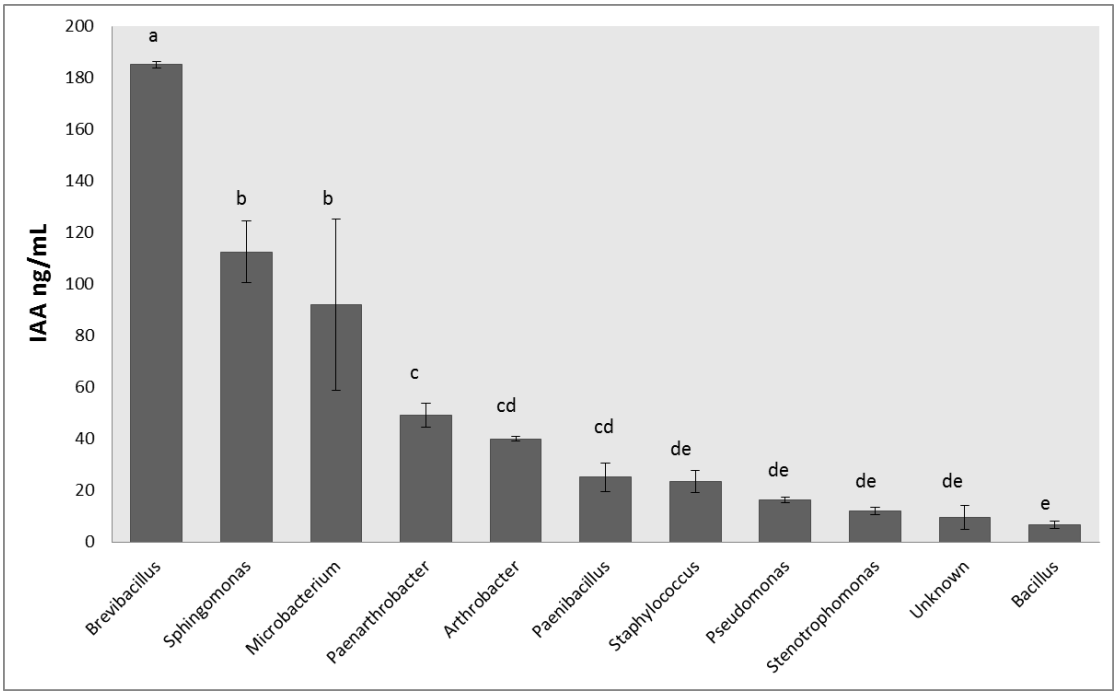

Figure 3: Production of indole-3-acetic acid by endophytic isolates according the genus. Indole-3-acetic acid production was measured in ng per mL after 20 hours of growth at 28 ºC in LB medium supplemented with Trp 5mM. Production was measured in triplicates for each isolate, and the final concentration was obtained after standardization. Bar errors represent the standard error. Letters represent the significant groups after ANOVA and HSD Tukey post-hoc tests. Signification threshold: $p \leq 0.05$.

The four different CKs detected (cZ, cZR, iP, and iPR) showed different dynamics in the isolates (Figure 4) and we have classified the CKs producers according to the total CKs production. This production could be highly positive (>30 pmol/mL, CKs great producers), positive or zero (0-30 pmol/mL, CKs low or no producers) or negative (<0 pmol/mL, CKs consumers). Most of the isolates consumed part of the iPR initially present in the culture broth, but they produced large amounts of iP (Figure 4). On the other hand, in general, isolates produce smaller quantities of cZR than cZ. CKs production appears as a diverse and essential trait for different endophytic taxa. There were 22% isolates with a high net production, ranging between 45-72 pmol/mL of total CKs. The most represented genera inside these CKs producers group were *Stenotrophomonas maltophilia* (14 isolates) strains (Table S1). In general, high CKs net production was accompanied by low levels of IAA production.

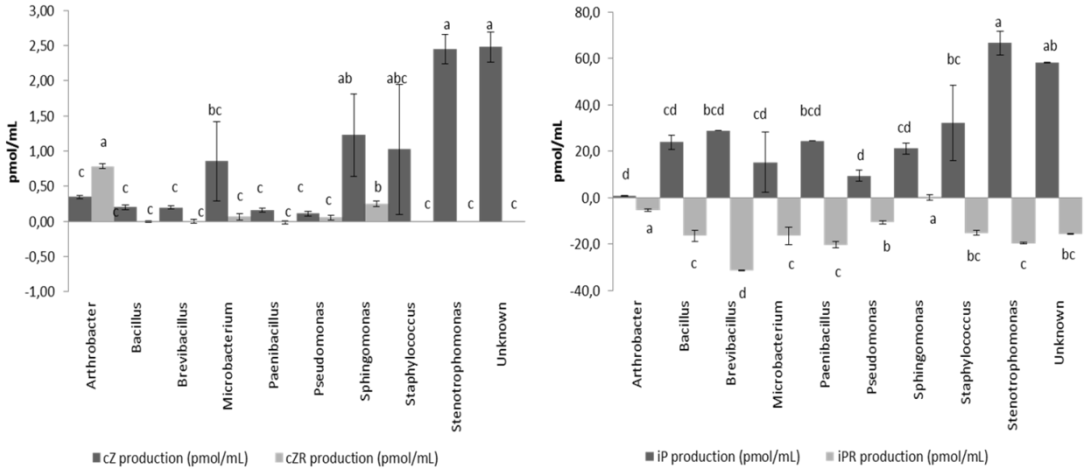

Figure 4: Production of cytokinins (cZ, cZR, iP and iPR) by endophytic isolates according the genus. Cytokinins production was measured in pmol per mL after 20 hours of growth at 28 ºC in LB medium supplemented with Trp 5 mM. Production was measured in triplicates for each isolate, and the final concentration was obtained after standardization. Bar errors represents the standard error. Letters represents the significant groups after ANOVA and HSD Tukey post-hoc tests. Signification threshold: $p \leq 0.05$.

4. Discussion

4.1. Description of the plant growth promotion traits of the most important endophyte taxa found in SHA-treated plants

It is generally assumed that endophytic communities are originated and shaped by the soil, being the roots the main entrance door (Hardoim et al., 2008; Hardoim et al., 2015; Reinhold-Hurek et al., 2015). In a previous study (de Hita et al., 2018), the

treatment with SHA was proven to induce changes in the populations of the endophytic microorganisms of cucumber plants grown hydroponically. Considering that the use of a hydroponic system limits the origin source of endophytes, the most plausible origin is the vertical transmission of this microbiome through the seeds (Johnston-Monje and Raizada, 2011; Hardoim et al., 2012; Truyens et al., 2013; Truyens et al., 2015; Khalaf and Raizada, 2016 and 2018; Nelson, 2018). Endophytes from seeds have been specially selected by evolutionary forces; thus, they are an interesting option for agriculture application. As mentioned above, the composition of SHA-treated cucumber endophytes was dominated by the isolates from the *Proteobacteria* phylum (44% of isolates), followed by *Actinobacteria* (24%), *Firmicutes* (31%) and *Bacteroidetes* (1%). That composition is, as a general rule, predominant in rhizospheres and endospheres of angiosperms plants (Santoyo et al., 2016), such as barley (Bulgarelli et al., 2015), rice (Hameed et al., 2015), wheat (Liu et al., 2017), the plant model *Arabidopsis thaliana* (Lundberg et al., 2012), and other plants (Fitzpatrick et al., 2018).

The phylum *Proteobacteria* was the most diverse, with five different families belonging to two major classes (*a*- and *y-proteobacteria*). The most common taxon was *Stenotrophomonas maltophilia*. That bacterial species is a well-known endophyte that has been widely used in agriculture for its lack of plant pathogeny (Johnston-Monje and Raizada, 2011; Berg and Martinez, 2015). In this work, most of the strains of *Stenotrophomonas maltophilia* present 4 out of the 5 PGP traits studied. *Stenotrophomonas* was previously known as siderophore producers (Egamberdieva et al., 2016; Singh and Jha, 2017), although not all strains were able to produce them. The same happened with the ability to grow in N-free medium. It would be possible that siderophore production and N fixation/scavenge are strain-dependent features and not phylogenetically related ones. Siderophore production is related to iron acquisition, and that also improves the competitiveness against pathogens and induce competitive plant defense (Beneduzi, Ambrosini, and Passaglia 2012; Ahmed and Holmström 2014). Nitrogen fixation/scavenging increases the plant disponibility of this nutrient. *Stenotrophomonas maltophilia* also produced significant amounts of CKs, mainly cZ and iP; but lower concentrations of IAA (12.2 ng/mL). Bacterial CKs production probably is an important trait in plant growth; but it is not common to find CKs analyses in scientific reports exploring new PGP bacteria. In this work, CKs and IAA were analyzed from the same aliquot of 5 mM Trp supplemented LB broth. The relationship between the concentrations of IAA and CKs (ratio IAA/CK) is essential for determining plant growth

direction (Timmusk et al., 1999; Dodd et al., 2010; Egamberdieva et al., 2016). Within the plant, an imbalance favoring CKs production (as it occurs with *Stenotrophomonas* strains) favors the formation of shoots, chloroplastic maturation, cell expansion, stomatic conductance, and meristematic tissues differentiation (Cassán et al., 2014). Therefore, a possible role of this bacterium inside the plant might be as a shoot growth promoter with the capability to increase the transport and disponibility of nutrients, as Fe and N. Also, it is possible to play an essential role in plant defense due to the competition for iron against pathogens.

Other endophytic *Proteobacteria* isolated were those pertaining to *Pseudomonas* genus (29% of *Proteobacteria* isolates, Table S1). *Pseudomonas* species have been already observed as PGP candidates (Browne et al., 2009; Bulgarelli et al., 2013; Malfanova et al., 2013) but also as plant pathogens (Großkinsky et al., 2016). That is relevant because endophytes have the potential of being pathogens if there are some environmental disturbances, plant diseases, or plant molecules (Hardoim et al., 2015). Maintaining the natural imbalance in bacterial communities could be essential for plant fitness (Hardoim et al., 2012; Vorholt, 2012; Hardoim et al., 2015), preventing the pathogeny of endophyte inhabitants. Some of the isolates of *Pseudomonas* genus were the only ones in the whole compendium of isolates under assessment in this study that showed all of the PGP traits measured. All the strains were able to grow in N free medium, solubilize inorganic phosphate (best solubilizers), produce IAA (16.5 ng/mL) and CK (low amounts of cZ, cZR and iP) and, with the exception of three isolates, also to produce siderophores, with the greatest MCI ratios (Table S3). *Pseudomonas* genus is well-known as siderophore producers, especially by the synthesis of the siderophore pyoverdin (Kloepper et al., 1980; Gamalero and Glick, 2011). Also, that genus is known for its diverse metabolism (Malfanova et al., 2013; Großkinsky et al., 2016; Sun et al., 2019); indeed, the ability of solubilizing inorganic phosphate has been already described in some strains (Browne et al., 2009). As our results point out, the role of *Pseudomonas* within the plant might be to increase the disponibility of nutrients (P, N, and Fe) rather than to induce plant growth through hormone regulators. Regarding their PGP behavior, the *Pseudomonas* strains here evaluated appear as the most promising candidates for future applications; but their potential as plant pathogens must be explored.

Another interesting *Proteobacteria* isolated from the SHA-preconditioned cucumber plants were the *Sphingomonas* strains. These strains were found to be N fixers/scavengers. The capability of biologically fixing/scavenging nitrogen could be an evolutionary

advantage in the predominately oligotrophic endosphere (Carvalho et al., 2014; Hardoim et al., 2015), with low O_2 and nutrient concentrations, but a less competitive environment than soil and rhizosphere; indeed, *Sphingomonas* endophytes would have evolved to take advantage of such environment by acquiring that ability. Molecular biology-based tests such as nifH PCR amplification, biochemical assays (acetylene assay or N^{15} marked isotopes) or *in situ* microscopy should be carried out to assess the biological nitrogen fixation activity of these isolates. The other main characteristic of *Sphingomonas* isolates was the plant hormone production. The isolates were consistently identified as great producers (Truyens et al., 2013; Asaf et al., 2017; Durand et al., 2018), with an IAA production in our work ranging between 90-131 ng/mL and a positive CKs net production, producing cZ, cZR, and iP, but not consuming iPR. In contrast with *Stenotrophomonas* isolates, *Sphingomonas* IAA/CK ratio tendency was inclined towards IAA. An increase in IAA promotes cell proliferation, enlarges the root system (increasing root biomass), enhances nutrient and water uptake efficiency, and changes the root architecture (Dodd et al., 2010; Egamberdieva et al., 2016; Gilbert et al., 2018). Accordingly, *Sphingomonas* genus seems to be an intimately plant related microbe due to the vital role of plant hormone in plant-microbe cross-talk (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011; Carvalho et al., 2014; Koul et al., 2015) and as previously said, the ability to grow in N free medium. The particular behavior of these *Sphingomonas* isolates makes them attractive candidates for future uses as PGP inoculum, mainly due to their high amount of IAA production combined with a moderated CKs production.

The second most abundant phylum was *Actinobacteria* with two different families of isolates: *Microbacteriaceae* and *Micrococcaceae*. All the isolates from *Microbacteriaceae* family were identified as *Microbacterium sp.* candidates; a diverse taxon that is commonly found in endophytic communities (Fidalgo et al., 2016; Khalaf and Raizada, 2016; Defez et al., 2017; López et al., 2018). These candidates were heterogeneous in their taxonomical assignation (six different species) and their performance as PGP endophytes. For instance, the isolate which produces the most considerable amount of IAA (254 ng/mL of IAA) belong to *Microbacterium* genus, but at the same time, another *Microbacterium* isolate was the greater producer of CKs. Also, some strains were able to fix/scavenge nitrogen and to solubilize phosphorus, but others only show one or none of those traits. All of these traits have been previously described for *Microbacterium* isolates (Dodd et al., 2010; Ji et al., 2014; Banik et al., 2016). Due to the *Microbacterium* heterogeneity, it is challenging to propose an ecological role to *Microbacterium* in plant endosphere. The

general pattern is the capability of producing phytohormones (mainly IAA), but not all *Microbacterium* strains produce similar levels, thus showing strain-production diversity.

In *Micrococcaceae* family, only one genus was found, *Arthrobacter*. Isolates were able to grow in N free medium and to produce moderate amounts of IAA, averaging 40 ng/mL. These two traits are the most prevalent within all the isolates of this work. Also, most of the isolates showed phosphate solubilization being, together with the *Pseudomonas* isolates, the main solubilizers found in this study. The solubilization of inorganic phosphate is an ecologically important trait because P is the second major limiting nutrient for plant growth (Weyens et al., 2009; Khalaf and Raizada, 2016). Phosphorus is abundant in soils, but in non-plant-available forms, such as tricalcium phosphate or phytate; so its biodisponibility directly depends on microbial activity (Mahanty et al., 2016).

The third most important phylum in the bacterial community isolated in this work was *Firmicutes*, represented by *Bacilli* class. Inside that class, the isolates belong to 4 different genera: *Bacillus* (the most diverse and abundant), *Brevibacillus*, *Staphylococcus,* and *Paenibacillus*. The different genera showed diverse trait patterns. For instance, *Bacillus* were predominantly N fixers/scavengers and low plant hormone producers, *Brevibacillus* only produce IAA (one of the higher producers), *Staphylococcus* isolates are heterogeneous, and *Paenibacillus* are mainly N fixers/scavengers but low IAA and CK producers. Only *Paenibacillus xylanexedens* strains were phosphate solubilizers. In contrast with previous reports where this class had been found as the most dominant cultivable group in *Cucurbitaceae* plants (Khalaf and Raizada, 2016) and highlighted as important bacterial community founders (López et al., 2018) due its wide diversity and ecological role; our *Bacilli* isolates did not perform particularly well in our PGP traits screening tests, especially in phytohormones production (except *Brevibacillus*). Although *Bacilli* still are an essential fraction of microbes in our plants due to their capability to overcome desiccation during seeds storage (Ali et al., 2014), they do not seem to play an important role in plant growth promotion. Probably, as suggested by Khalaf and Raizada (2016) and López et al. (2018), the ecological role of this group is to fund bacterial communities during plant germination (Chimwamurombe et al., 2016; Afzal et al., 2017; Batista et al., 2018).

Finally, only one *Bacteroidetes* phylum isolate was obtained from SHA-treated cucumber plants, identified tentatively as *Dyadobacter fermentans* as the sequence did not

reach 97% of identity with the BLAST match. *Bacteroidetes* are frequent in molecular techniques-based studies of endophytic communities (Santoyo et al., 2016; de Hita et al., 2018), but they are rarely mentioned in reports using endophytic culture-based methods. The lack of isolates of this phylum points to a non-adequate culture medium formulations. In spite of the increasing efforts made in microbial ecology research in the last decade, little has been done to improve non-cultivable bacteria isolation. The *Bacteroidetes* isolate only showed the ability to grow in N-free medium, but few could be said of the ecological role of this microbe in cucumber endosphere.

4.2. Relationships between the main plant growth promotion traits of culturable endophytes in SHA-treated plants and the main steps involved in the mechanism of action of SHA enhancing plant growth.

Mora et al. (2010) reported that SHA is able to promote nitrate root uptake and nitrate reductase activity in cucumber. Other studies showed that humic acids extracted from different sources were able to induce the expression of genes directly involved in nitrate transport and further assimilation (Trevisan et al., 2010; Jannin et al., 2012; Olaetxea et al., 2018). All these effects are potentially reciprocal with the ability to obtain inorganic nitrogen from the medium found in some bacterial isolates from SHA-treated plants.

Other studies have shown that the treatment of plants with humic (HA)-metal-phosphate complexes was associated with an increase in the internal utilization of P by plants (Urrutia et al., 2014; Jindo et al., 2016). This fact was related to the presence of higher concentrations of soluble phosphate in plant tissues. These studies indicated that HA might play a relevant role in this process (Urrutia et al., 2014). On the other hand, the results presented here show that several families of endophytes isolated from SHA-treated plants were able to mobilize P from water-insoluble calcium phosphate. This action has been normally associated with P mobilization in the rhizosphere but in the case of endophytes it might also play a very relevant role in solubilization of P fractions precipitated with Fe or Ca in the cell apoplast. Therefore, both SHA and endophytes could participate in a simultaneous way in the mobilization of internal fractions of precipitated P. The same reasoning might be applied for Fe plant nutrition and the activity of those endophytes isolated in SHA-treated plants with the ability to produce siderophores. Various studies have shown the ability of SHA to improve Fe root uptake and further assimilation in cucumber (Aguirre et al., 2009; Zanin et al., 2019). These effects were accompanied by a significant activation of Fe-deficiency root responses even under Fe sufficient conditions (Aguirre et al., 2009), and increases of the so-called active

Fe within the plant were also observed (Aguirre et al., 2009). In this framework, those endophytes producing siderophores could contribute to this process through the solubilization of Fe precipitated in cell apoplast. Thus, as in this case of P, the effects observed upon SHA treatment improving Fe plant nutrition are compatible with a positive action of specific endophytic groups.

