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MÓNICA YORLADY ALZATE ZULUAGA

EFEITO DE BACTÉRIAS ASSOCIATIVAS E DO ESTRESSE SALINO SOBRE A FUNCIONALIDADE DA RIZOSFERA E O DESENVOLVIMENTO VEGETAL

Londrina
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Orientador: Prof. André Luiz Martinez de Oliveira

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RESUMO

A inoculação com bactérias promotoras do crescimento vegetal (BPCV) representa um método eficiente para melhorar a disponibilidade de nutrientes e a produção agrícola, além de proporcionar uma maior tolerância contra estresses como a salinidade. O objetivo deste trabalho foi investigar a modulação da exsudação radicular de tomate (*Solanum lycopersicum*) e da atividade metabólica do microbioma associado à rizosfera, como resposta à inoculação com duas bactérias benéficas (*Enterobacter* sp. 15S e *Pseudomonas* sp. 16S) e à imposição de estresse salino (NaCl 100 mM). O uso da abordagem metabolômica UHPLC/QTOF-MS permitiu identificar padrões metabólicos distintos dos exsudatos radiculares de tomate, com acúmulos diferenciais impostos por cada bactéria ou pela salinidade. Compostos cruciais do metabolismo primário e secundário tais como aminoácidos, ácidos orgânicos, mas principalmente a exsudação de compostos fenólicos, flavonoides, fitohormônios e fitosideróforos, foram relacionados a mecanismos de colonização e interação planta-bactéria, promoção do crescimento vegetal e tolerância ao estresse por salinidade. Adicionalmente, o uso da metologia de Biolog EcoPlate para avaliar os perfis fisiológicos da comunidade microbiana (PFCM) permitiu observar uma preferência metabólica diferencial do rizomicrobioma associado ao tomate, na qual carboidratos, ácidos carboxílicos e aminoácidos desempenharam um papel decisivo no domínio das diferenças. Além disso, diferenças significativas no aumento da biomassa vegetal foram observadas, em função da estirpe bacteriana utilizada e da condição ambiental avaliada. Enquanto a inoculação com *Pseudomonas* 16S mostrou um efeito mais pronunciado no desenvolvimento do tomate sob estresse salino, aumentando a tolerância do tomate à salinidade, a inoculação com *Enterobacter* 15S mostrou-se mais eficaz em condições sem estresse.

Palavras-chave: Bactérias Promotoras do Crescimento Vegtal (BPCV). Microbioma associado à rizosfera. Metabolômica. Biomarcadores. Salinidade.

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ABSTRACT

Inoculation with plant growth-promoting bacteria (PGPB) represents an efficient method to improve nutrient availability and crop production, besides to providing greater tolerance against stresses such as salinity. The aim of this work was to investigate the modulation of tomato root exudation, as well as the metabolic activity of the rhizosphere-associated microbiome, in response to the inoculation with two beneficial bacteria (*Enterobacter* sp. 15S and *Pseudomonas* sp. 16S) and salt stress imposition (100 mM NaCl). On the one hand, the use of the UHPLC/QTOF-MS metabolomic approach allowed to identify distinctive metabolic patterns in tomato root exudates, with different accumulations dependent either on each bacterium or salinity. Crucial compounds from primary and secondary metabolism such as amino acids, organic acids, but mainly the exudation of phenolics, flavonoids, phytohormones and phytosiderophores, were related to mechanisms involving colonization and plant-bacteria interactions, promotion of plant growth and tolerance to saline stress. On the other hand, the use of the Biolog EcoPlate method to assess the community level physiological profiling (CLPP) allowed to identify a differential functional activity of the rhizomicrobiome, in which carbohydrates, carboxylic acids and amino acids played a decisive role in dominating the differences. In addition, significant increases in tomato biomass were observed as a function of the bacterial strain used when compared with untreated plants ($p < 0.05$). In this regard, while inoculation with *Pseudomonas* 16S showed a more pronounced effect on tomato development under salt-stress imposition, increasing salt tolerance in tomato, inoculation with *Enterobacter* 15S proved to be more effective under normal conditions without salinity imposition.

Key-words: Plant growth-promoting bacteria (PGPB). Rhizosphere-associated microbiome. Metabolomics. Biomarkers. Salinity.

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1 INTRODUÇÃO

A rizosfera pode ser definida como a zona ao redor da raiz onde acontecem importantes processos para o crescimento e a saúde das plantas, e abriga uma alta densidade de diversos organismos (BAKKER et al., 2013; MENDES; GARBEVA; RAAIJMAKERS, 2013). A manipulação dos processos que acontecem na rizosfera reflete mudanças dinâmicas na biologia e química da rizosfera relacionadas com as interações entre plantas, solo e microrganismos, proporcionando uma abordagem eficaz para melhorar a eficiência do uso de nutrientes, a produtividade das culturas e a resolução de alguns problemas críticos que o planeta enfrenta, incluindo a sustentabilidade agrícola, o melhoramento da qualidade da água, a mitigação das mudanças climáticas e a preservação da biodiversidade (SHEN et al., 2013; TRABELSI; MHAMDI, 2013).

Através das raízes, as plantas liberam na rizosfera uma enorme variedade de compostos orgânicos e inorgânicos biologicamente ativos denominados de rizodepósitos ou exsudatos radiculares (UREN, 2007), constituídos principalmente por compostos de baixo peso molecular, como aminoácidos, ácidos orgânicos, açúcares, fenólicos e compostos de alto peso molecular, como polissacarídeos e proteínas (BAIS et al., 2006; BADRI; VIVANCO, 2009). Esses exsudatos são conhecidos por apresentarem multiplicidade de funções, agindo como moléculas chave na mobilização e aquisição de nutrientes, assim como em processos de sinalização entre planta-microrganismos ou mesmo entre planta-planta (BAETZ; MARTINOIA, 2014; BOWSHER et al., 2016). Variações na concentração e composição dos exsudatos podem exercer efeitos estimulantes ou inibitórios sobre o crescimento e desenvolvimento das plantas, e desempenham um papel valioso na configuração do microbioma da rizosfera (CANARINI et al., 2019). Sabe-se também, que a atividade microbiana na rizosfera aumenta quando a taxa de exsudação é alta, favorecendo o crescimento de bactérias copiotróficas, capazes de metabolizar grandes quantidades de substratos simples, como açúcares, aminoácidos e ácidos orgânicos liberados pelas plantas na rizosfera, principalmente no período em que as plantas são fotossinteticamente ativas (HAASE et al., 2007; PHILLIPS; FINZI; BERNHARDT, 2011).

Adicionalmente, condições ambientais externas e diversos estresses bióticos e abióticos alteram o metabolismo primário e secundário tanto das plantas como do seu fitomicrobioma associado (KARLOWSKY et al., 2018; SKLIROS et al., 2018), levando a uma reprogramação metabólica da planta que permita manter o seu metabolismo essencial nas condições de estresse prevalecentes (OBATA et a., 2012). Nesse sentido, há uma diversidade de metabólitos que potencialmente podem estar envolvidos na regulação das respostas as condições de estresse. Por outro lado, a inoculação de plantas com bactérias benéficas tem mostrado um grande potencial para modular as respostas fisiológicas das plantas numa dada condição de estresse, contribuindo com o aumento da tolerância e sobrevivência das plantas, além de contribuir com o acumulo de metabólitos secundários na planta (XIE et al., 2019).