The complementarity between SHA and endophytic microbiota in the whole mechanism responsible for the shoot- and root- growth enhancing effect of SHA in cucumber plants would appear clearer in the case of plant hormone action. Several studies have shown that the shoot- and root-growth promoting action of SHA in cucumber is regulated by IAA, ABA and some families of CKs, principally trans-zeatin (tZ) and adenine-based CKs (Mora et al., 2010; Olaetxea et al., 2015 and 2018). The results presented here show that a number of endophytes found in SHA-treated plants were able to increase the production of some of these phytoregulators. As discussed above, many of the main endophytic families isolated from SHA-treated cucumber plants were able to produce significant amounts of IAA (Figure 3). This fact is in line with a potential cooperation between the biochemical action of SHA and bacterial endophytic activity in plant tissues. Regarding CKs, however, endophytes promoted the synthesis of cZ and not tZ, which is the main CK involved in the SHA shoot growth promoting effect. However, recent studies have shown that the cZ:tZ ratio plays a very relevant role in the regulation of plant responses to P deficiency (Silva-Navas et al., 2019). It is therefore possible that the ability of endophytes to produce cZ has some influence in the improvement of the adaptation of SHA-treated cucumber plants to low concentrations of available P in the nutrient solution.

5. Conclusions

In conclusion, this work shows that endophytic microorganisms could be a promising tool to improve the crop production due to its natural presence in plants tissues which confer them an ecological advantage against rhizosphere microorganisms. Furthermore, the ecological role of endophytes, through the improvement of nutrient acquisition and plant hormone regulation, makes them a suitable choice for sustainable agriculture. Even, our study highlights the importance of seed microbiome as the second major source of endophytes but the primary source of microbial community founders due to the hydroponic system used to grow the plants (Johnston-Monje and Raizada,

2011; Hardoim et al., 2012; Truyens et al., 2013; Khalaf and Raizada, 2016 and 2018; Nelson, 2018).

On the other hand, cultivable endophytes isolated from plants treated with a sedimentary humic acid present a relevant capacity to affect some processes related to plant mineral nutrition and hormonal signaling pathways. In addition to that, all these plant growth promotion traits can be evolved in a complementary, additive or synergetic way with the main mechanisms activated upon SHA application. Thus, the ability of some endophytes to mobilize P or Fe precipitated within the plant, for example in cell aplopast, through the production of polycarboxylic acids and siderophores is similar to the SHA ability to improve P and Fe use efficiency within the plant. Also the capacity to obtain inorganic N from the medium showed for most endophyte isolates found in SHA-treated plants could be related with the SHA capacity to improve N transport within the plant. This relationship between endophyte activity and SHA effect is especially clear in plant hormone activity, since most isolates were able to produce IAA and CKs, which are two phytoregulators directly involved in the shoot and root growth promoting action of SHA. One of the perspectives to explore in depth in future works would be the role of HS as bacterial community activity modulator.

Finally, our results are in line with the hypothesis proposed by Khalaf and Raizada (2016 and 2018) that crop selection made by agricultural activity across the history has selected not only the most producing plants but also their endophytic community with most plant growth promotion traits in contrast with the evolutive selection of wild vegetation (Gilbert et al., 2018). For all those reasons, we consider that some bacteria isolated in this work could be used in as a new alternative like a single plant inoculum or a microbial consortium.

6. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author contributions

The study was conceptualized and designed by DDH, MF and JMGM. Experimental work was performed by DDH and YR. Hormone detection was assessed

by AMZ. Statistical analysis was done by DDH. Data analysis was carried out by DDH, MF and JMGM. Manuscript was prepared by DDH, MF, JMGM and AMZ.

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10. Data Availability Statement

All datasets generated in this study are included in the manuscript and the supplementary files. Isolates sequencing reads were deposited in GenBank under accession numbers MN512151 to MN512214.

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CHAPTER IV

The microbiome of barley root endosphere was modified by the application of sedimentary humic substances under different nitrogen fertilization regimes

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In preparation

GENERAL DISCUSSION

Nowadays, agriculture must face not only the feeding of a growing population (in 2050 the demand of crop production will double) but also an increase in the interest for more sustainable practices (Sanz et al., 2017; Gazzola et al., 2019; Pukalchik et al., 2019). The ´60s agricultural practices, such as an excessive use of chemical fertilizers and pesticides, changes in land use, ecologically aggressive agricultural practices (tillage or monocropping), or a gradual loss of cultivars diversity, have led to deleterious effects on soil physicochemical and biological properties (Sergaki et al., 2018, and references therein). The degradation of agroecosystems impeded balanced food consumption for all humans and domesticated animals, and the shortage in N and P fertilizers will be common soon. Furthermore, the actual climate change scenario is expected to worsen the situation (Yuan et al., 2018).

Therefore, there is a growing interest in the development and implementation of new sustainable land management practices. In this dissertation, we have proposed the use of organic amendments such as HS (specifically an SHA) as plant growth promoters and also as plant endophytic microbiome inductors. Traditionally, most of the studies in SHA have been focused in the direct application to the roots, but in agriculture the foliar application is an extended practice. Hence, as a first step, with the aim of evaluating which is the best application strategy for promoting plant growth, in Chapter 1, we unravelled how the SHA foliar application promoted the plant development in comparison with root application. Then we assessed in Chapter 2 how the SHA application strategy affected the plant endophytic microbiome. Besides, we identified which bacterial and fungal taxa were enriched or depleted by SHA application and also according to the application strategy. Finally, in Chapter 4, we have experimented with more realistic conditions (in soil) with a highly consumed crop (barley) and testing different N fertilization regimes combined with SHA application (root application), for assessing how the endophytic microbiome would behave in a long term field scenario.

Additionally, we have explored in Chapter 3 the potential of endophytes isolated from plants pre-conditioned with the same SHA as PGP bacteria.

Differences between foliar and root SHA application: effects in plant and endophytic microbiome composition.

Foliar and root SHA application can increase the root and shoot DW of cucumber plants grown in hydroponics and similar experimental conditions. We have observed these effects both in short and medium-term applications, at different doses (40 mg L^{-1} and 100 mg L^{-1}), and they are in concordance with previous studies (Aguirre et al., 2009; Mora et al., 2010; 2012; 2014; Rose et al., 2014; Canellas et al.; 2015; Olaetxea et al., 2015; 2019). However, we proved that the plant response was different for each application strategy.

The main mechanistic differences were (Figure 1):

i. In short-term experiments, the foliar treatments, does not promote lateral and adventitious root proliferation as root application does (Nardi et al., 2002; Canellas et al., 2012; Mora et al., 2012; García et al., 2016). In our experimental model, this effect is related to ABA concentrations in the root, which is essential to lateral root promotion (Olaetxea et al., 2019). Nevertheless, foliar treatment increased primary and secondary roots number.
ii. Any of the tested doses of foliar applied SHA activated the PM H^+-ATPase as root application did in short-term experiments. The activity of this enzyme is essential in the promotion of root nutrient uptake caused by the root application of SHA (Olaetxea et al., 2019). As a result, the increment in leaf nutrient concentrations commonly found in SHA root-treated plants was absent in foliar-treated plants.
iii. Foliar treatment, in short term experiments, increases the root concentration of IAA. This increment, as well as in root applications, is commonly associated with a shoot growth (Mora et al., 2014). In root application, this shoot growth is commonly accompanied by a rise in plant root ABA concentration (Olaetxea et al., 2015; 2019); however, no effect was found in ABA concentration by foliar treatment.
iv. Foliar application increases the concentration of some CKs in root and leaves like root treatment did, but this action was not accompanied by an increase in PM H^+-ATPase activity, which usually is related with a root-shoot CKs translocation (Olaetxea et al., 2019).

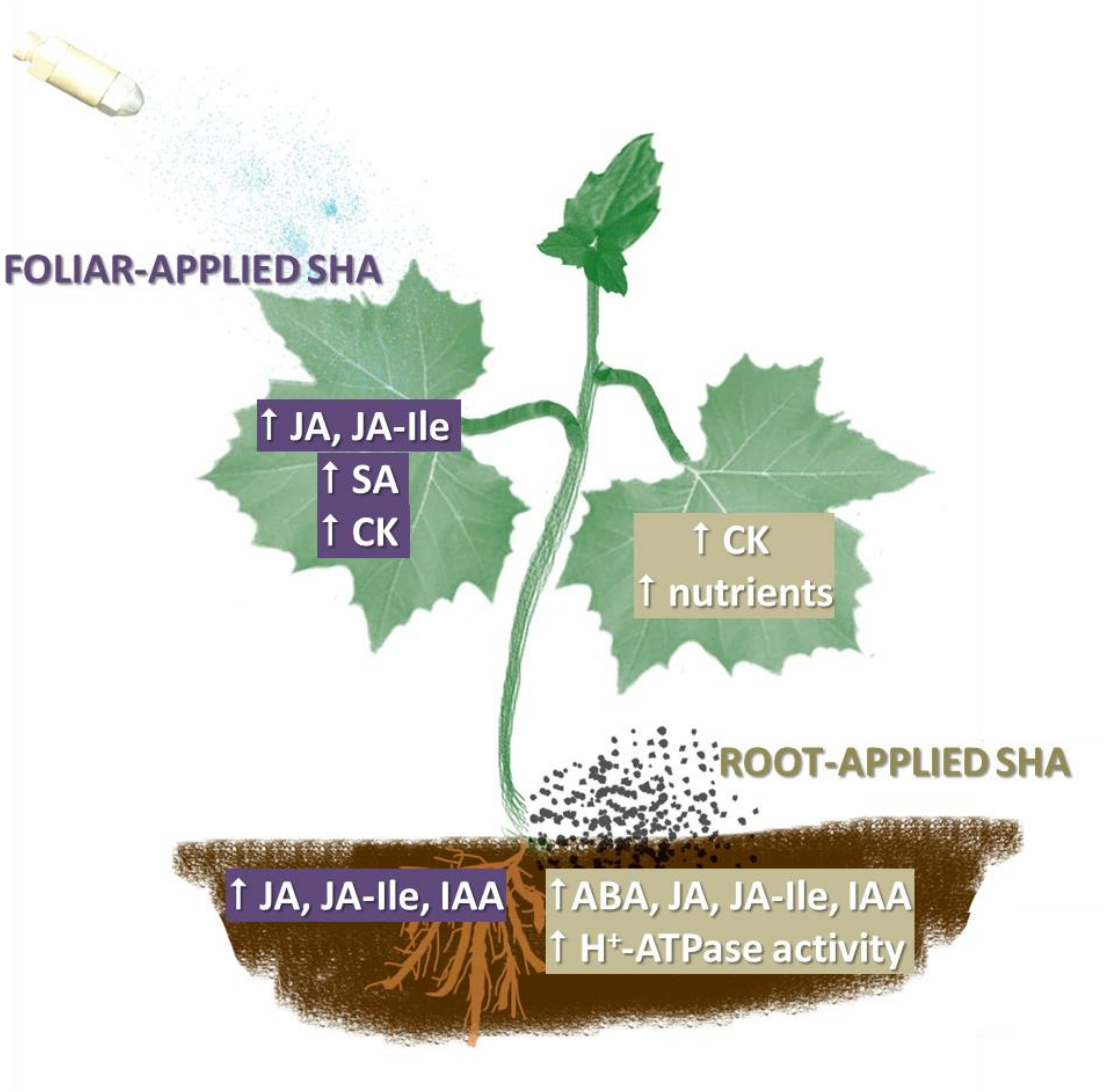

Figure 1: Main effects of SHA according to the application strategy (purple-foliar application, light brown-root application).

The treatment with SHA, regardless the type of application, increased root concentrations of JA and JAIle (the active form of JA). The fact that the foliar application also promotes the JA and SA signaling pathways in shoots could reflect that these plants are perceiving the foliar application as an external damage (Wasternack and Hause, 2013; Nazar et al., 2017). In this line are the changes caused in leaf morphology, as observed in SEM/TEM images (Figures 11 and 12, in Chapter 1), showing a reduction in trichome density, changes in leaf cuticle, and also a decrease in soluble cuticle waxes. Also, we found a decline in leaf mesophyll starch accumulation, which might be related with a mobilization of carbohydrates, that it is just the opposite result found in the root SHA application (Jannin et al., 2012).

But, is it possible that these differences in SHA mechanism of action affect differentially the plant endophytic microbiome? Is it related the SHA application, especially the foliar type, with enhancement of plant ISR? And may the SHA application effects, such as changes in root architecture, leaf surface or hormonal balance, modify the endophyte colonization patterns?

For answering these questions, we carried out the experiment detailed in Chapter 2. As it was demonstrated in that chapter, both foliar or root SHA applications increased the bacterial richness and reduced the fungal richness in the endophytic microbiome of cucumber. These effects were more pronounced in foliar-treated plants. Also, the plant compartment where the SHA was applied showed lower microbial richness than the other plants

Particularly, the SHA application influenced both in the endophytic bacterial and fungal microbiotas of cucumber plants growing in a hydroponic system. The dominant phylogenetic groups found in the microbiome (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* for Bacteria kingdom; and *Ascomycota* and *Basidiomycota* for Fungi kingdom), were similar to those found in other studies (Lundberg et al., 2012; Fürkrantz et al., 2012; Bulgarelli et al., 2013; 2015; Edwards et al., 2015; Lee Taylor and Sinsabaugh, 2015; Coleman-Derr et al., 2016; Khalaf and Raizada, 2016; Adam et al., 2018; Brinkmann et al., 2019), in the barley experiment of Chapter 4, and also similar to the distribution of cultivable endophytes described in Chapter 3. However, some bacterial and fungal taxa were differentially promoted according to the SHA treatment, and even the SHA application type influenced them:

i. Some genera from *Actinobacteria* phylum, like *Micrococcus*, *Streptomyces,* or *Nocardia*, increased their relative abundances in SHA-treated cucumber root endosphere. This group of bacteria is well-known as antimicrobial compound producers and recalcitrant compounds degraders (Ventura et al., 2007; Barka et al., 2016), that commonly appear colonizing metabolically active cells (Bulgarelli et al., 2013). Such a situation can be promoted by the SHA application with the concomitant stimulation of plant growth (García et al., 2016; Olaetxea et al., 2018). Several isolates from Chapter 3 belonged to this group of bacteria.
ii. *Pseudomonales* order was also promoted in cucumber roots by SHA application. Many members of this order have been characterized as PGP, showing traits

like P solubilization, siderophore production, BNF, and IAA and CKs production (Gamalero and Glick, 2011; Cabanás et al., 2014, Großkinsky et al.,2016). *Pseudomonas* isolates from Chapter 3 are a good example of the PGP potential of this bacterial order.

iii. Order *Sphingobacteriales* declined in roots from SHA-treated plants in cucumber. *Bacteroidetes* cultivable isolates from Chapter 3 were scarce. On the contrary, SHA promoted this order in barley plants (Chapter 4), what may be indicative of a possible genotype-dependent effect of SHA on the endophytic microbiome; this highlights the importance of studying how the SHA affects the endophytic microbiome functioning (Lemanceau et al., 2017). Could the SHA application be modifying the microbiome functionality, rather than the taxonomy?

iv. Foliar SHA application showed a deleterious effect in some fungal taxa as *Microbotryomycetes* (in root endosphere), *Sordariales* (root and stem compartments), and *Dothideomycetes*, *Agaricomycetes,* and *Saccharomycetes* (in leaves).

The pronounced antifungal effect of foliar SHA application might be due to different mechanisms:

i. ISR induction by SHA application and/or endophytic populational changes.
As we observed in Chapter 1, there is a promotion of JA and SA signaling pathways. The simultaneous effect of both defensive hormones is rarely found because their effects have been reported as inverses (Traw and Bergelson, 2003). In fact, SA commonly is produced by plants when they are attacked by biotrophic and hemibiotrophic pathogens, and the JA signalling pathway is promoted in necrotrophic pathogens attacks. However, it has been reported the ability of endophytes to induce both signalling pathways at the same time, protecting at the same time against two different pathogens (Conn et al., 2008).

ii. Changes in bacterial microbiota can modify the interkingdom balance.
It is known the capability of certain bacteria to produce antifungal compounds or compete against fungi by the same ecological niches (Durán et al., 2018; Hassani et al., 2018). We have seen that SHA application increased the relative abundance of *Actinobacteria*, which have the potential to produce antifungal compounds when they reach certain populational densities (*quorum*

sensing) (Barka et al., 2016), and of *Pseudomonales*, which are able to produce siderophores. These molecules have an antifungal effect by Fe competition (Beneduzi et al., 2012; Ahmed and Holmström, 2014).

iii. Toxic effect of SHA over fungal cells.

In vitro assays have shown that SHA has a growth inhibitory effect in fungi (Pukalchik et al., 2019, and references therein). This inhibition can be linked to the stimulation of antagonistic fungi for saprophytic/pathogenic fungi or to a toxic effect of SHA, probably due to the structure and functional groups of these substances (de Melo et al., 2016; Wu et al., 2016).

Despite we cannot refuse any option or the simultaneous action of all mechanisms, the ISR induction would be the most probable, or the one with a greater influence upon microbial communities. As above-mentioned, the SHA application reduced the richness of fungal OTUs but also reduced the bacterial OTUs in the plant compartment where the SHA application was done. Some authors have proposed that an increase in JA concentration can decrease the endophytic colonization by roots (Miché et al., 2006; Carvalhais et al., 2013; Liu et al., 2017). Hence, the application of SHA would enhance defense systems in plants (Chen et al., 2009; Wang et al., 2015), hindering the bacterial colonization but also reducing the number of fungal OTUs in the endosphere, especially when SHA was applied to the leaves. Conversely, SA is known to modify bacterial microbiome, attracting and increasing the population of *Proteobacteria* (for instance, *Pseudomonales* order) and *Actinobacteria* phyla (Lebeis et al., 2015).

Nitrogen fertilization effect in endophytic microbiome composition and interaction with SHA application

The PERMANOVA analysis for the endophytic microbiome showed that N fertilization affects differently to fungal and bacterial communities. On the one hand, according to PERMANOVA, the fungal community was not influenced by N fertilization; neither the SHA application significantly modified the fungal endophytic microbiome composition of barley plants. It is known that fungal colonization is essentially dependent of stochastic events and biogeography (Lekberg et al., 2012; Peay and Bruns, 2014; Coleman-Derr et al., 2016; Hassani et al., 2018); nevertheless, our barley plants were grown in the same greenhouse under similar conditions. Moreover, several

authors have demonstrated that N fertilization is not a driving factor to fungal communities (Sommermann et al., 2018; Fulthorpe et al., 2019; Revillini et al., 2019).