Nos últimos anos, uma grande variedade de metodologias multidisciplinares tem sido desenvolvidas com a finalidade de ter uma melhor compreensão dos mecanismos fisiológicos e moleculares que auxiliam na compreensão da dinâmica resultante da interações entre plantas e microrganismos. Por exemplo, a metabolômica não direcionada é descrita como a abordagem mais adequada para a análise de interações complexas, facilitando a compreensão dos processos metabólicos que acontecem nas plantas e na rizosfera (MHLONGO et al., 2018). Por outro lado, como a disponibilidade de fontes de carbono é o principal fator que domina o crescimento microbiano no solo, o perfil fisiológico de comunidades microbianas (PFCM) baseado em padrões únicos de utilização de fontes de carbono, fornece um método eficaz, simples e rápido para medir a atividade metabólica funcional do microbioma do solo e da rizosfera (LLADÓ; BALDRIAN, 2017).

Nesse contexto, a exploração dos processos ancorados na rizosfera, especialmente as relações entre as plantas, a matriz do solo e sua microbiota associada, bem como o papel dinâmico da exsudação de plantas no estabelecimento da microbiota da rizosfera, poderiam fornecer novas abordagens de manejo para a agricultura, aumentando a eficiência do uso de nutrientes pelas plantas e mitigando as tensões ambientais (GUPTA et al., 2015; ZHALNINA et al., 2018). Nesse sentido, com base num estudo anterior descrito por Zuluaga et al., (2020), duas bactérias, *Enterobacter* 15S e *Pseudomonas* 16S, foram selecionadas com base nas suas habilidades de produção de fitohormônios e fitosideróforos, como uma alternativa viável para promover o crescimento vegetal e auxiliar na tolerância do tomate ao estresse salino. Para estudar o impacto da inoculação com *Enterobacter* 15S e *Pseudomonas* 16S, e o efeito da imposição do estresse salino na rizosfera do tomate (*Solanum lycopersicum*), duas abordagens integrativas foram utilizadas neste estudo: (1) metabolômica não direcionada por UHPLC-ESI/QTOF-MS para elucidar o perfil metabólico dos exsudatos radiculares de tomate, visando compreender as interações planta-microrganismo e planta-microrganismo-estresse na rizosfera e; (2) Biolog EcoPlates para obter e comparar o perfil fisiológico de comunidades microbianas, caracterizando a diversidade metabólica da comunidade microbiana associada à rizosfera do tomate como resposta aos tratamentos de inoculação e à salinidade.

Este trabalho será apresentado em seções na forma de artigos em inglês que serão enviados para publicação em revistas internacionais. Para manter a característica original dos artigos, os mesmos foram mantidos em inglês quando inseridos na tese. Dessa forma, esta tese esta dividida da seguinte forma:

Seção 2 – São apresentados o objetivo geral e objetivos específicos da tese.

Seção 3 – Apresenta um artigo de revisão, equivalente a revisão bibliográfica da tese.

Seção 4 – Artigo de pesquisa no qual foram discutidas as mudanças no perfil de exsudação radicular de tomate e na atividade metabólica funcional da comunidade microbiana associada à rizosfera, em resposta à inoculação com duas bactérias benéficas (*Enterobacter* 15S e *Pseudomonas* 16S).

Seção 5 – Artigo de pesquisa no qual, após os resultados obtidos descritos no artigo anterior, decidiu-se avaliar se a inoculação bacteriana com *Enterobacter* 15S e *Pseudomonas* 16S, seguida pela imposição do estresse salino (NaCl 100 mM), conduzem a importantes mudanças no perfil de exsudação radicular na atividade metabólica funcional da rizomicrobiota de tomate, com o intuito de identificar biomarcadores envolvidos na tolerância ao estresse salino.

Seção 6 – Apresenta uma conclusão geral da tese.

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2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar as mudanças no perfil metabólico de exsudatos radiculares e a atividade metabólica funcional do microbioma associado à rizosfera de tomate como resposta à inoculação com duas bactérias benéficas associativas (*Enterobacter* sp. 15S ou *Pseudomonas* sp. 16S) com ou sem a imposição de estresse salino (NaCl 100 mM).

2.2 OBJETIVOS ESPECÍFICOS

- Elucidar o perfil metabólico dos exsudatos radiculares de tomate utilizando uma abordagem metabolômica não direcionada por UHPLC-ESI/QTOF-MS em condições de inoculação bacteriana e/ou sob imposição de estresse salino.
- Agrupar e discriminar os compostos envolvidos na resposta do tomate à inoculação bacteriana e salinidade, por meio de análise estatística multivariada.
- Identificar os metabólitos responsáveis pela discriminação entre os tratamentos e avaliar a razão da expressão (fold-change) em relação ao controle.
- Detectar possíveis moléculas biomarcadoras envolvidas tanto nas interações de colonização pelas bactérias benéficas como na tolerância à salinidade.
- Avaliar a versatilidade metabólica da comunidade microbiana associada à rizosfera do tomate em função da inoculação bacteriana e do estresse salino, utilizando o método Biolog EcoPlate.
- Utilizar ferramentas de análise multivariada para determinar as fontes de carbono preferencialmente metabolizadas pelo microbioma associado à rizosfera de tomate.

3 REVIEW PAPER

ENGINEERING THE RHIZOSPHERE: IMPLICATION OF PLANT-EXUDED BIOMOLECULES ON THE SOIL-MICROBIOME

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ABSTRACT

The rhizosphere is the soil region that extends closely from root surface and has modulated its physical, chemical and biological characteristics by the compounds released from the plant roots and associated microbiota. Plants under normal conditions of growth are extremely active in releasing organic compounds from their roots, also referred as rhizodeposits, encompassing a wide variety of biomolecules released in the rhizosphere. These molecules ranges from low (e.g., carbohydrates, amino acids, organic acids, phenolics) to high molecular weight compounds (e.g., polysaccharides, proteins), and are thought to be involved in key processes which modulates the plant-environment interactions, including the plant interactions with the soil microbiota. The release of biomolecules from plant roots induces modifications in the rhizosphere microbiome, by decreasing the microbial diversity but resulting in an increase of specific microbial communities, distinct from those found in the non-rhizospheric soil (bulk soil), a phenomenon known as rhizosphere effect. It is currently assumed that the plant microbiome engineering can improve the phytosanitary and productivity status of commercial crops, once promoting the dominance of the plant microbiome by beneficial microorganisms can optimize functions such as crop productivity and plant tolerance against environmental stresses. As plant-microbe communications play major roles in defining the composition of rhizosphere microbial communities, a better understanding of chemical and biological importance of root exudates in shaping the plant microbiome is fundamental, and in this sense, metabolomic approaches are of ultimate importance to reveal the metaorganisms (plant and associate microorganisms together) behavior. This review intends to summarize the current comprehension on rhizosphere composition and the putative functions for the main biomolecules exuded by plant roots over the soil microbial community, as well as to highlight the importance of the rhizosphere engineering as a tool to aid the increase of beneficial microorganisms. Methodological concepts related to the collection and analysis of root exudates with focus on metabolomic approaches are also discussed. Once different techniques can be devised to study the rhizosphere metabolomics, this review does not intend to exhaust this subject. Instead, we attempt to highlight milestone studies using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) on rhizosphere metabolomics.

Key-words: root exudates, rhizomicrobiome, plant-microbe interactions, beneficial microorganisms, sustainable agriculture.

3.1 INTRODUCTION

The importance of root exudates in rhizosphere formation has been increasingly demonstrated. However, the quali-quantitative determination of the chemical composition of root exudates is still an analytical challenge, due to its intrinsic complexity and the difficulty to accessing by adequate sampling, in addition to the labile character of its compounds which are usually found in low concentrations (RUGOVA et al., 2017). The continuous improvement of analytical techniques aimed to large-scale analysis of the chemical profiling in complex mixtures has led to the evolution of high-throughput approaches which have been applied to study the pool of biomolecules present in organic fluids, where metabolomics lies. Metabolomic studies have been successfully used to improve the knowledge of dynamics and regulation of processes taking place in multi-molecular environments, such as those found in the rhizosphere (WHITE et al., 2017; RAZZAQ et al., 2019). A huge amount of interactions between plants and its surrounding environment take place at the rhizosphere, and the biomolecules constantly released and modified in this environment mediate signaling events between the biota and the soil. Increases in carbon flow on the rhizosphere also promote the maintenance of high activity and microbial biomass in this environment, differing from those found in the bulk soil due to the phenomena known as “rhizosphere effect” and “rhizosphere priming” (BERENDSEN; PIETERSE; BAKKER, 2012; PAUSCH; KUZYAKOV, 2018).

Root exudation in the rhizosphere is the major pathway by which plants and soil microbial communities are connected, and play a key role in ecosystem response to environmental change. A wide set of evidence shows that the composition of root exudates is altered by a high variety of biotic and abiotic environmental factors (HASSAN; MCINROY; KLOEPPER, 2019). In this sense, the identification of the compounds that influence the soil microbial community structure and function can help to build novel strategies for improving plant performance and increasing crop yield and sustainability (HUANG et al., 2014). Thus, the implementation of rhizosphere metabolomics studies is an important strategy for understanding the mechanisms behind the functional relationships in this habitat. For instance, by using untargeted metabolomics, Scherling et al. (2010) reported that variation in light or nutrient availability, induce physiological adaptations of individual plant species. Moreover, other metabolomics studies have reported that increased production of signaling molecules such as plant hormones, osmolytes and secondary compounds originated from the phenylpropanoid pathway, in response to different stressors, may be considered as important biomarkers of stress tolerance (GUPTA; DE, 2017; KARLOWSKY et al., 2018; SKLIROS et al., 2018). Composition of root exudates can also shape root microbiome assembly to promote plant health, as reported in *Arabidopsis*, where the excretion of antimicrobial coumarins stimulated the presence of pathogen resistance-inducing and growth-promoting rhizobacteria (STRINGLIS; DE JONGE; PIETERSE, 2019). Additionally, Hu et al. (2018) showed that root exudates metabolites released by maize (*Zea mays*) roots alter root-associated fungal and bacterial communities, increase jasmonate signalling and plant defences, and suppress herbivore performance in the next plant generation. Other studies have also reported root exudates as the main mechanisms influencing microbial nitrogen cycling independently of other physicochemical characteristics of the rhizosphere (SCHMIDT et al., 2019) apart from key components for the regulation of soil organic matter dynamics under climate change scenarios (XIONG et al., 2019), facilitating C allocation and soil macroaggregation (BAUMERT et al., 2018). Hereof, it is evident that plants can modulate rhizosphere processes by altering their root exudation in response to any environmental situation, which can positively impact their future adaptability.

On the other hand, beneficial bacteria (also known as plant growth-promoting rhizobacteria, PGPR) present in the rhizosphere microbiome are able to trigger specific changes to the plant transcriptome through the secretion of an array of signaling metabolites or molecules favorable to the host plant (MHLONGO et al., 2018). The communication between PGPR and plant roots is interdependent and it can be root–root or root–microbe in which both exchange nutrients for survival. In the root-microbe interactions, plants use pattern-recognition receptors (PRRs) to detect particular microbe-associated molecular patterns (MAMPs) which are molecular conserved signatures of microorganisms (VENTURI; KEEL, 2016; SALWAN; SHARMA; SHARMA, 2019). Therefore, it is clear that rhizosphere metabolic properties can shape the microbial ecology in this habitat. In the last years, advanced technologies are deepening our biochemical knowledge on rhizodeposition, connecting exudate chemistry with microbial metabolism, thus identifying that diverse microbial strains have evolved discrete patterns of substrate uptake in order to colonise specific metabolic niches in the rhizosphere (ZHALNINA et al., 2018; JACOBY; KOPRIVA, 2019). Moreover, plant-associated rhizomicrobiome has a huge potential to improve plant resilience and yields in farming systems. Thus, the use of beneficial microorganisms, e.g., PGPR can enhance nutrient uptake and yield, control pests and alleviate plant stress responses (TRIVEDI et al., 2017). In this regard, emerging efforts exploring rhizosphere engineering could lead to beneficial microbes and chemical biomarkers that can enhance crop productivity.

In rhizosphere studies, where a complex mixture of metabolites is typical in the samples, it is suggested the use of high-precision and wide dynamic-range technologies, which allow the correct identification of these molecules (OBURGER; SCHMIDT, 2016; WHITE et al., 2017). Metabolomics has arisen as a powerful tool by which to gain a comprehensive perspective of how metabolic processes in the rhizosphere are regulated and has also an immense potential in the field of genetic breeding (RAZZAQ et al., 2019). In this sense, separation methods coupled with mass spectrometry (MS) such as gas chromatography (GC-MS), liquid chromatography (LC-MS) or capillary electrophoresis (CE-MS) and other technologies such as nuclear magnetic resonance and Fourier transform infrared spectroscopy (FT-IR) have become the analytical technologies commonly used in metabolomic studies, including those related to the rhizosphere (TUGIZIMANA; PIATER; DUBERY, 2013; ERNST et al., 2014; RUGOVA et al., 2017; RAZZAQ et al., 2019). In this review, in addition to the conceptual focus, we highlight recent studies about biological and chemical rhizosphere composition, with particular attention to chemical composition, since several metabolites released by roots are involved in key processes modulating interactions between plants and microorganisms (YEVDOKIMOV, 2013; ROLFE; GRIFFITHS; TON, 2019). Accordingly, several studies targeted to elucidate functions of the main organic compounds exuded by plant roots such as mucilage, carbohydrates, amino acids, organic acids, flavonoids, strigolactones, phytosiderophores and benzoxazinoids are discussed. This review also highlights some analytical technologies commonly used in metabolomic studies of the rhizosphere.