However, the N fertilization increased the fungal number of different OTUs in barley roots, although this promotion was not observed in those plants also treated with SHA root application. Besides, as observed in Chapter 2 in cucumber, the root application of SHA decreased the number of fungal OTUs in the endosphere of barley roots (SHA fungicide effect) (Pukalchik et al., 2019, and references therein), while increasing the fungal richness in the shoot compartment, though this effect was less pronounced when the SHA application was combined with the N fertilization. Although N fertilization improved the fungal richness in roots, any fungal taxa was especially promoted by this treatment. Only in combination with SHA application, the *Gibberella* genus was significantly promoted (more than 90% of reads) in barley root endospheres. Meanwhile, in the shoot endosphere, the N fertilization increased the abundance of *Gibberella* (65% of reads). Both results could indicate that the N disponibility is essential for the colonization of the endosphere by *Gibberella* fungi. Some members of this genus are known as barley pathogens, *Gibberella zeae* as the causal agent for Fusarium Head Blight (Yuen and Schoneweiss, 2007). Despite this disease can reduce the grain yields (Yuen and Schoneweiss, 2007) several studies have reported that *Gibberella* fungi are common endophytes inhabiting herbaceous crops without relevant disease symptoms (Bacon and Hinton, 1996; Bokati et al., 2016; Pili et al., 2016). Thus, the N fertilization, like other modern agricultural practices, could induce a soil/plant microbiome dysbiosis facilitating the presence of this fungal genus (van Oberbeek and Saikkonen, 2016; Jha et al., 2019).

On the other hand, according to PERMANOVA results, root bacterial communities were significantly influenced by the combination of N fertilization and SHA application. Also, the SHA application alone modified the bacterial composition of root and shoot endospheres. In concordance with PERMANOVA results, we observed that the combination of treatments HA+N+ significantly increased the relative abundance of *Rhizobium* genus in those plants. *Rhizobium* bacteria are well-known N_2 fixers as legume endosymbionts (Carvalho et al., 2014; Jha et al., 2019), but also as a free-living endophytes in grass plants as sugarcane (Yanni et al., 2001; Carvalho et al., 2014; Yeoh et al., 2016). This result was unexpected because, based in previous works (Tan et al., 2003; Carvalho et al., 2014; Yeoh et al., 2016; Jha et al., 2019), we have presupposed that this group of bacteria (N_2 fixers) should decline in N fertilized plant endospheres, and

increase by plant recruitment in unfertilized plants due to the absence of nitrogen. Besides, the combined treatment of SHA and N also increased the bacterial OTUs richness in plant roots; in fact, the N fertilization had a promotion effect in bacterial richness in the root endosphere. Additionally it seems to be concomitant with the shoot growth (FW and DW); in fact it has been suggested that higher microbial diversities can confer plant defense improvements and increase the plant growth (Podolich et al., 2014; Hameed et al., 2015; Berg and Koskella, 2018). Therefore, our results contradict the general statement indicating that N fertilization decreased bacterial diversity (Fuentes-Ramirez et al., 1999; Oliveira et al., 2003; Yeoh et al., 2016; Jha et al., 2019), although Liu and Ludewig (2019) also found an increment of *Rhizobium* in N-fertilized *Miscanthus* plants.

In barley shoot endosphere, the number of fungal OTUs was similar among all the treatments, with a slight increment in the OTU number richness in those plants treated with SHA (HA+N- and HA+N+), as previously observed in SHA-treated cucumber plants (Chapter 2). N fertilization did not have any effect in shoot OTUs richness. In barley shoot endosphere, the *Pseudomonales* order (genus *Acinetobacter*) was the only one significantly promoted by the SHA application. We previously had observed an enhancement of *Pseudomonales* in cucumber roots treated with SHA in Chapters 2 and 3. Genus *Acinetobacter* is a well-known PGP bacteria commonly found in plant rhizospheres and endospheres (Sachdev et al., 2010; Zhang et al., 2017). Furthermore, its plant colonization can be related to changes in SA signaling pathways (Wang et al., 2015), like the changes observed in Chapter 1.

Finally, we have observed that those barley plants treated with the combination of SHA and N fertilization showed the highest shoot DW after 30 days since the SHA application. This result confirmed that the SHA plant growth promotion effect, like the obtained in Chapter 1 and other studies (Mora et al., 2010; 2012; 2014; Olaetxea et al., 2015; 2016; 2019), occurs in plants grown under nutrient sufficiency. However, the SHA application did not promote the plant growth in N-deficient barley plants. This result highlights the importance of N as essential nutrient for plants. Besides, the SHA application was unable to increase the recruitment of BNF microorganisms; and as a result, unfertilized plants showed the lowest DWs. It is possible that as it happens with P nutrition, the application of SHA only improves the nutrition in suboptimal conditions but not in deficient conditions (Urrutia et al., 2014).

Other factors affecting endophytic microbiome composition

Many studies have highlighted the importance of plant host factors like age, genotype, habitat, developmental stage or plant compartment; but also climate factors as temperature, UV light, soil pH, soil type, nutrients soil content, pollutants or pathogens, in the endophytic microbiome composition (Müller et al., 2016; Afzal et al., 2019).

Thus, in Chapters 2 and 4, we have confirmed the great impact of plant compartment (type of tissue) on microbiome composition. That supports the idea of a niche specialization of the microbiome due to the specific conditions of each plant region, as it occurs in the human microbiome (Grice and Segre, 2011). For instance, in both barley and cucumber endophytic microbiomes, the fungal community in aboveground tissues was more phylogenetically diverse (high number of OTUs) than in root endospheres, whereas bacterial communities seem to be more diverse in roots, decreasing the number of OTUs in shoot endosphere. Reinhold-Hurek et al. (2015) described the plant roots as bacteria filters; as a consequence, endophytic root bacterial populations would be a subpopulation from the rhizosphere, and at the same time, endophytic shoot bacterial population would be a subpopulation of the root endosphere (Figure 2). However, fungal colonization, as already mentioned, would be influenced by biogeography (plant location, habitat, weather) and stochastic events as well (Lekberg et al., 2012; Peay and Bruns, 2014; Coleman-Derr et al., 2016; Hassani et al., 2018); therefore, as our results suggested, fungal communities in plants would be a subpopulation of the environmental fungal community with a certain grade of exchange between plant endospheres (Figure 2).

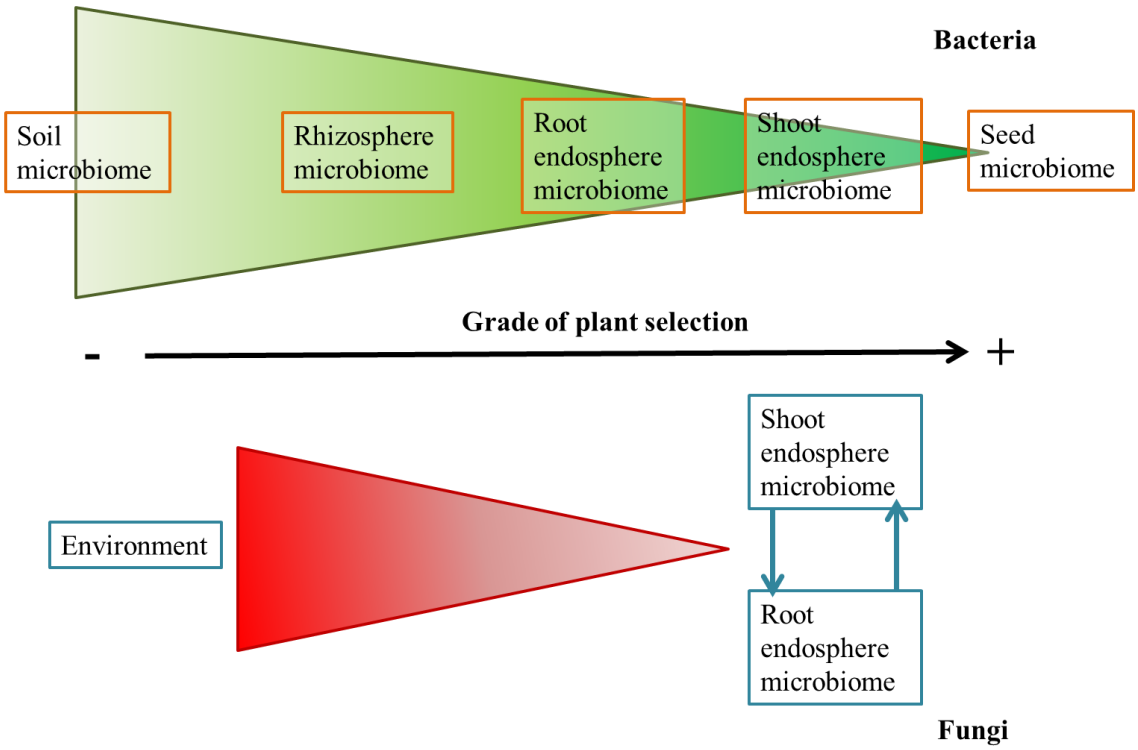

Figure 2: Plant colonization flux for bacterial communities (up, green triangle) and fungal communities (down, red triangle). In the green triangle, the triangle represents the bacterial diversity for each microbiome and the arrow the importance of plant selection grade for the microbiome. The red triangle represents the fungal diversity from the environment to microbiomes, and the blue arrows the OTUs exchange between endophytic microbiomes. In the fungal microbiome, the environment influence for each endosphere can be different.

Also, in Chapter 2, we observed that the composition of microbiomes is dynamic. Thus, the time variable or developmental stage, explained about 5% of microbiome composition variability, both in fungal and bacterial communities. For instance, *Bacteroidetes* phylum in cucumber roots increased in SHA-treated plants along the time, but decreased in control group; or *Basidiomycota* fungi reduced their relative abundances in aboveground cucumber tissues, especially in those plants treated with SHA (Figures 7 and 11 in Chapter 2). Microbiome natural dynamics has been little explored, thus the changes in microbial composition across the time have been little underrated. For instance, Copeland et al. (2015) observed in plant phyllosphere that the bacterial diversity decreased during the growing season of three important crops: soybean, canola and bean; moreover, these authors observed that *Actinobacteria* phylum had a tendency to increase over time, while *Firmicutes* decreased across the time. Although the microbial groups may change between plant genotypes it is important to highlight that microbiomes are dynamic and evolve according to the plant developmental stage.

Moreover, it is important to highlight that, in spite of the similarity between endospheres, the plant species is an important source of diversity between microbiomes. Observing the relative abundances of the main bacterial and fungal phyla and classes, we can figure out which taxa are differentially selected by monocots and dicots. For example, as we observed in Chapters 2 and 3, *Firmicutes* phylum (especially the class *Bacilli*) was an essential part of the microbiome; while in barley (Chapter 4), *Firmicutes* decreased their importance, especially in root barley endosphere. In contrast, *Bacteroidetes* phylum performed conversely for barley (high relative abundance) and cucumber (low abundance). The same happened in fungal communities, being the barley endospheres dominated by *Ascomycota* fungi, while in the cucumber endospheres, *Basidiomycota* fungi were more common to find than in barley. Despite the environment influence (hydroponics-growth chamber vs. soil-greenhouse) should be take into account, several authors have shown in multiple studies that plant genotype is the determining factor in endophytic microbiome composition (Lundberg et al., 2012; Bulgarelli et al., 2013; 2015; Lemanceau et al., 2017; Durán et al., 2018; Hassani et al., 2018).

Potential of SHA application and endophytes as biostimulants

In the framework of this thesis, in which the relationship between SHA plant growth promotion effect and the endophytic microbiome composition has been explored, we have observed the parallelism among the effects exerted by SHA in plants and PGP endophytic bacteria traits. For instance, the root SHA application promotes the root nitrate uptake, the nitrate reductase and the expression of those genes involved in nitrate transportation and assimilation (Mora et al., 2010; Trevisan et al., 2010; Jannin et al., 2012). Furthermore, root SHA application increases the bioavailability of P and Fe and other metals (Urrutia et al., 2014; Jindo et al., 2016; Olaetxea et al., 2018; Zanin et al., 2019), but also rises and regulates the phytohormone concentrations (IAA, ABA, ET, and CKs) (Mora et al., 2010; Olaetxea et al., 2018; 2019).

Potentially, all of these SHA actions could also be mediated by PGP endophytes. As it was observed in Chapter 2 and 4, the SHA application in cucumber and barley plants promoted certain bacterial groups (mainly *Actinobacteria* and *Pseudomonales*) that could display interesting agronomic effects. In fact, we conclude that SHA application could enhance the isolation of interesting PGP endophytes. Endophytes can be used as bacterial inoculum or consortia in combination with SHA or alone, to promote crop

yields as biofertilizers (Mahaffee and Kloepper, 1997; Compant et al., 2010; Bhattacharyya and Jha, 2012; Sarathambal et al., 2015; Kumar et al., 2017; Gouda et al., 2018; Lopez et al., 2018). Their use has been suggested as a promising strategy to improve sustainable agriculture, the main advantages or endophytes are their capability to colonize plant tissues avoiding the competition with soil microbes, and their heritability to following plant generations (Johnston-Monje and Raizada, 2011; Hardoim et al., 2012; Truyens et al., 2013; Khalaf and Raizada, 2016; 2018).

Thus, in Chapter 3, we isolated several endophytic PGP candidates. The isolates belonged to the four most common phyla in plants (*Proteobacteria*, *Actinobacteria*, *Firmicutes,* and *Bacteroidetes*). From all isolates only a few bacterial genera appeared as promising candidates for PGP use:

iv. *Stenotrophomonas* isolates, taxon commonly found in endospheres and not plant pathogenic (Johnston-Monje and Raizada, 2011; Berg and Martínez, 2015), showed 4 out 5 PGP traits. Regarding their traits, we can say that *Stenotrophomonas* could promote the N and Fe nutrition, would act against pathogens (by siderophore production), and they will promote the shoot growth (IAA/CKs ratio displaced to CKs action).

v. *Pseudomonas* isolates are potentially plant pathogens, but our isolates presented low IAA production levels, which indicate low bacterial pathogenicity (Rashid et al., 2012). In addition, these strains were able to live in N free medium, solubilize inorganic P, and produced siderophores like pyoverdin (Gamalero and Glick, 2011). All these traits, in combination with SHA, would enhance enormously the plant nutrition. As it is previously said, the SHA application can increase the relative abundance of these bacteria in the endophyte microbiome.

vi. *Sphingomonas* strains mainly showed balanced phytohormone production (IAA and CKs). The effect on plants of a treatment combining these isolates with SHA could increase the plant growth by the concomitant hormonal boost.

vii. The isolates from the *Micrococcaceae* family (mainly *Arthrobacter* genus) could survive in an N free medium, produced moderate quantities of IAA, and solubilize P. These Gram + bacteria, moreover, were promoted in cucumber root endosphere by SHA application, as we described in Chapter 2.

Future investigations should consider combining one or several isolates with SHA, but also the deep characterization of the most promising candidates. HS are potential microbial carriers that could enhance the survival of the inoculated microbes (Canellas et al., 2015; Olivares et al., 2017). Also, future experiments should check which application strategy is the best, improving the microbial endosphere colonization (Canellas et al., 2015; Van Oosten et al., 2017; Olaetxea et al., 2018; Justi et al., 2019). Finally, a key point in living microorganism applications is the plant developmental stage. Early stages are known to improve the PGP effects and the colonization, and even, these stages favored the endophytic inheritance by seeds colonization (Mitter et al., 2017).

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GENERAL CONCLUSIONS

General Conclusions

The main conclusions of this research about the effect of SHA application on endophytic microbiomes and its potential for developing sustainable agricultural practices are:

1. Foliar application of sedimentary humic acids (SHA) improves the plant development by different structural and biochemical signaling pathways than the root application of these substances.

2. The foliar application of SHA differs from root application in the absence of enhancement of PM H^+-ATPase activity, the lack of promotion of lateral and adventitious root or of changes in ABA concentrations, and the increase in SA concentration. Furthermore, JA and JA-Ile concentrations were increased in leaves and roots, when root application of SHA only increased the concentration of this hormone in roots.

3. The application of SHA in leaves modified their structure. This implied the reduction in trichome density, changes in leaf cuticle thickness, a decrease in the quantity of soluble waxes in cuticle and also a decline in leaf mesophyll starch accumulation.

4. Endophytic microbiome composition was mainly affected by the plant compartment, what underscore the importance to analyze roots and aboveground tissues separately. Then, microbiome composition was also influenced by time, plant genotype and growth system (hydroponics or with soil).

5. The endophytic microbiome composition was modified by the use of SHA regardless of the type of application, the plant genotype, the plant compartment and the growth system.

6. Bacterial communities from plant endosphere were dominated by *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. The endophytic fungal microbiome was mainly composed by *Ascomycota*. Fungal communities were highly variable among individuals.

7. Bacterial endophytic communities were promoted by the use of SHA; certain taxa as *Nocardia*, *Streptomyces* and *Micrococcus* (from *Actinobacteria* phylum) or the order *Pseudomonales* were consistently promoted. Moreover, in N fertilized environments *Rhizobium* bacteria were promoted inside barley tissues by the application of SHA.

8. Fungal endophytic communities were inhibited by the SHA application, especially in foliar application strategy. We propose that this deleterious effect might be due to an ISR induction by SHA and/or endophytes, a modification in the interkingdom balance by changes in bacterial endophytes or by a possible SHA toxicity for fungal cells, or a combination of them.

9. Nitrogen fertilization promoted the barley shoot growth. Besides, fertilization incremented the bacterial and fungal richness in root endosphere. However N fertilization was not a driving factor in endophytic microbiome composition as we observed in PERMANOVA analysis.

10. Endophytes isolated from SHA pre-treated cucumber plants showed PGP traits such as P solubilization, siderophore production, the ability to grow in N-free medium and the production of IAA and CKs. The most promising candidates belonged to *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas* and *Arthrobacter* genera.

SUPPLEMENTARY INFORMATION
DATA AVAILABILITY

All the appendices with supplementary information relevant to this thesis are stored in electronic format in Zenodo (https://zenodo.org).

Links will be accessible during revision and evaluation of this manuscript. Afterwards, all the accessible contents will be subject to the same embargo as the PhD thesis.

A relation of all the supplementary material is listed below, with a brief caption:

Chapter II

The folders can be accessed using the following link: https://doi.org/10.5281/zenodo.3585627

Supplementary Information 1. OTUs tables with the relative abundances of bacterial and fungal endophytes from cucumber plants grown in hydroponics according to the treatment applied and the harvesting time.

Supplementary Information 2. Bacterial and fungal core microbiomes maintened across the time by plant compartment.

Supplementary Information 3. Pairwise comparisons of the relative abundances of bacteria communities among treatments within each taxonomical level (phylum, class, order and family). F-Test, *p≤0.05, **p≤0.01, ***p≤0.001, NaN=absence of data

Supplementary Information 4. PERMANOVA analysis for the bacterial microbiome. Post-hoc test between SHA treatments (Control, Foliar and Root). PERMANOVA analysis for bacterial community by plant compartment and harvest.

Supplementary Information 5. Pairwise comparisons of the relative abundances of fungi communities among treatments within each taxonomical level (phylum, class, order and family). F-Test, *p≤0.05, **p≤0.01, ***p≤0.001, NaN=absence of data

Supplementary Information 6. PERMANOVA analysis for the fungal microbiome. Post-hoc test between SHA treatments (Control, Foliar and Root). PERMANOVA analysis for fungal community by plant compartment and harvest.

Chapter III

The folders can be accessed using the following link: https://doi.org/10.5281/zenodo.3585692

Table S1. Identification and PGP characterization of endophytes extracted from SHA-treated cucumber plants. The identification used as reference databases the BLASTn and RDP Seq-Match assignment of 16S rRNA sequences.

Table S2. Inorganic phosphate solubilization (IPS) ratios for those isolates able to solubilize the inorganic phosphate after seven or fourteen days after incubation.

Table S3. Metalic chelation index (MCI) ratios for those isolates producing siderophores after seven days of incubation.

Chapter IV

The folders can be accessed using the following link: https://doi.org/10.5281/zenodo.3585648

Supplementary Information 1. OTUs tables with the relative abundances of bacterial and fungal endophytes from barley plants grown in greenhouse conditions according to the treatment applied and the plant compartment.

Supplementary Information 2. Barley core endophytic microbiomes for bacterial and fungal communities in the whole plant and by plant compartment.

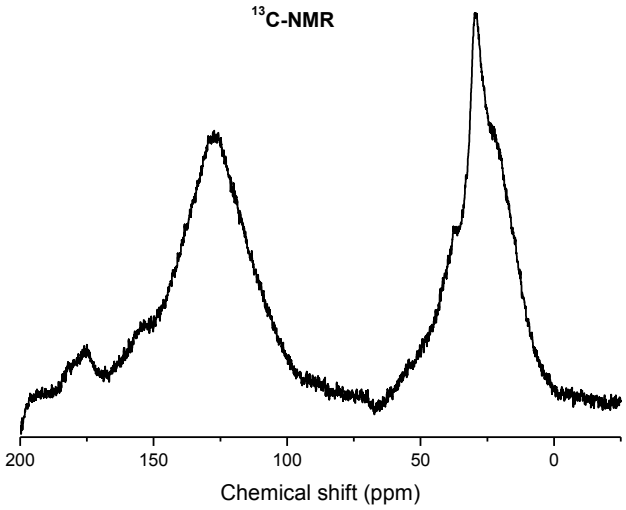
Supplementary Information 3. Pairwise comparisons of the relative abundances of bacterial communities in barley between treatments within each taxonomical level (phylum, class, order, family and genus) by plant compartment. F-Test, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, NaN=absence of data

Supplementary Information 4. PERMANOVA analysis for the bacterial endophytic microbiome in barley roots according to the SHA application and the N-Fertilization. PERMANOVA analysis for the bacterial endophytic microbiome in barley shoots according to the SHA application and the N-Fertilization. PERMANOVA analysis for the bacterial endophytic microbiome in barley plants. The analysis was constrained according to the plant compartment to detect the importance of SHA application and N-fertilization in the entire endophytic bacterial community.

Supplementary Information 5. Pairwise comparisons of the relative abundances of fungal communities in barley between treatments within each taxonomical level (phylum, class, order, family and genus) by plant compartment. F-Test, *p≤0.05, **p≤0.01, ***p≤0.001, NaN=absence of data

Supplementary Information 6. PERMANOVA analysis for the fungal endophytic microbiome in barley roots according to the SHA application and the N-Fertilization. PERMANOVA analysis for the fungal endophytic microbiome in barley shoots according to the SHA application and the N-Fertilization. PERMANOVA analysis for the fungal endophytic microbiome in barley plants. The analysis was constrained according to the plant compartment to detect the importance of SHA application and N-fertilization in the entire endophytic fungal community.

SUPPLEMENTARY INFORMATION
CHAPTER I



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

Figure. S1. ^{13}C-NMR for SHA

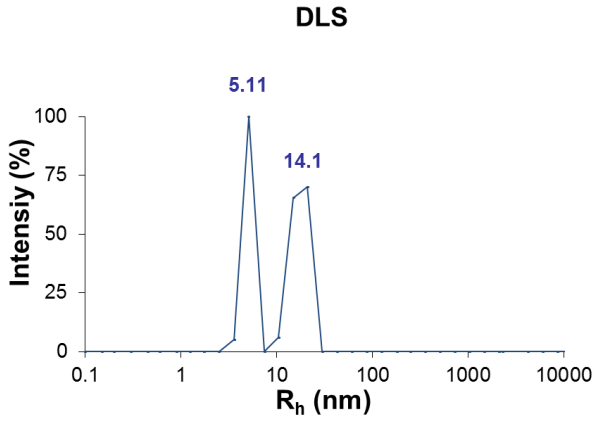


Figure S2. Dynamic light scattering (DLS) analysis of size SHA populations in solution.

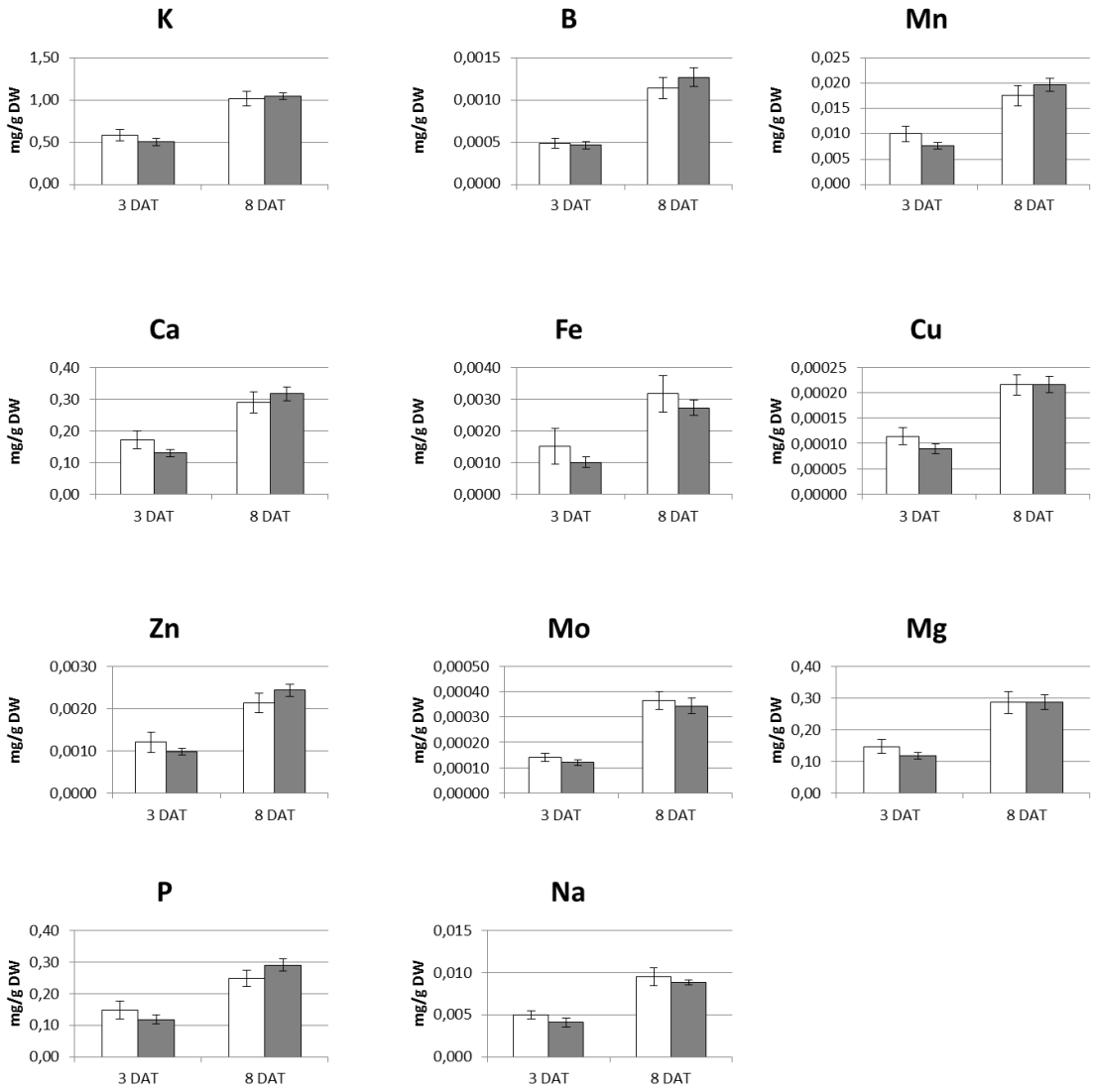


Figure S3. Concentration of mineral nutrients in the leaves of control and 100 mgL-1 SHA foliar sprayed cucumber plant after 3 and 8 days from the onset of treatments.

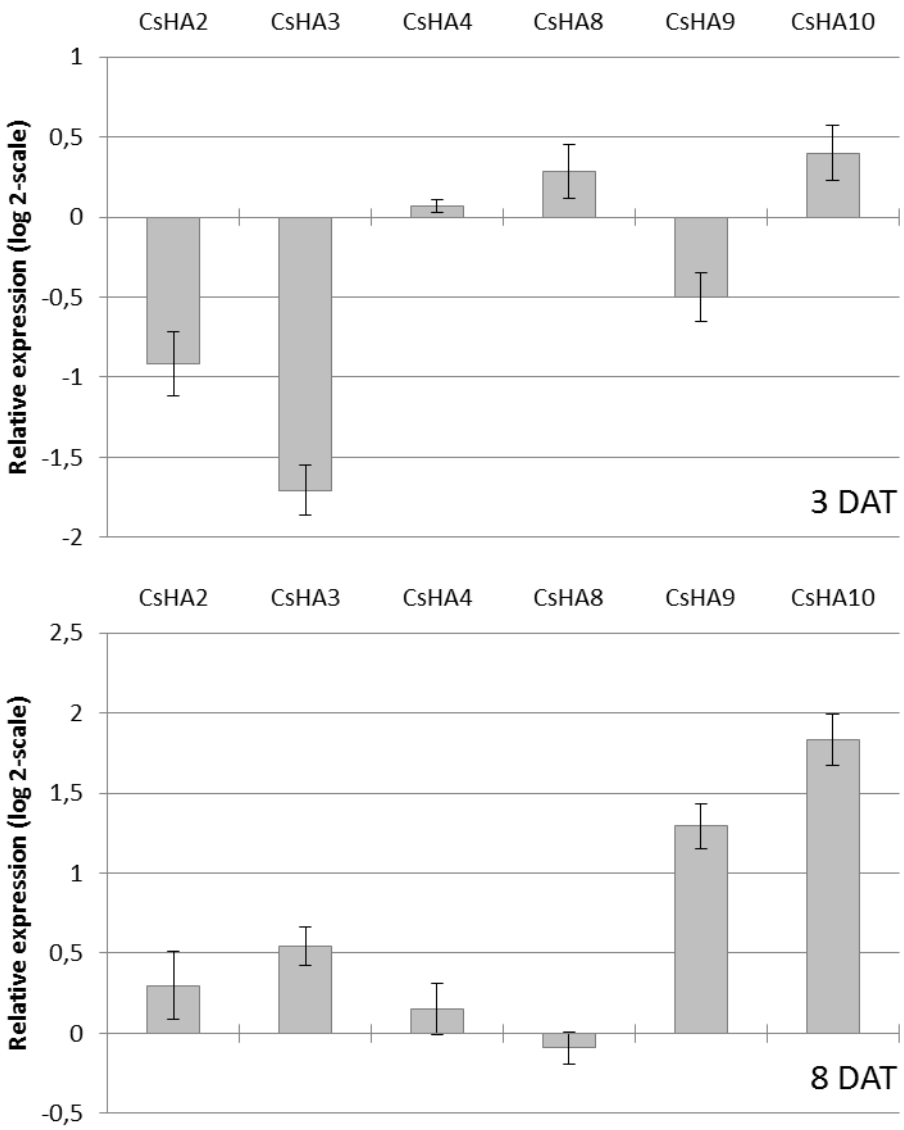


Figure S4. Expression of genes codifying root PM-H$^+$- ATPase isoforms.

Table S1. Elemental Analysis of SHA

	%C	%H	%N	%S	%O*
SHA	48.2	2.99	0.98	0.14	47.7

* by difference

Table S2. Primer sequences used to amplify CsHA isogenes.

Gene name	Primer pairs
CsHA2	For 5`-ACCCGAGTCGACAAACATCT-3` Rev 5`-CTTGGCACAGCAAAGTGAAA-3` (Santi et al., 2005)
CsHA3	For 5`-AAGTTTCTGGGGTTCATGTGGAAT-3` Rev 5`-GTAACAGGAAGTGACTCTCCAGTC-3` (Mlodzinska et al., 2010)
CsHA4	For 5`-CTACAGCTTGGTAACATACATTC-3` Rev 5`-GTTGTAGTCCATGTAATGTCCTC-3` (Wdowikowska & Klobus, 2016)
CsHA8	For 5`-CTCATGCGCAAAGAACATTAC-3` Rev 5`-CTGAATTGTGTCAATGTCAAGTC-3` (Wdowikowska & Klobus, 2016)
CsHA9	For 5`-AAACCAGAAGTGCTGGAG-3` For 5`-CTCAGCACCCTCACTAGTAA-3` (Wdowikowska & Klobus, 2016)
HEMA1	For 5`-GGATGATCTTAGTCGTGGTATAGT-3` For 5`-TGTTCTCCAAGGTTTCACTTAAAG-3`
α-tua	For 5`-GGATGATCTTAGTCGTGGTATAGT-3` For 5`-TGTTCTCCAAGGTTTCACTTAAAG-3`

SUPPLEMENTARY INFORMATION
CHAPTER II

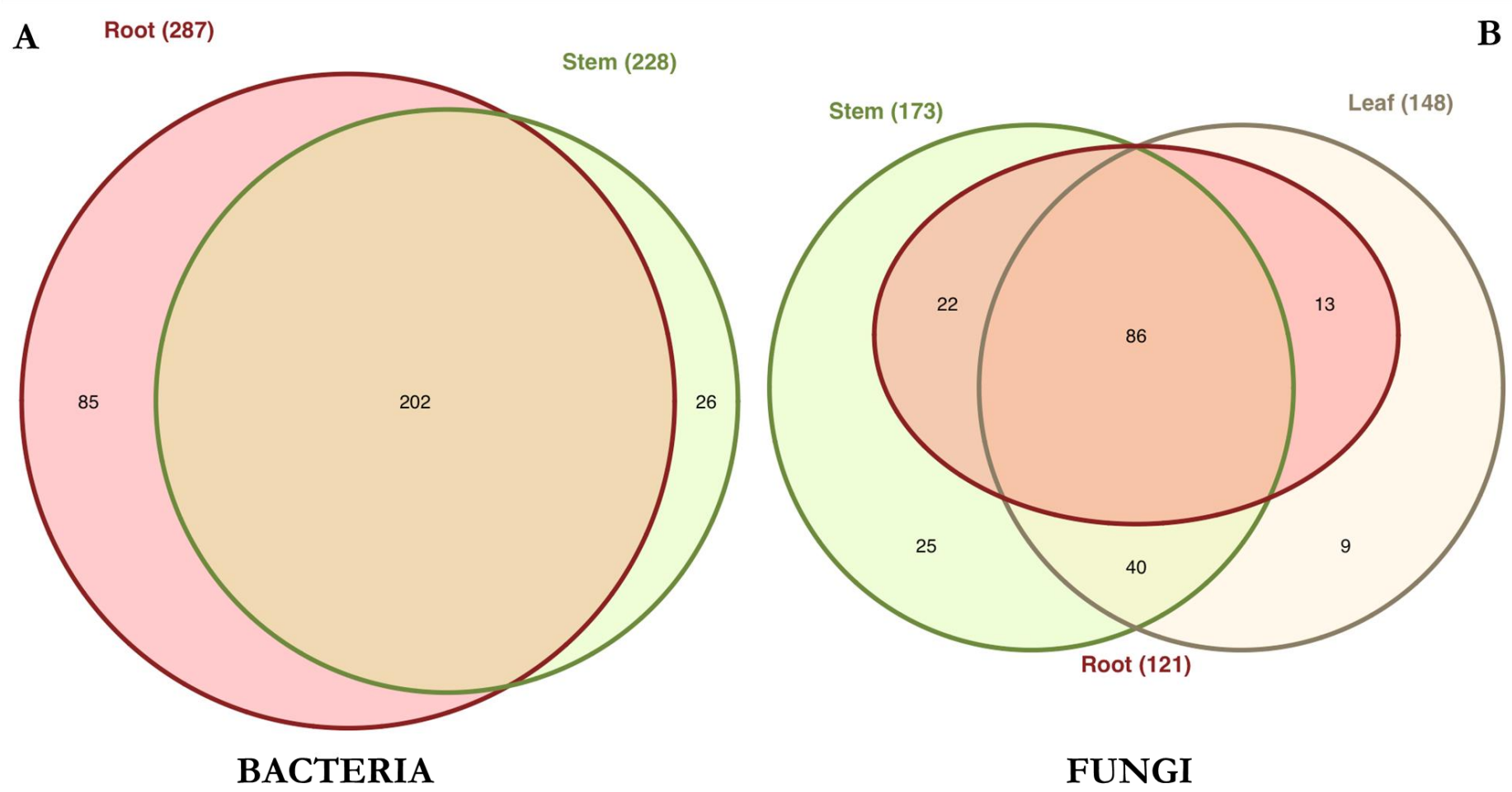


Figure S1. Venn diagrams of shared OTUs between the plant compartments. (A) Shared bacterial endophytic OTUs in cucumber plants. (B) Shared fungal endophytic OTUs in cucumber plants.