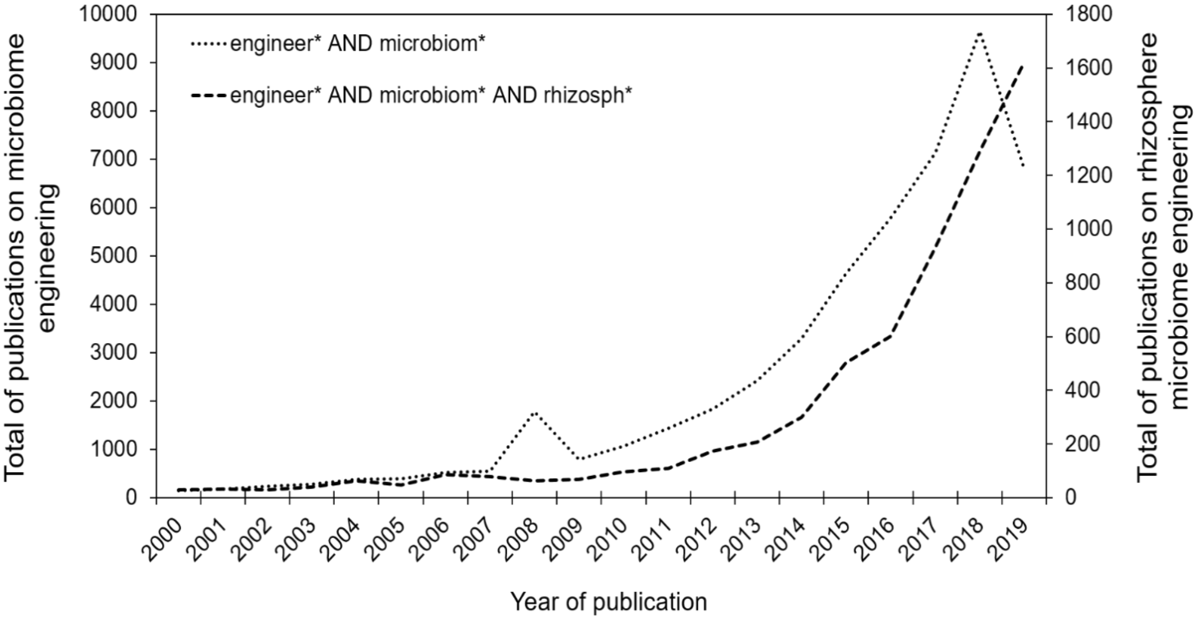
3.2 THE RHIZOSPHERE

The rhizosphere is defined as the portion of soil located in the vicinity of plant roots, being under the direct influence of plant metabolism and which differs physically, chemically and biologically from the bulk or non-rhizospheric soil (WHITE et al., 2017). The rhizosphere is not a region of definable size or shape since is constructed as a consequence of rhizodeposits which in turn vary according to the plant species and age, and environmental (biotic, abiotic) conditions (HARTMANN; ROTHBALLER; SCHMID, 2008). The enrichment of rhizosphere soil with an enormous variety of biologically active organic and inorganic compounds (UREN, 2007), leads to a complex and highly dynamic environment where countless interactions between the biotic and abiotic components of this ecosystems take place and may affect the biogeochemical cycles and plant growth (OBURGER; SCHMIDT, 2016). The high concentrations of easily degradable carbon sources in the rhizosphere, due to the large amount of nutrients released by the roots, such as lysates and exudates, a phenomenon called rhizodeposition (DESSAUX; GRANDCLÉMENT; FAURE, 2016), contrast with the general soil condition, where microbial community exist in a state of constant nutritional limitation. The release of these compounds favors the maintenance of high microbial communities in this habitat (about 10 to 1000 times greater than those found in bulk soil), as well as a metabolic activity up to 50 times greater (BAUDOIN; BENIZRI; GUCKERT, 2003; PAUSCH; KUZYAKOV, 2018). Thus, the quality and quantity of metabolites excreted by plant roots promote a selective effect (called the rhizosphere effect) on microbial groups compatible with the existing root exudates (WALKER et al., 2003a; PRASHAR; KAPOOR; SACHDEVA, 2014; MUSILOVA et al., 2016).

Rhizodeposits released from plant roots into the rhizosphere includes substances from older and sloughed-off cell lysates, secretions such as mucilages, proteins and volatiles actively transported from roots into the soil, and the root exudates which mainly consists of passively leached compounds from damaged and intact cells, such as ions, amino acids, organic acids, sugars and phenolics (BAIS et al., 2006; BADRI; VIVANCO, 2009; DENNIS; MILLER; HIRSCH, 2010; VAN DAM; BOUWMEESTER, 2016; PAUSCH; KUZYAKOV, 2018). Rhizodeposits are known to have a multitude of functions, acting as key molecules for mobilizing and acquiring of soil nutrients, mediate signaling processes (plant-plant and plant-microorganisms), and modulate the composition of rhizosphere microbiome, which in turn can function as an extended plant genome, exerting stimulatory or inhibitory effects on plant growth and development (BAIS et al., 2006; BAETZ; MARTINOIA, 2014; RASMANN; TURLINGS, 2016; SANCHEZ-CANIZARES et al., 2017). Through the diversity of exuded molecules, plants can exert an active role in modelling the rhizosphere functioning and biodiversity. The provisioning of readily useful carbon sources and signaling molecules contributes to the increase of compatible microbial populations in the rhizosphere, and in turn, these microorganisms provides signaling molecules and soluble inorganic nutrients (CHAPARRO; BADRI; VIVANCO, 2013; MENDES; GARBEVA; RAAIJMAKERS, 2013)

An extensive collection of physical (e.g., water and nutrient flux, temperature, aeration), chemical (e.g., pH, dissolution, precipitation, adsorption and desorption of ions) and biological (e.g., metabolic activity, intra and inter-species communication, exudation of biomolecules by living and death cells) processes arise from the rhizosphere, with importance for the establishment, growth and development of the plant and its associative microbiome (ZHANG et al., 2004; BLAGODATSKAYA; KUZYAKOV, 2008; RASMANN; TURLINGS, 2016; SHEORAN; SHEORAN; POONIA, 2016; RUGOVA et al., 2017). These processes are though interconnected on the rhizospheric environment, in a complex space-time dynamic that results from the interaction between the biotic (plant and microbiome) and abiotic (edaphoclimatic conditions) components, which most clearly observed consequences are the rhizosphere priming and the rhizosphere effect (JONES; HINSINGER, 2008; PHILIPPOT et al., 2013; TRABELSI; MHAMDI, 2013; DOTANIYA; MEENA, 2015). From the myriad of interactions taking place at plant rhizosphere, one of the most intriguing ecosystems globally found arises, whose complexity is illustrated by constitutive variations among plant species, plant age and from the bulk soil, for a given edaphoclimatic condition. Nevertheless, only recently proper attention has been given to the broad comprehension of rhizosphere dynamics and functioning, aiming to conscientiously interfere on this environment for the improvement of agricultural crops (**Fig. 3.1**).