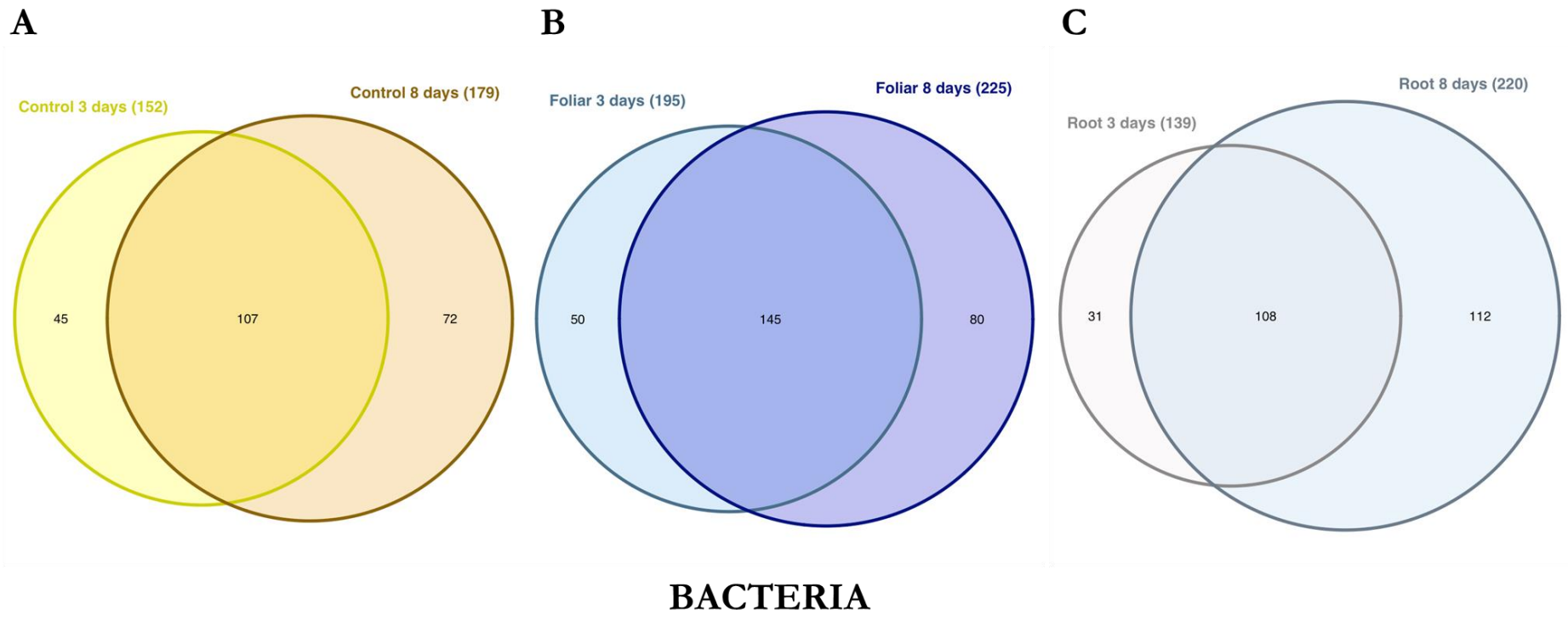


Figure S2. Venn diagrams of shared bacterial endophytic OTUs in root endosphere between the two harvesting times for each SHA treatment. (A) control-yellow, (B) foliar-blue and (C) root-grey.

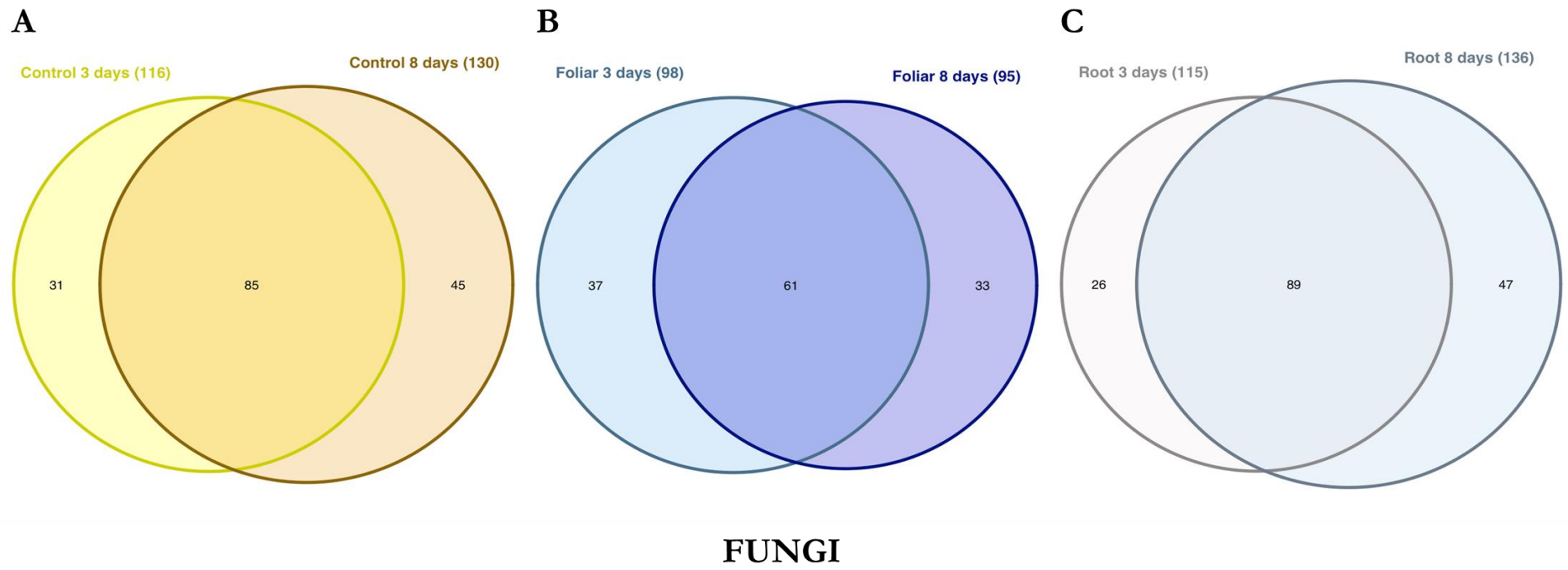


Figure S3. Venn diagrams of shared fungal endophytic OTUs between the two harvesting times for each SHA treatment. (A) control-yellow, (B) foliar-blue and (C) root-grey..

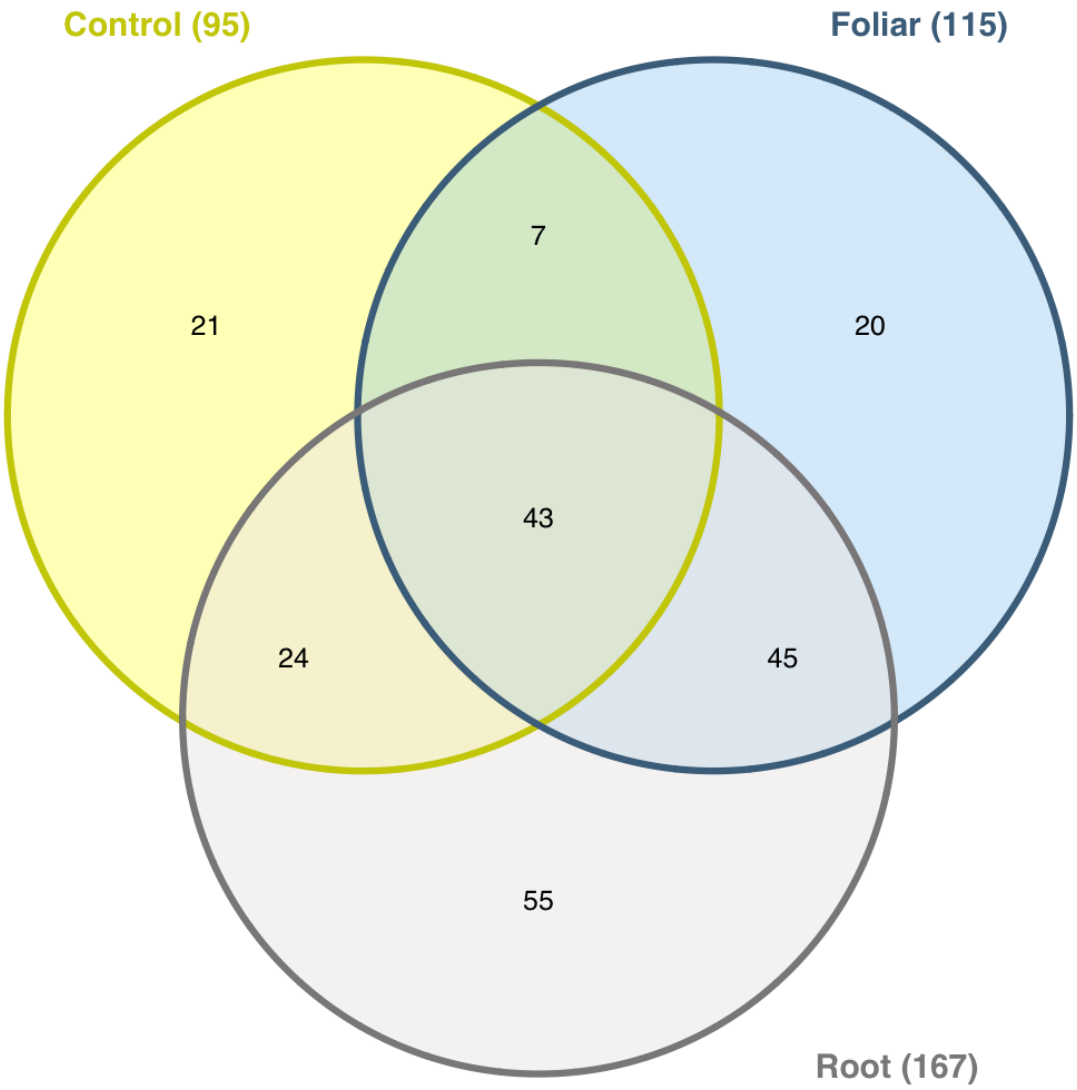


Figure S4. Venn diagram of the number of endophytic bacterial OTUs at 8 days harvest and the three treatments (control-yellow, foliar-blue and root-grey SHA applications) inside the cucumber stem endosphere

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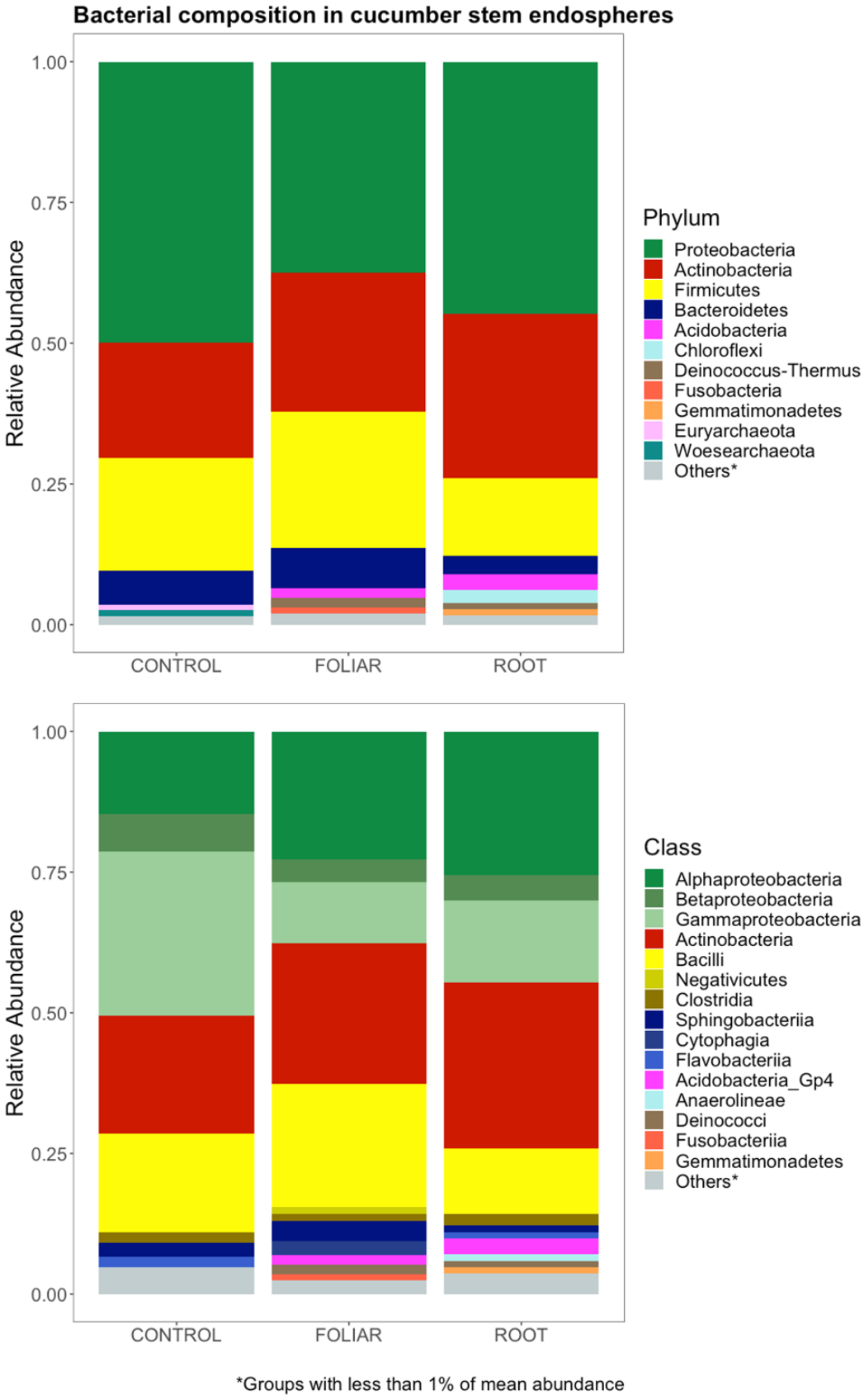

Figure S5. Bacterial phyla and classes relative abundances in stem endosphere for each SHA treatment in cucumber plants.

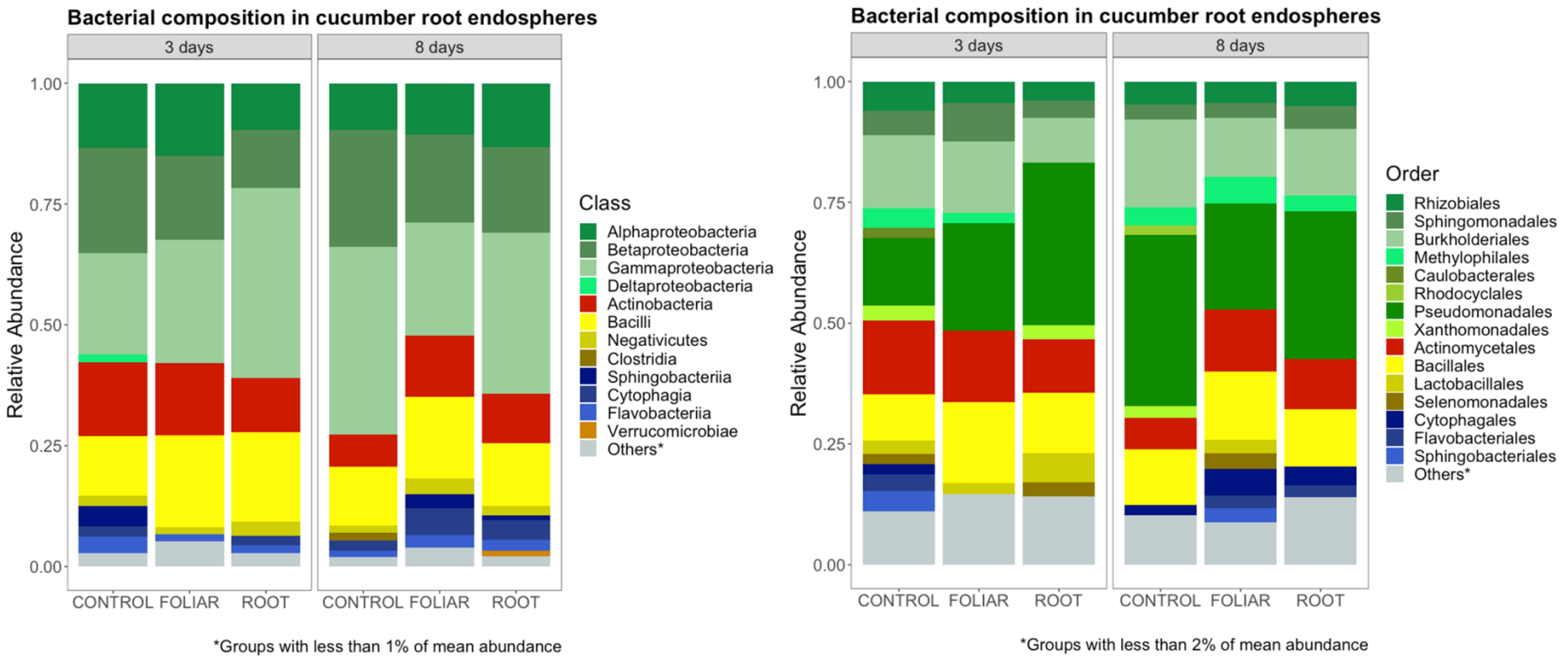

Figure S6. Bacterial class and order level relative abundance bar plots in root endophytic communities by harvesting time and SHA treatment.