Fig. 3.1. Bibliographic survey on a search for studies related to microbiome engineering (engineer* AND microbiom*) and rhizosphere microbiome engineering (engineer* AND microbiom* AND rhizosph*). Results from the boolean search on Google Scholar, PubMed and Web of Science tools, using the above terms, were merged.



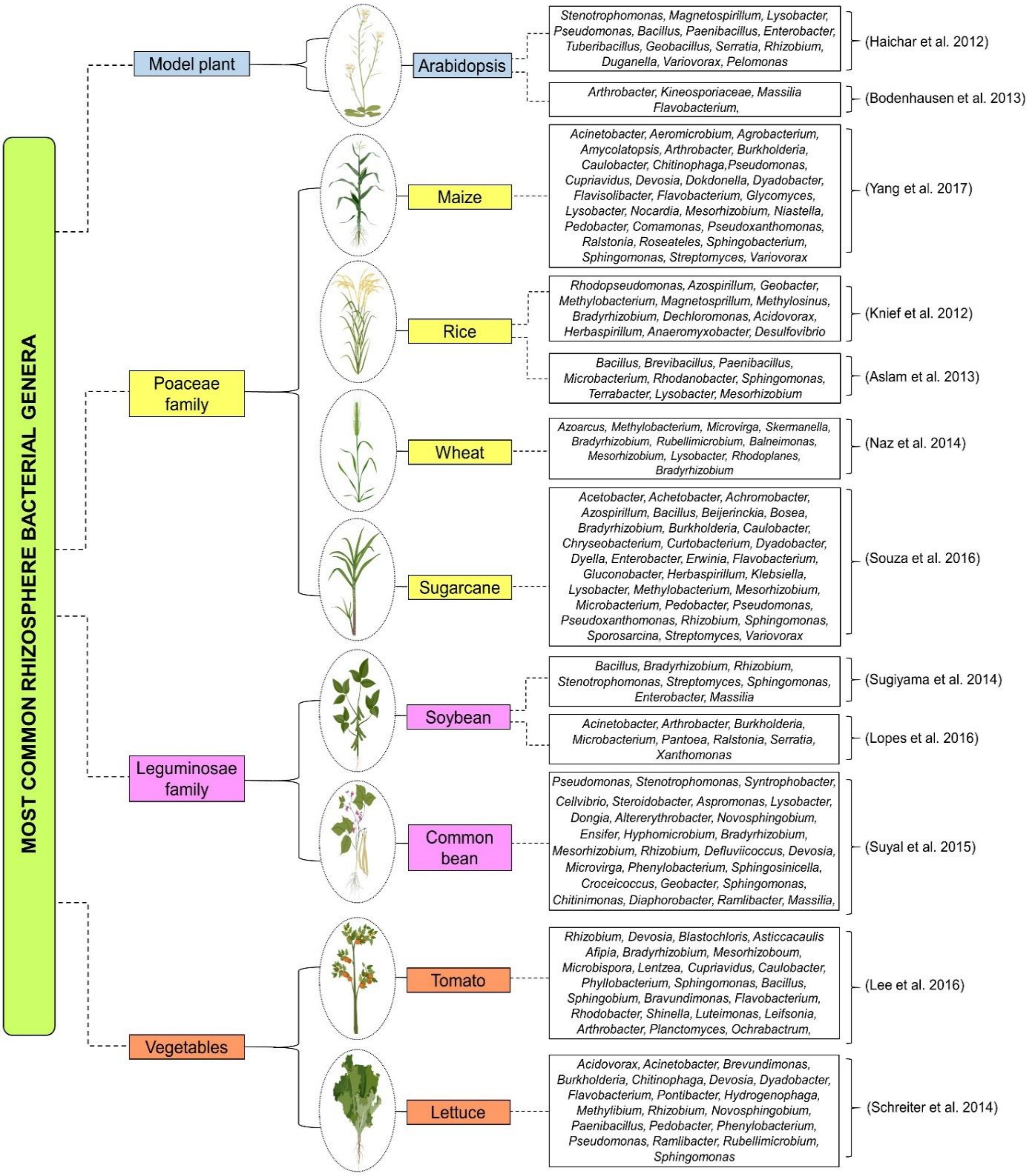
The manipulation (engineering) of the rhizosphere has been pointed out as a potential tool to alleviate critical problems taking place in this planet, for instance, the increasing of CO_2 in the atmosphere, the need for more sustainable agricultural and forestry practices, the improvement of water quality and the preservation of biodiversity (TRABELSI; MHAMDI, 2013; QUIZA; ST-ARNAUD; YERGEAU, 2015; BENDER; WAGG; VAN DER HEIJDEN, 2016). Rhizosphere engineering can be virtually achieved through all three components: the soil by its physical-chemical properties; the plants by classical or molecular breeding; and microbial populations by introducing new or favoring populations of endemic species (BENDER; WAGG; VAN DER HEIJDEN, 2016; DESSAUX; GRANDCLÉMENT; FAURE, 2016). On the other hand, as pointed by Machado et al. (2017), microbiome engineering can not be faced always in a positive perspective since the knowledge gap associated to the harms and risks of microbiome management are far to be filled; there is a need to increase the knowledge on ecosystems biodiversity and the role of associate microbiomes on its functioning.

3.2.1 Biological Composition of the Rhizosphere

In addition to the differences in physical and chemical properties of rhizospheric soil as compared with the non-rhizospheric soil, the biological structure of rhizosphere is indeed very distinct, and although its species composition resembles those found in the non-rhizospheric soil, the rhizosphere microbiome is far less diverse but also richer in populations size and metabolic activities (BERG; SMALLA, 2009; PHILIPPOT et al., 2013; OFAIM et al., 2017). Biological diversity in the rhizosphere includes several species of bacteria, archaebacteria, fungi, protozoa, algae, nematodes, arthropods and virus (MENDES; GARBEVA; RAAIJMAKERS, 2013; PHILIPPOT et al., 2013). Among these organisms are those known to promote beneficial effects on growth and health of plants, such as the plant growth-promoting bacteria (PGPB), arbuscular mycorrhizal fungi (AMF), biocontrol microorganisms and protozoa. On the other hand, in the rhizosphere also inhabits organisms that cause detrimental effects on plant development, such as pathogenic fungi, oomycetes, bacteria, nematodes and virus (MENDES; GARBEVA; RAAIJMAKERS, 2013). Equilibrium in the plant-associated microbial community is therefore essential for ecosystem function but indeed to hold down pathogenic organisms in competitive disadvantage for the access and use of rhizosphere nutrients, in relation to the beneficial organisms.

The rhizosphere microbiome is reported to be represented by up to 10^9 microbial cells and more than 30,000 different prokaryotic species per gram, which can cover up to 15% of the root surface with biofilms and microcolonies (VAN LOON, 2007; BERENDSEN; PIETERSE; BAKKER, 2012). Bacterial diversity found in the rhizosphere is mainly represented by species belonging to the phylum Proteobacteria, Acidobacteria, Actinobacteria and Firmicutes (MENDES et al., 2011; BERENDSEN; PIETERSE; BAKKER, 2012; GAO et al., 2019) and the most common genera reported through rhizosphere metagenome in some of the most important crops worldwide are shown in **Fig. 3.2**.