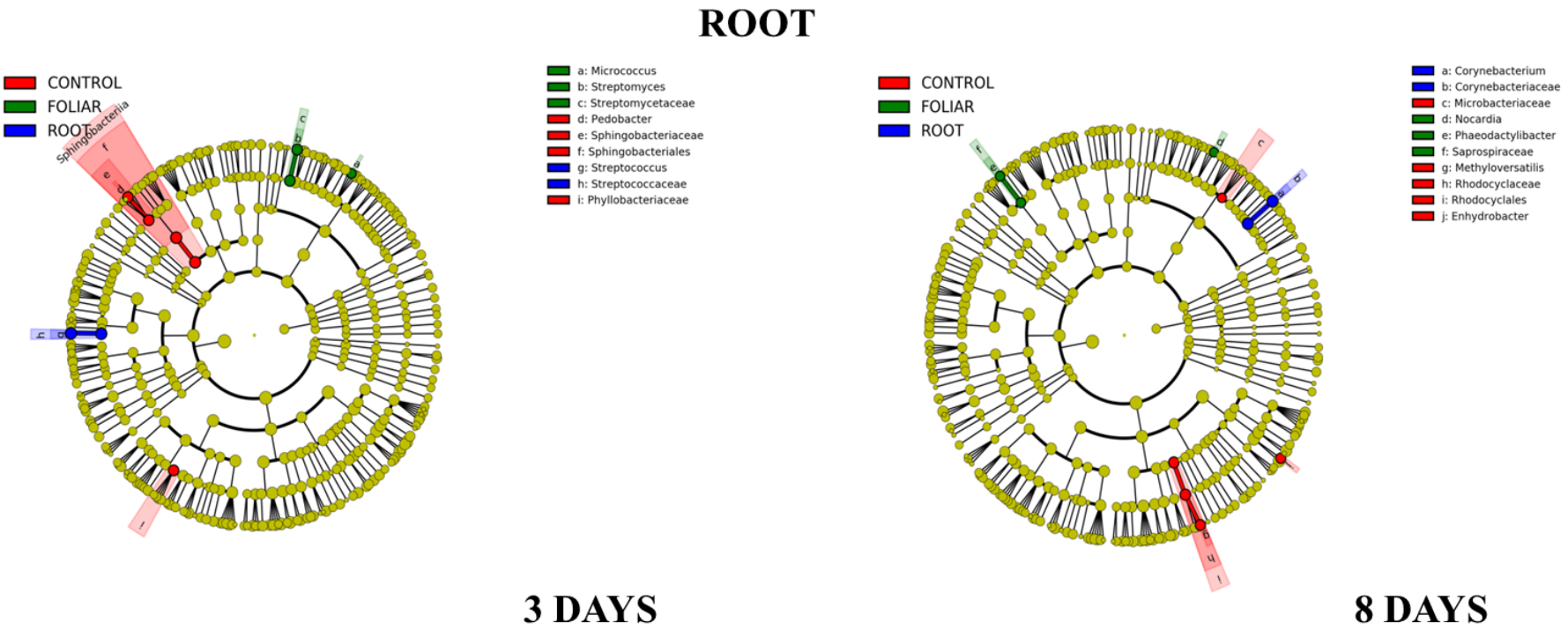

Figure S7. LEfSe biomarker analysis results for bacterial microbiome in root endosphere at each harvesting time.

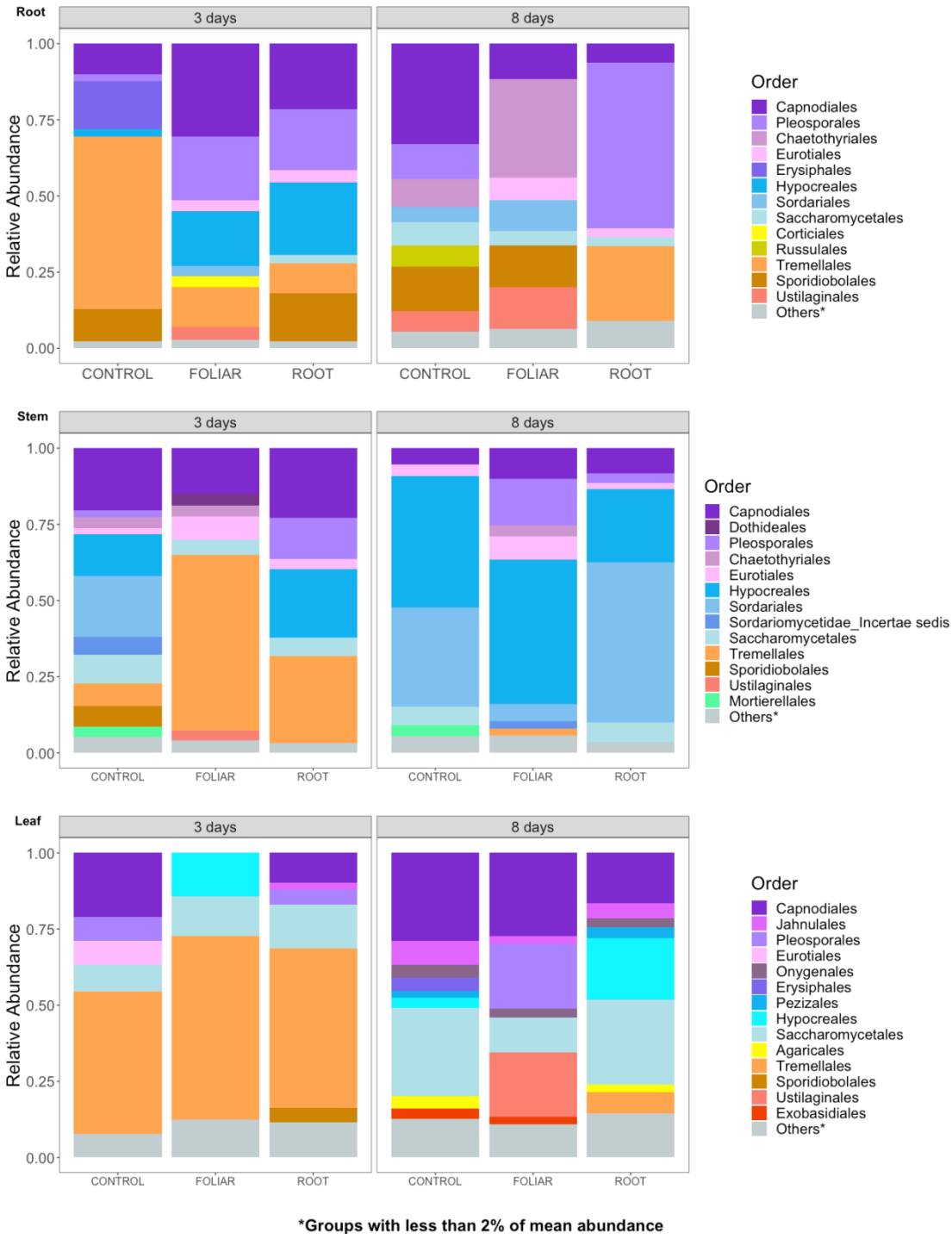

Figure S8. Fungal orders relative abundance bar plots in root, stem and leaf endophytic communities by harvesting time and SHA treatment

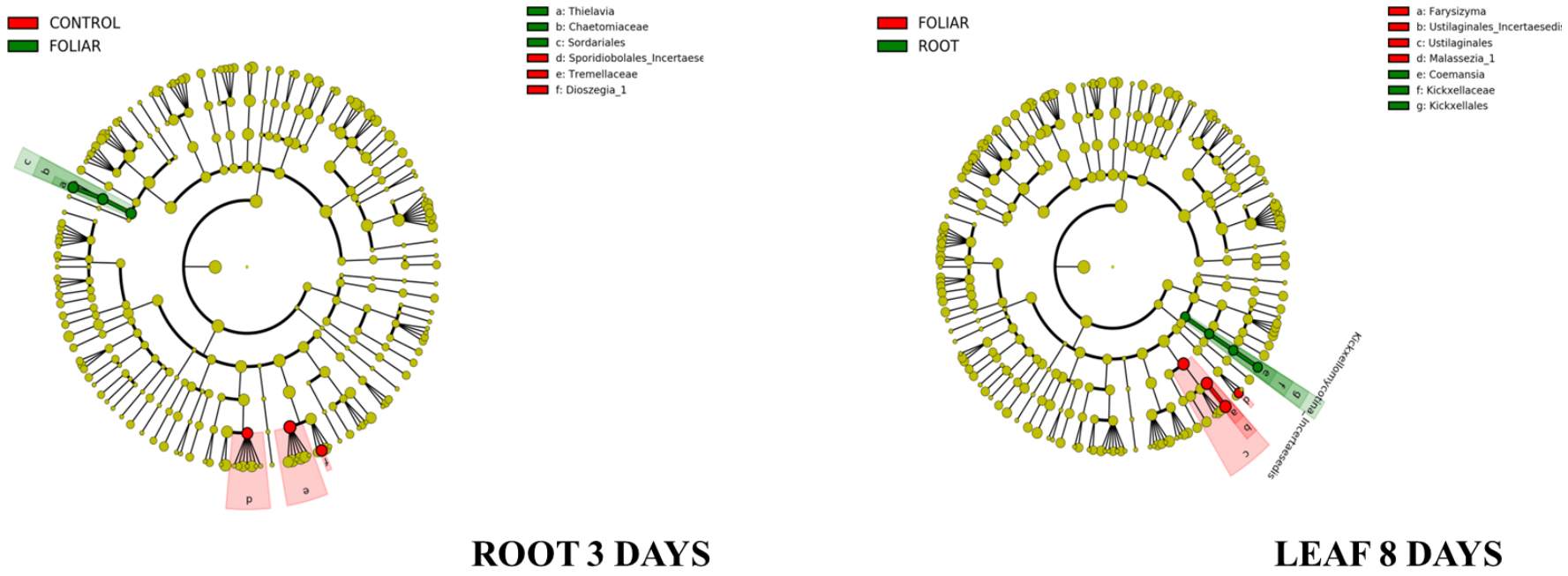

Figure S9. LEfSe biomarker analysis results for fungal microbiome. Only significant differences were found when foliar and root SHA applications were compared. These differences appeared only at 3 days harvest in root endosphere and at 8 days harvest in leaf endosphere.

SUPPLEMENTARY INFORMATION
CHAPTER III

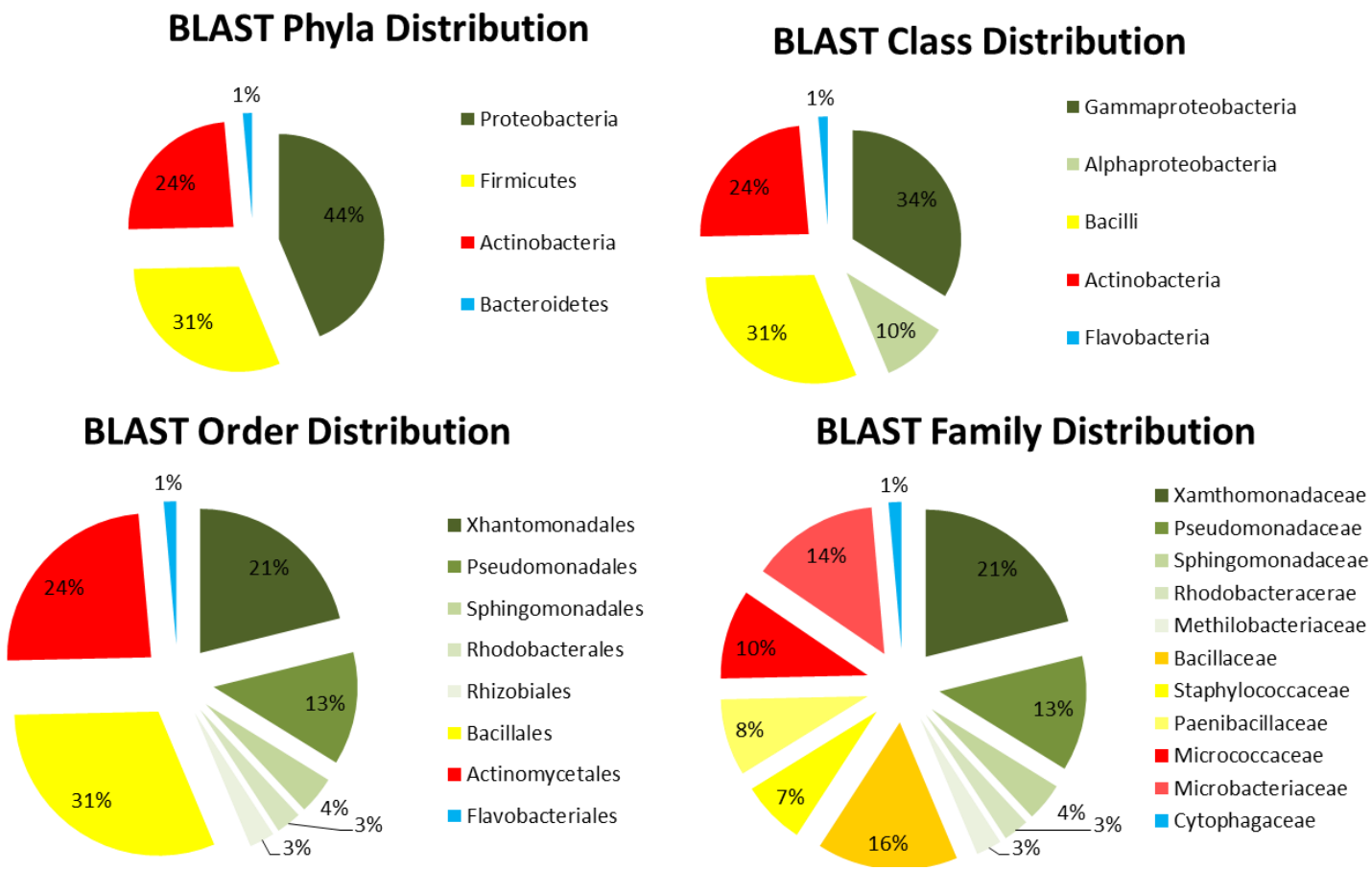

Figure S1. Isolates distribution by phylum, class, order and family.

SUPPLEMENTARY INFORMATION
CHAPTER IV

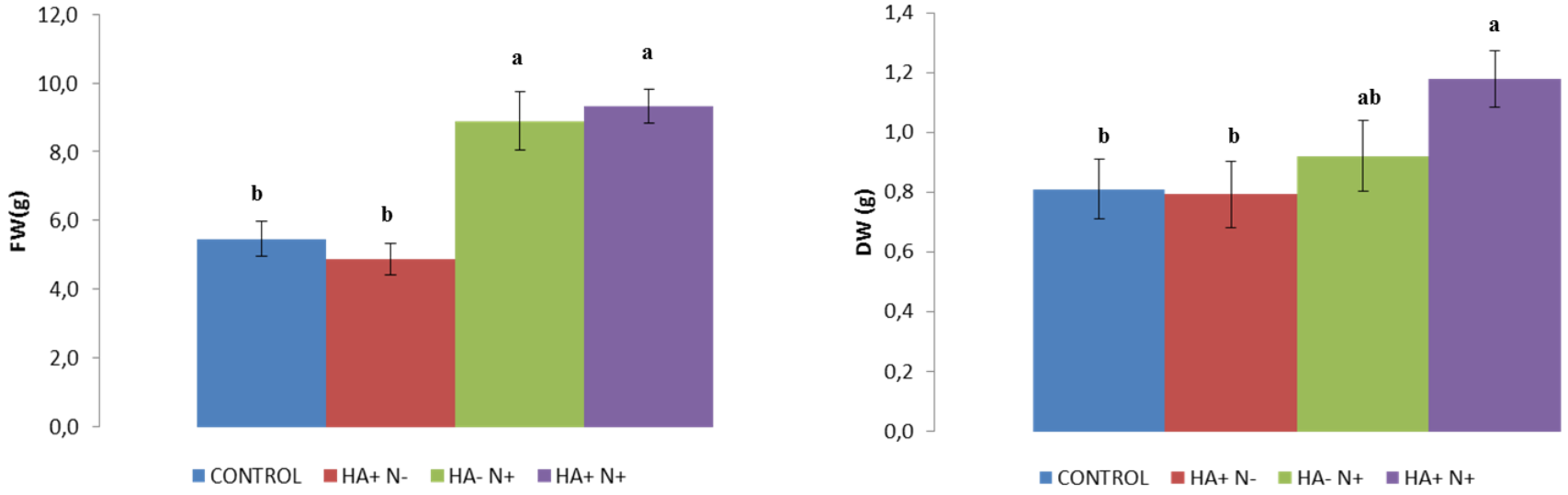

Figure S1. Bar plot for the average FW (g) and DW (g) in barley plants according to the treatments. Error bars represented the standard error (SE); letters represent the statistical significance ($p \leq 0.05$).

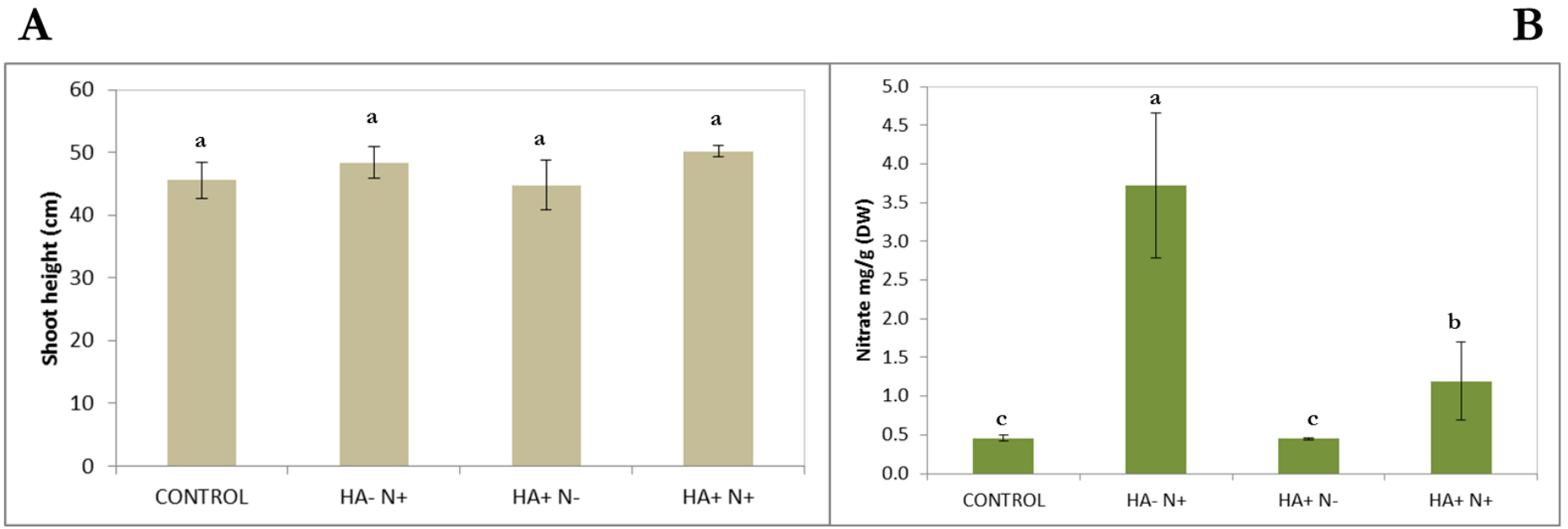

Figure S2. (A) Bar plot for the average length (cm) in barley plants according to the treatments. Error bars represented the standard deviation (SD); letters represent the statistical significance ($p \leq 0.05$). (B) Bar plot for the nitrate content (mg g^{-1} DW) in barley leaves according to the treatments. Error bars represented the standard deviation (SD); letters represent the statistical significance ($p \leq 0.05$).