Fig. 3.2. The most common rhizospheric microbial genera revealed through rhizosphere metagenome of eight important crops worldwide along with *Arabidopsis* as a model plant (References between parentheses indicate studies conducted after 2012).



As rhizosphere is enriched with easily metabolizable substrates, resident microbial populations are expected to trigger a battle with intend to dominate the available resources and to surpass in population size of the other competing species, in a way that populations with increased competence to metabolize the available substrates and those characterized as r-strategists are thought to prevail (HIBBING et al., 2010). In fact, fast-growing bacterial genera able to use a wide range of carbon sources such as *Pseudomonas* and *Burkholderia*, are reported to predominate in the rhizosphere of several plant species (PRASHAR; KAPOOR; SACHDEVA, 2014; YANG et al., 2017). Nevertheless one can not ignore the unsteady state of rhizosphere resources, beacuse it presents a complex food-web where its microbiome not only uses the available nutrients but also transform them and secrete other metabolites on this environment, in addition to compositional variations of rhizodeposition related to different plant species, plant ages and edaphoclimatic conditions (YEVDOKIMOV, 2013).

In barley (*Hordeum vulgare*), for example, the rhizosphere was enriched with Bacteroidetes as compared with bulk soil community, but significant differences from bulk soil were found only for the wild genotype (*H. vulgare* ssp. *spontaneum*) (BULGARELLI et al., 2015). However, Proteobacteria, Acidobacteria, Cyanobacteria, Firmicutes and Planctomycetes were found as the most abundant phyla in the rhizosphere of cotton (*Gossypium hirsutum*), but a higher diversity was found in the bulk soil. Besides, bacterial community structure varied significantly with soil type, plant genotype and developmental stage (QIAO et al., 2017). On the other hand, no significant differences in the bacterial community at phylum level was observed along three different growth periods of maize (YANG et al., 2017) nor in rice (*Oryza sativa*) grown under greenhouse conditions or in the field (EDWARDS et al., 2015). In grapevine (*Vitis vinifera*), the bacterial and fungal microbiome associated with its rhizosphere was significantly shifted by plant genotype, predominantly composed by Proteobacteria and Actinobacteria (bacterial phyla) and Ascomycota and Basidiomycota (fungal phyla) (BERLANAS et al., 2019). Hawkes et al. (2007) by using the 16S rDNA gene, reported that the rhizosphere of 14 plant species (nine herbaceous dicotyledons, two woody dicotyledons and three grasses) was mainly represented by Proteobacteria, Actinobacteria, Firmicutes and Acidobacteria phyla. However, the number of phyla revealed in this analysis, suggest that the composition of microbial communities in the rhizosphere may be more complex than the simple response of microorganisms to the quantity and quality of rhizodeposits released by the roots. Moreover, the high dominance of Proteobacteria and Actinobacteria phyla in the rhizosphere of plants might be associated with their role in the carbon biochemical cycle and their production of secondary metabolites (ZHAO et al., 2014).

At the genus level, microbial communities can be host-specific, but can also colonize the rhizosphere of different plant species. For instance, in the spring season, rhizospheres of wheat (*Triticum aestivum*) and canola *(Brassica napus*) were enriched with bacterial copiotrophs including *Pseudomonas*, *Janthinobacterium*, *Flavobacterium, Oxalobacteraceae,* and *Sphingobacteriaceae* (16S rDNA gene), in addition to the saprotrophic fungi such as *Ulocladium*, *Mortierella*, *Cryptoccocus*, *Chaetomium*, *Penicillium* and *Trichoderma* (ITS). However, the relative abundance was observed to be season-dependent (SCHLATTER et al., 2019). A metatranscriptome sequencing study conducted by Pathan et al. (2018) showed that two contrasting maize lines with different nitrogen use efficiency shaped not only microbial communities but also conditioned the microbial functions and the N cycle in their rhizospheres. They described that *Proteus, Nitrosopumilus, Conexibacter, Nitrospira* and *Arthrobacter* were the genera which more contributed to the differences. Since rice cultivation is a large contributor to agricultural methane emissions due to the anaerobic conditions resulting from flooded fields, Edwards et al. (2015) examined the microbial communities associated with methane cycling in rice fields by amplifying fragments of the 16S rRNA gene and observed a high relative abundance of methanogenic archaea including the genera *Methanocella, Methanosarcina, Methanosaeta* and *Methanobacterium*. Paungfoo-lonhienne et al. (2015) reported that fungal communities in the rhizosphere of field-grown sugarcane involves mainly two phyla, Ascomycota and Basidiomycota and that *Clonostachys* and *Basidiomycete* genus were amongst the most promoted.

Among the bacteria commonly found in the rhizosphere, there are several beneficial species, known as plant growth-promoting rhizobacteria (PGPR), which are capable to colonize and promote plant growth (GLICK; KARATUROVÍC; NEWELL, 1995; PRASHAR; KAPOOR; SACHDEVA, 2014) by both direct and indirect mechanisms. Direct mechanisms usually involve processes which facilitate resource acquisition (nutrients and water) or modulate plant hormone levels while the indirect mechanisms are related to the decrease of inhibitory effects of pathogens on plant growth and development, that is, by acting as biocontrol agents (GLICK, 2012; AHEMAD; KIBRET, 2014). A bacterium in particular may use one or more of these mechanisms, or may act synergistically with other microorganisms to stimulate the growth of the host plant (VESSEY, 2003). Furthermore, bacteria can provide different benefits at different times during the plant life cycle (GLICK, 2005). Gontia et al. (2017) studied the diversity of 1-aminocyclopropane-1- carboxylate (ACC) deaminase producing PGPR from wheat rhizosphere and reported that belonged to different species of genera *Klebsiella, Enterobacter, Acinetobacter, Chryseobacterium, Pseudomonas, Citrobacter, Flavobacterium, Stenotrophomonas*, *Empedobacter*, *Escherichia*, *Alcaligenes* and *Bacillus* showing multiple PGP traits such as IAA production, phosphate, zinc and potassium solubilization and siderophore production. Arruda et al. (2013) reported that *Klebsiella*, *Pseudomonas, Stenotrophomonas*, *Enterobacter, Serratia, Pantoea, Citrobacter* and *Burkholderia* were the genera most abundantly found in rhizosphere of maize and some of them showed multiple PGP traits. Islam et al. (2016) described that PGPR isolated from cucumber (*Cucumis sativus*) rhizosphere were mainly members of genus *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* that possessed PGP attributes and antagonism against phytopathogens.

In the rhizosphere can also be found several microorganisms known to form symbiotic associations with a wide variety of plants. The most studied symbiotic interactions are those between leguminous and rhizobia, plants and AM fungi, and between actinorhizal plants and *Frankia* actinobacteria. A common feature of these symbiotic associations is the ability of these microorganisms to make available nutrients that limit plant growth and the plant, in return, provides carbon sources derived from photosynthesis (OLDROYD, 2013).

Chibeba et al. (2017) reported that 105 isolates were obtained from soybean nodules of which 75% were assigned to the *Bradyrhizobium* genera and the remaining to the *Agrobacterium* or *Rhizobium* genera. Ribeiro et al. (2013) reported the isolation of nineteen *Rhizobium* strains from root nodules of common bean and Rouhrazi and Khodakaramian (2015) described that representative isolates from root nodules of chickpea were closely related to *M. mediterraneum*, *M. ciceri,* and *Agrobacterium tumefaciens*. Furthermore, some species of the recently described genus *Paraburkholderia* (belonging to the *β* subclass of Proteobacteria: *β*-rhizobia) are able to nodulate and fix nitrogen in symbiosis with legumes (TAULÉ et al., 2012; SAWANA et al., 2014). Agnol et al. (2017) reported that most strains isolated from *Mimosa pudica* and *Phaseolus vulgaris* in the brazilian atlantic forest, were assembled in the genus *Paraburkholderia*, including *P. sabiae* and *P. nodosa*. The establishment of legume-rhizobia symbiosis results in formation of root nodules, which main function is to produce an environment conducive for biological nitrogen fixation, restricting the free flow of oxygen that otherwise limits the nitrogenase responsible for nitrogen fixation (OLDROYD et al., 2011; VENKATESHWARAN et al., 2013). This relationship is achieved by chemical communication between rhizobia bacteria in the rhizosphere and the root of the host plant. Flavonoids released by legumes roots act as signals for the rhizobia which in turn produce nodulation factors (Nod factors) recognized by the host plant to activate a symbiosis signaling pathway initiating the formation of nodules (OLDROYD, 2013; UDVARDI; POOLE, 2013).

However, in recent years several studies reported that rhizobia can also to associate with non-leguminous plants without forming nodules. For example, species of the genus *Rhizobium* have been described associated with plants such as rice (YANNI et al., 1997; ZHAO et al., 2017), radish (ANTOUN et al., 1998), maize (CHABOT; ANTOUN; CESCAS, 1996), strawberry (COSTA et al., 2006), sweet potato (REITER et al., 2003), sunflower (AMBROSINI et al., 2012), lettuce (NOEL et al., 1996) and potato (TURNBULL; LIU; LAZAROVITS, 2012). Other studies have shown the effect of rhizobia inoculation in non-legumes and its potential to be used as PGPR (SINGH; MISHRA; JAISWAL, 2005; MEHBOOB; NAVEED; ZAHIR, 2009). García-Fraile et al. (2012) showed that *Rhizobium* strains colonize the roots of tomato and pepper plants promoting their growth in different production stages increasing yield and quality of seedlings and fruits; Flores-Félix et al. (2013) described that *R. leguminosarum* was able to colonize the roots and promote growth of lettuce and carrots and Jiménez-Gómez et al. (2016) reported that *Rhizobium* sp. colonized spinach root surfaces, producing changes in root hairs and an increase in plant growth.

In contrast, AMF are obligate root symbionts of more than 80% of all land plants, including most agricultural crops (VOS et al., 2012). Soteras et al. (2016) revealed that members of family Glomeraceae, Pacisporaceae, Acaulosporaceae and Gigasporaceae were found colonizing the rhizosphere of perennial *Polylepis australis* tree. Senés-guerrero and Schüßler (2016) studied the composition of the AMF community colonizing potato roots and reported that this crop was mainly colonized by a conserved group of AMF species of the genera *Acaulospora*, *Cetraspora*, *Claroideoglomus* and *Rhizophagus* simultaneously, which appeared to be main colonizers of potato in the Andean region. The association of plants with AM fungi does not lead to the formation of macroscopic structures. Instead, the plant facilitates fungal colonization in the root cortex, forming heavily branched structures called arbuscules that mediate nutrient exchange (OLDROYD, 2013). For the establishment of these symbiotic relationship, plants release strigolactones (SLs) which are perceived by fungus and promotes spore germination and hyphal branching. The fungal hyphae in turn produce diffusible molecules known as Myc factors that are perceived by plant roots activating the symbiosis signaling pathway and promoting the root invasion (MOHANTA; BAE, 2015). AMF not only improve plant growth by increasing the absorption of available phosphorus (P) and other nutrients, but also have been reported to alleviate the stress caused by biotic and abiotic factors and to increase the stability of soil aggregates (GIANINAZZI et al., 2010).

Symbiotic associations also occur between nitrogen-fixing actinobacteria of the genus *Frankia* and the roots of actinorrhizal plants. Phylogenetically, *Frankia* strains can be divided into four clusters. Cluster I include *Frankia* strains which form nodules with plants of Betulaceae, Myricaceae, and Casuarinaceae families, while cluster II contains *Frankia* associated with *Ceanothus* of the Rhamnaceae, Coriariaceae, Datiscaceae and Rosaceae families. Cluster III consists of bacteria that form effective nodules on members of the Myricaceae, Rhamnaceae, Elaeagnaceae, and *Gymnostoma* of the Casuarinaceae, while cluster IV contains a variety of strains that were isolated from nodules but cannot induce nodule formation (GHODHBANE-GTARI et al., 2010; SELLSTEDT; RICHAU, 2013). Based on the *nifH* gene sequence, Polme et al. (2014) distinguished 43 *Frankia* OTUs, belonging to the *Frankia alni* complex, from root nodules of 22 *Alnus* species from four continents. In these associations, *Frankia* colonizes the root and induces the root hair curling, which trapping the bacteria activating the production of nodules comparable to those observed in legumes, although with important structural differences (OLDROYD, 2013; HAICHAR et al., 2014).

Pathogenic organisms are also found in the rhizosphere. Plant parasitic nematodes are responsible for global agricultural losses, estimated at $ 157 billion annually (SINGH; SINGH; SINGH, 2015), seriously affecting many economically important agricultural crops around the world (ESCUDERO; LOPEZ-LLORCA, 2012). The nematode *Meloidogyne incognita* is able to infect the roots of almost all cultivated plants, becoming perhaps the most damaging of all pathogens of agricultural crops (TRUDGILL; BLOK, 2001). Root nematodes have an intimate interaction with their hosts. Within the host root, adult females induce the redifferentiation of the root cells into specialized giant cells, which provide a source of nutrition for the nematode (ABAD et al., 2008). This process of infection is known to interfere with the acquisition and translocation of nutrients by the plant and may result in the death of the root tissue (TREONIS et al., 2007). However, when nematodes damage root tissue, the turnover rate of these tissues is altered, resulting in increased rhizodeposition of organic compounds that act as a source of nutrients for the microorganisms in the rhizosphere (TREONIS et al., 2005; BONKOWSKI; VILLENAVE; GRIFFITHS, 2009; WURST et al., 2010). In this sense, nematode root infection can alter the C and N inputs into the soil, shaping the microbial communities in the rhizosphere and influencing the processes of nutrient cycling and plant growth (TU; KOENNING; HU, 2003; HAASE et al., 2007). For instance, Zhou et al. (2019) described that soils non-infested with *Meloidogyne* spp. had more microbial diversity than the infested soils from all plant rhizospheres assessed. Further, inoculation of tomato roots with microbiome of non-infested soils was associated with a reduction in the number of root galls, indicating that rhizosphere enrichment with specific microbial groups may be a way to control root-knot nematodes.