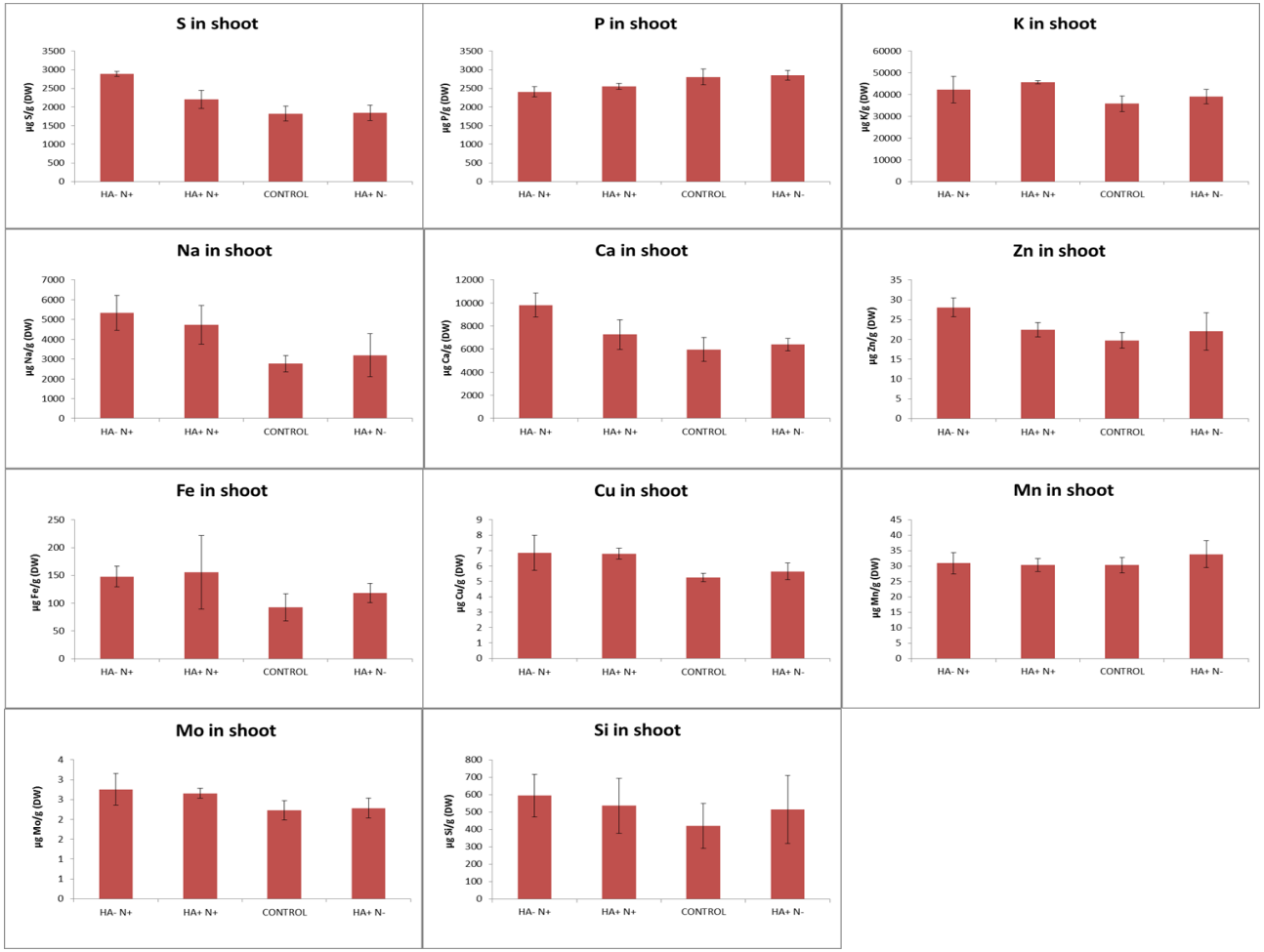

Figure S3. Concentration of main mineral nutrients in the leaves by treatment.

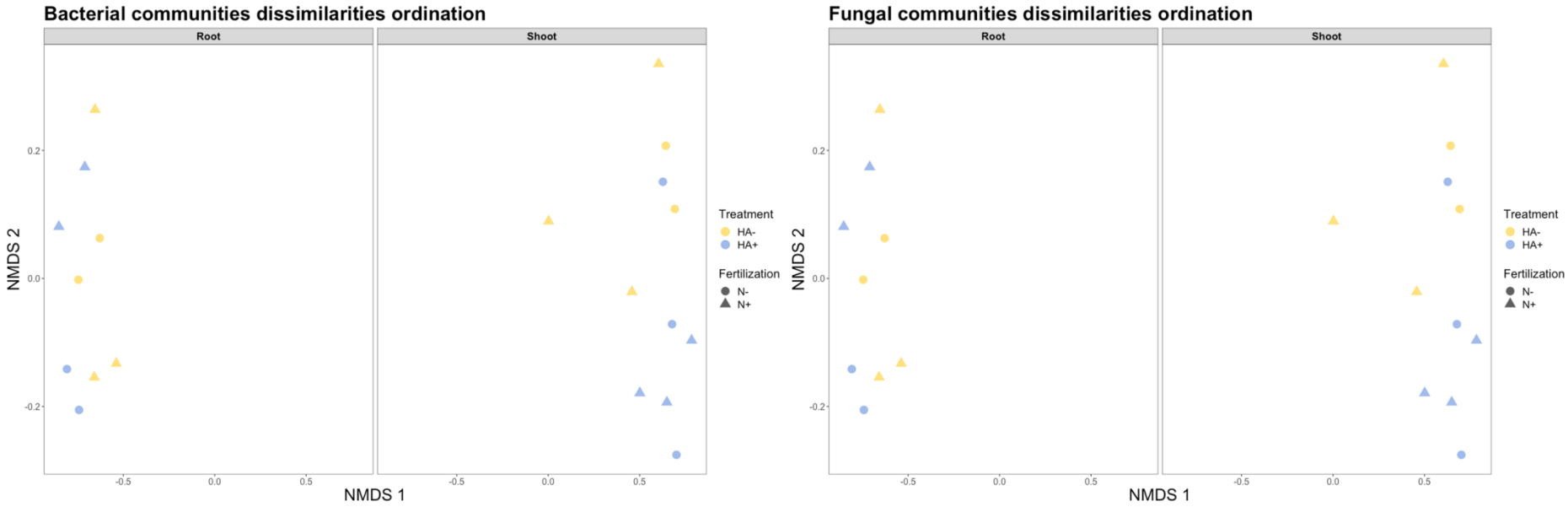

Figure S4. Nonmetric multidimensional scaling (NMDS) plots for Bray–Curtis distances of the bacterial and fungal endophytic communities.

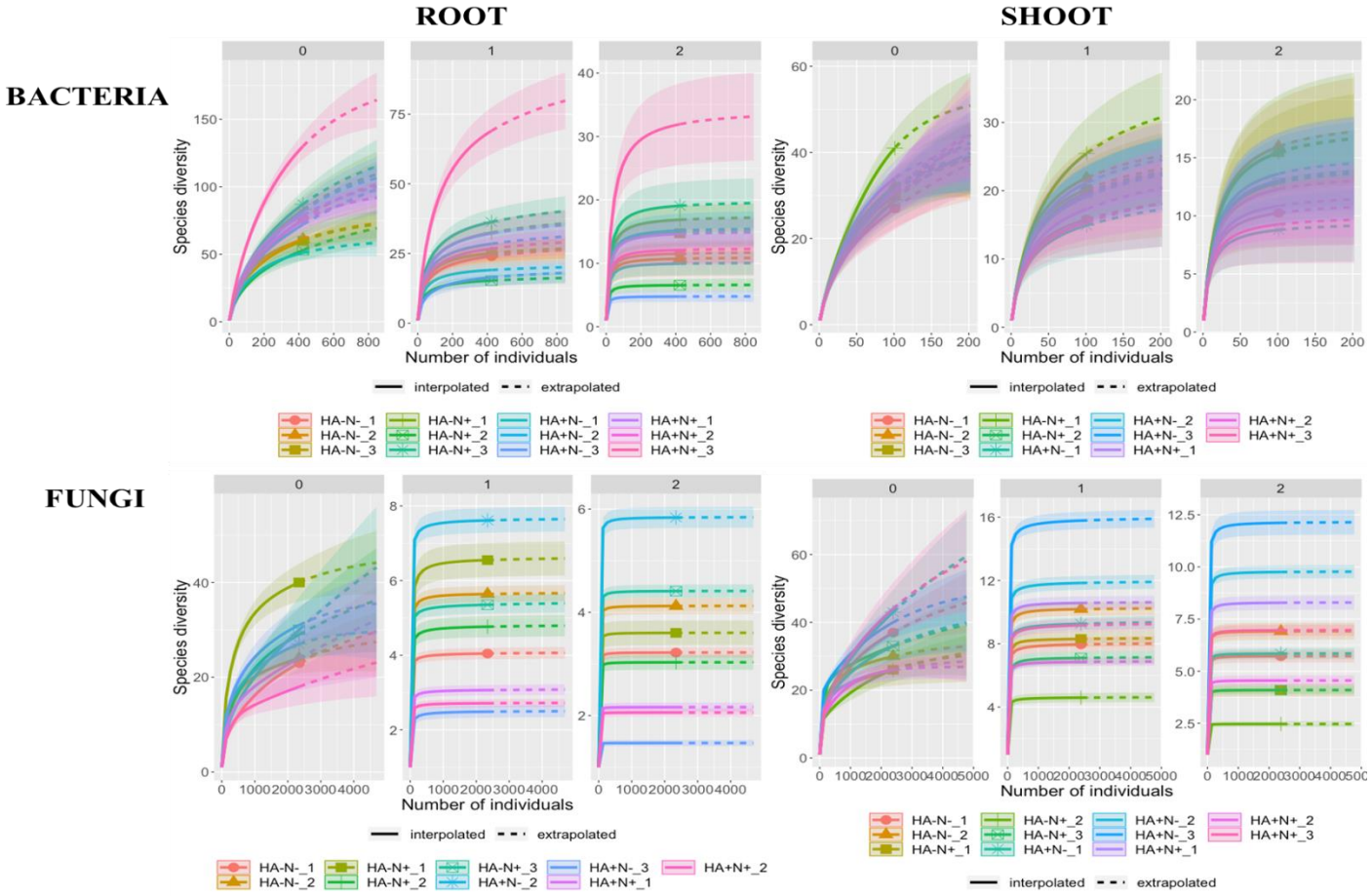

Figure S5. Rarefaction curves for each sample according the microbial community (bacteria or fungi) and plant endosphere (root or shoot). The Hill numbers (q0, q1, q2) measure the effective number of OTUs for richness, Shannon diversity index and Inverse Simpson index.

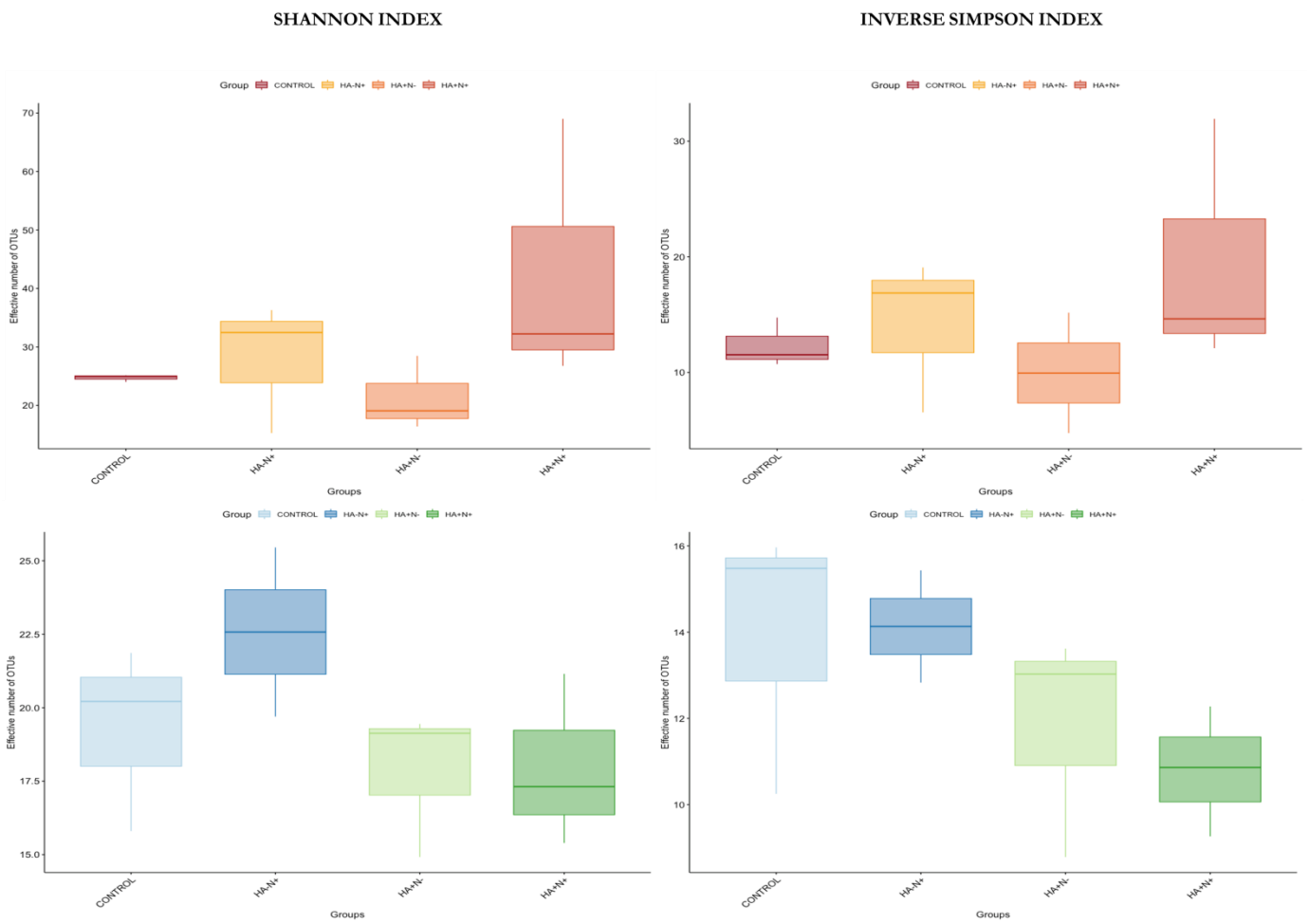

Figure S6. Boxplots of the estimated effective number of OTUs for Shannon index (H') and Inverse Simpson (D) in the bacterial communities of each plant compartment (root-warm colors, shoot-cold colors) of barley plants by treatments applied.

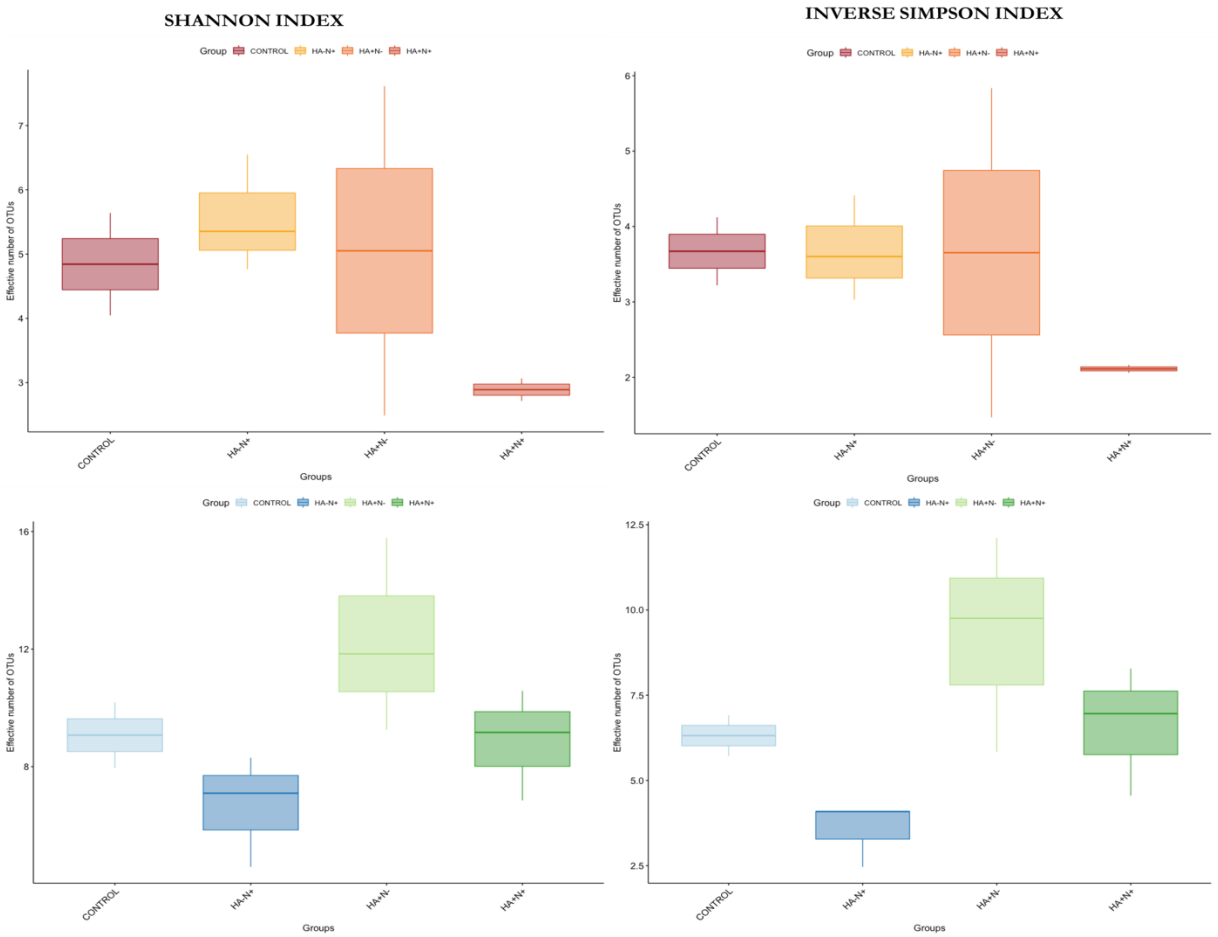

Figure S7. Boxplots of the estimated effective number of OTUs for Shannon index (H') and Inverse Simpson (D) in the fungal communities of each plant compartment (root-warm colors, shoot-cold colors) of barley plants by treatments applied.

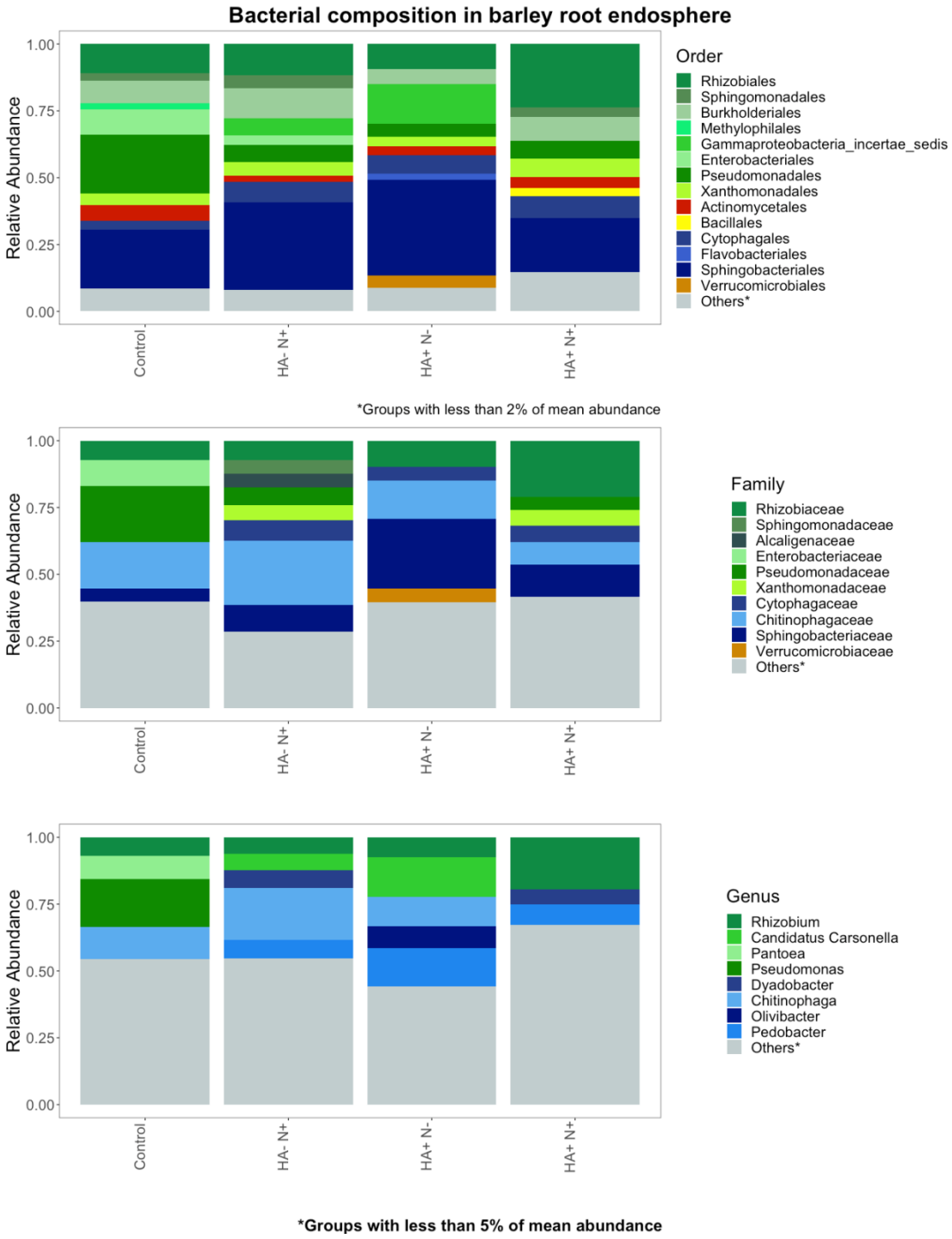

Figure S8. Bacterial order, family and genus levels relative abundances bar plots in barley root endosphere by treatment.

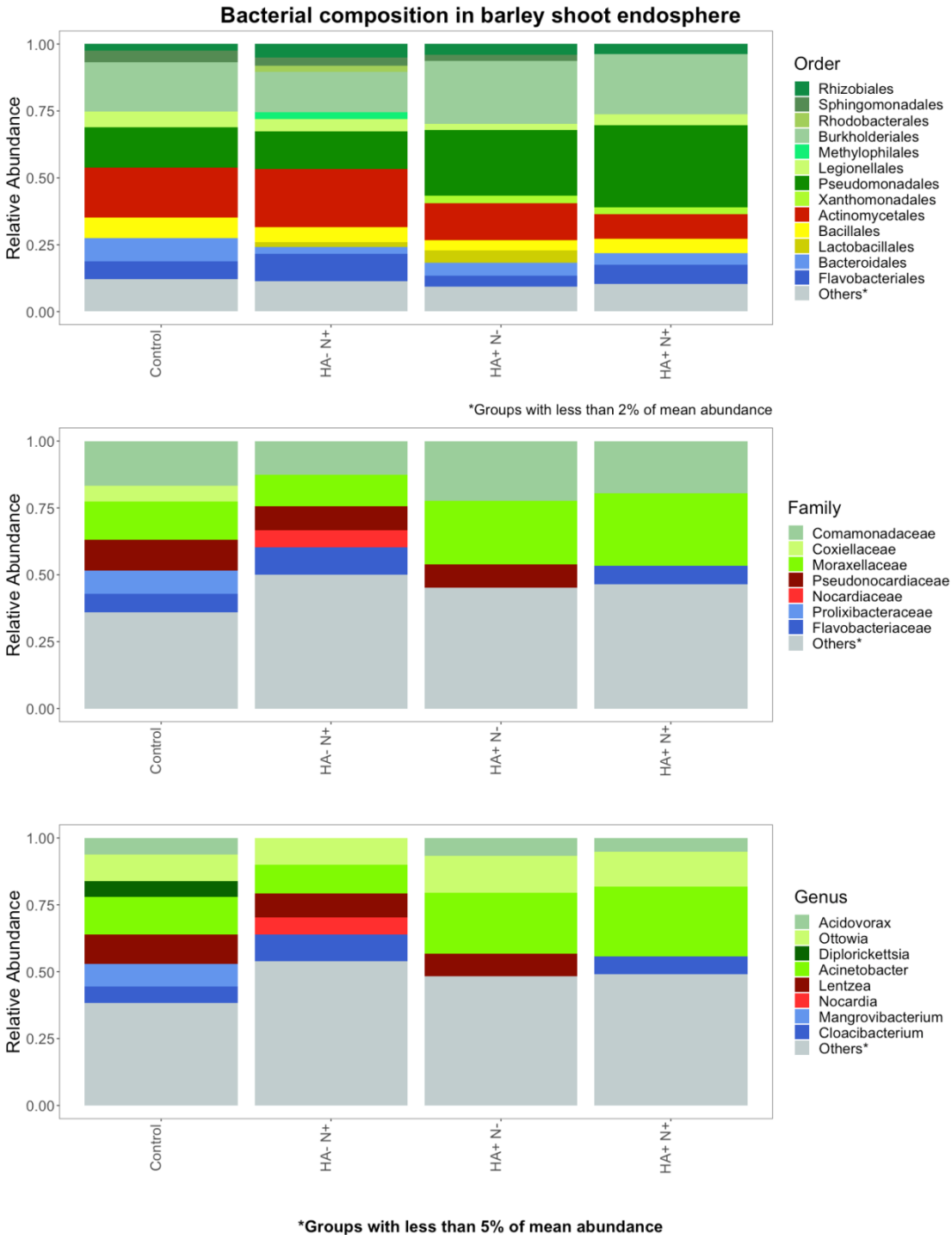

Figure S9. Bacterial order, family and genus levels relative abundances bar plots in barley shoot endosphere by treatment.

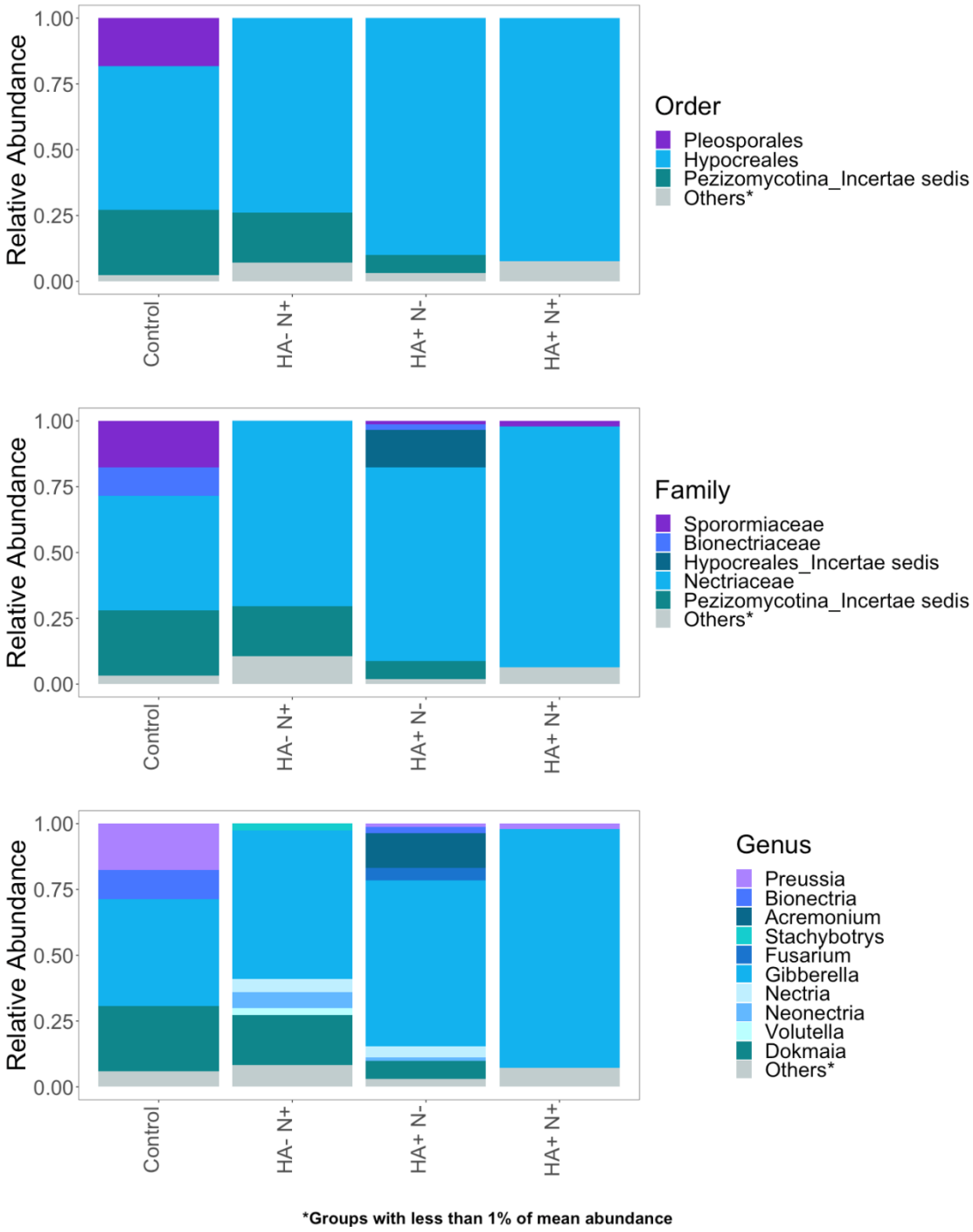

Figure S10. Fungal order, family and genus levels relative abundances bar plots in barley root endosphere by treatment.

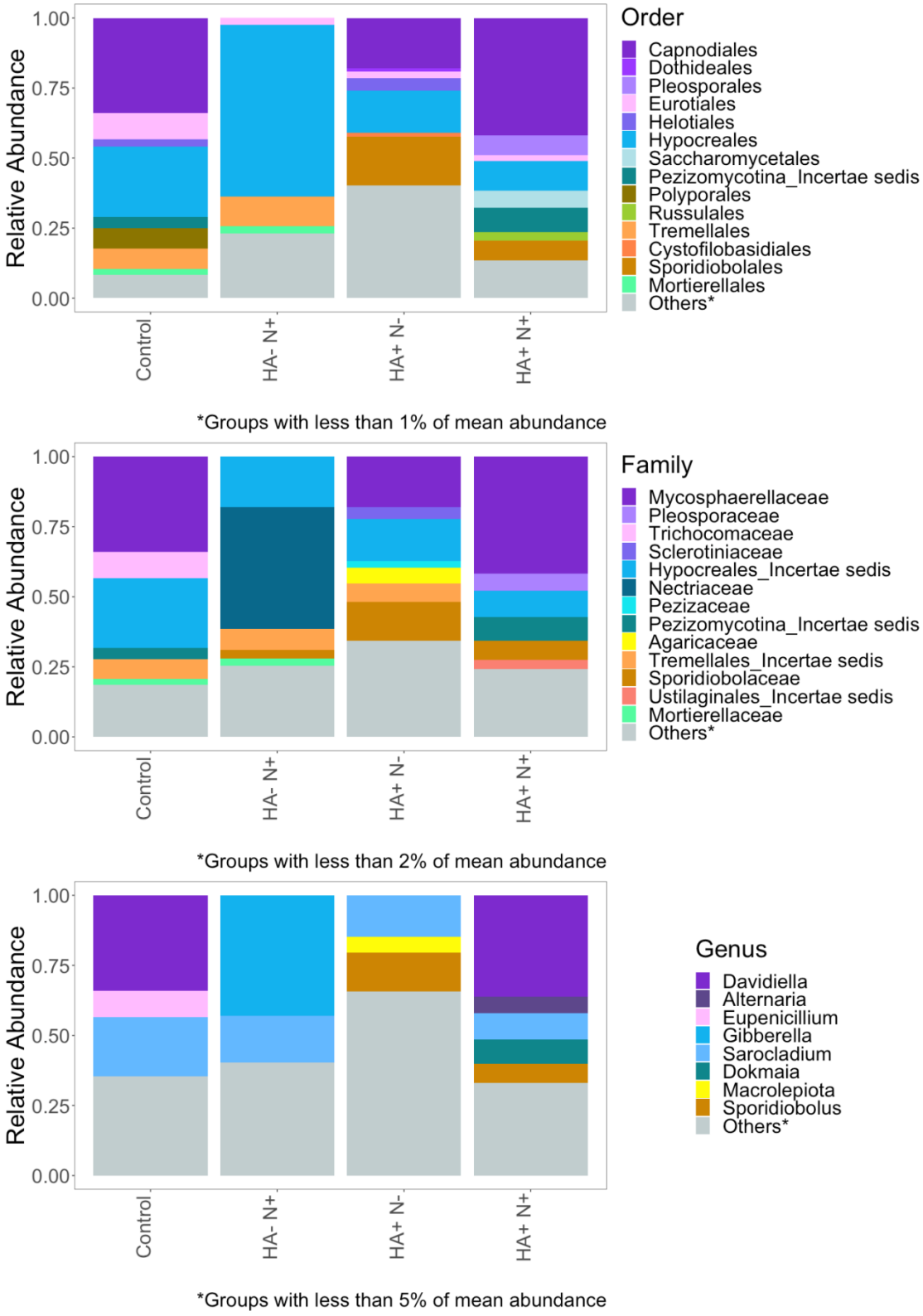

Figure S11. Fungal order, family and genus levels relative abundances bar plots in barley shoot endosphere by treatment.

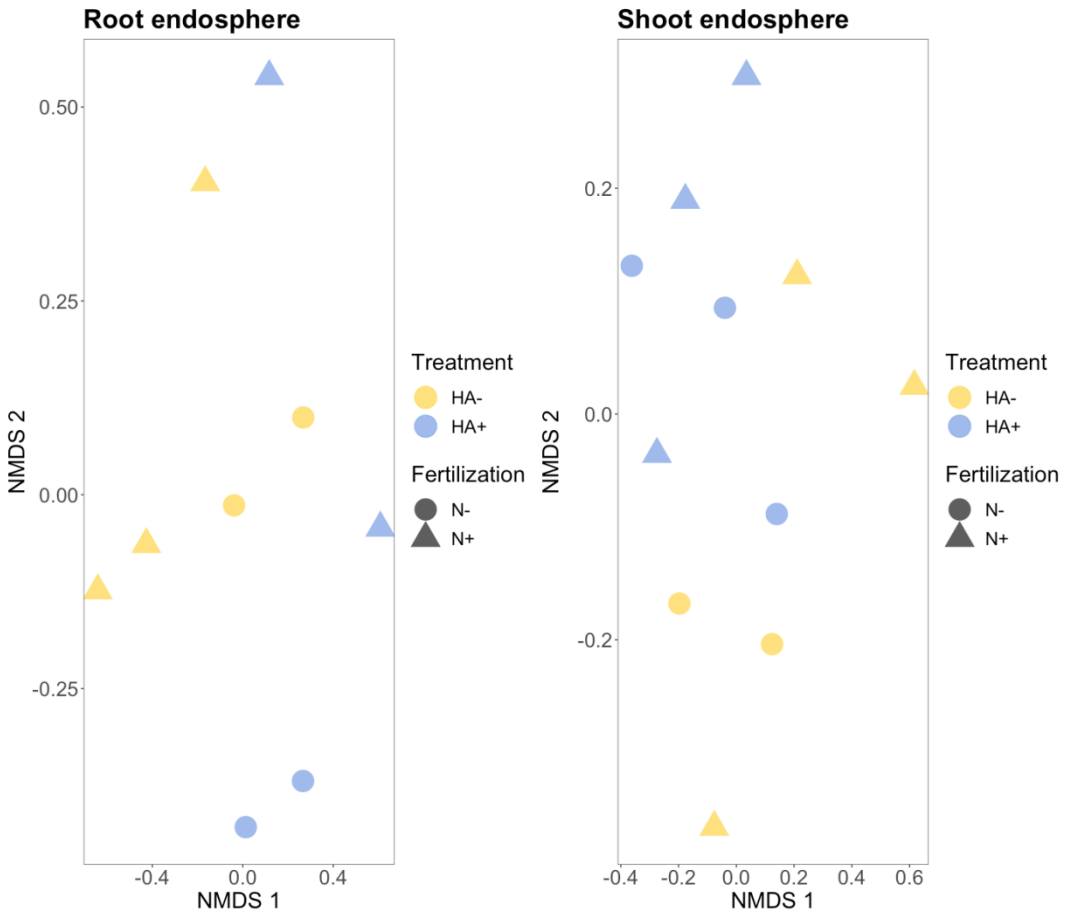

Figure S12. Nonmetric multidimensional scaling (NMDS) plots for Bray–Curtis distances of the fungal endophytic communities within each plant compartment and treatment applied.