3.2.2 Rhizodeposition and Chemical Composition of the Rhizosphere

During plant growth, carbon compounds are released by the roots and undergo various processes of transformation in the soil. It is estimated that between 20 and 60% of the photoassimilated carbon is allocated to the roots and up to 70% of this carbon can be released into the rhizosphere (NEUMANN; RÖMHELD, 2007). The release process of these compounds has several functions in plant nutrition and soil ecology and is often referred to as rhizodeposition (HÜTSCH; AUGUSTIN; MERBACH, 2002).

The rhizodeposition comprises a wide range of processes by which organic carbon enters the soil including lysates released by autolysis of detached cells and tissues, intact cells of the root apex, gas losses, secretion of insoluble polymers from living cells (mucilage) and release of root exudates (HASSAN; MCINROY; KLOEPPER, 2019). However, not all the compounds released by the roots are organic since they can also release H^+, inorganic ions, free oxygen, water and electrons (BERTIN; YANG; WESTON, 2003; NANNIPIERI et al., 2007).

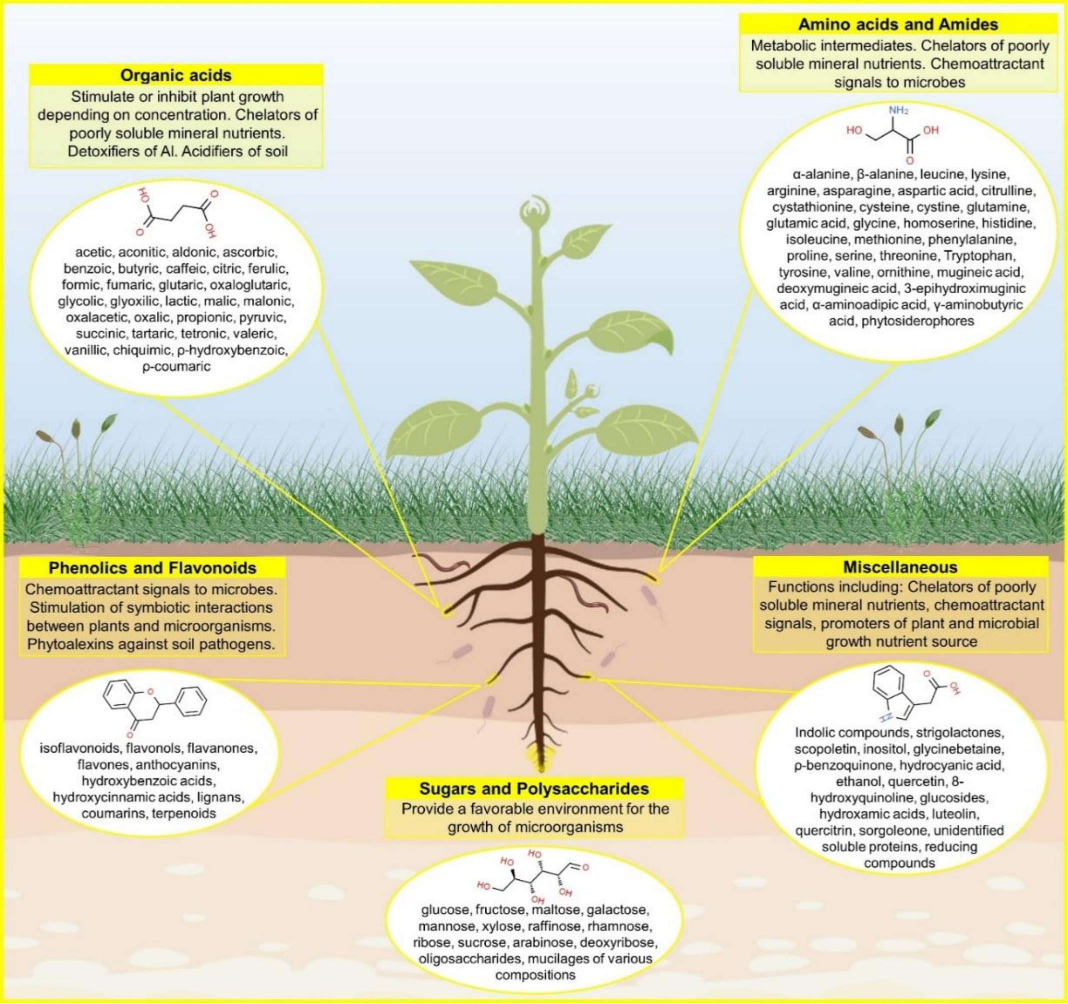
Several of these compounds released into the rhizosphere are able to improve the availability of plant nutrients (DAKORA; PHILLIPS, 2002; HINSINGER et al., 2011; RICHARDSON et al., 2011; SHEN et al., 2011), stimulate the increase of the population density of several microbial species, attracting microorganisms from the soil to the rhizosphere (NARULA; KOTHE; BEHL, 2009; NEAL et al., 2012; ZHANG et al., 2014) and promote the establishment of symbiotic relationships between legume-rhizobia, plants-mycorrhizal fungi and actinorhizal plants-*Frankia* (MANDAL; CHAKRABORTY; DEY, 2010; HAICHAR et al., 2014). In many soils, aluminum toxicity is an important factor limiting the productivity of crops, thus, the exudation of certain types of compounds can reduce the toxic effects of this element (RANGEL et al., 2010; YANG et al., 2011; CHEN; WANG; YEH, 2017). Other compounds have a well defined role, such as phytosiderophores, which are non-protein amino acids acting mostly as chelators of some micronutrients, mainly Fe (III) (OBURGER et al., 2014). Besides, the releasing of exudates acting as allelochemicals allow many plant species to resist potential phytopathogenic agents (LI et al., 2010; KONG et al., 2019).

Several biotic and abiotic factors influence the rhizodeposition. They include the plant species (VAN DER KRIFT et al., 2001; JONES; HODGE; KUZYAKOV, 2004), plant age and stage of development (LUGTENBERG; KRAVCHENKO; SIMONS, 1999; NGUYEN, 2003), soil texture (BOEUF-TREMBLAY; PLANTUREUX; GUCKERT, 1995; GROLEAU-RENAUD; PLANTUREUX; GUCKERT, 1998), presence of specific microorganisms (VAN DE MORTEL et al., 2012; PLANCHAMP; GLAUSER; MAUCH-MANI, 2014; D'ANGIOLI et al., 2016), atmospheric CO_2 concentration (PATERSON et al., 1997; BASLAM et al., 2014), light intensity (HODGE et al., 1997; WANG et al., 2010), temperature (KRASENSKY; JONAK, 2012; USADEL et al., 2008), soil pH (MEHARG; KILLHAM, 1990; LIANG et al., 2013), biotic and abiotic stress factors such as salinity, drought, toxicity caused by metals, infection by phytopathogenic organisms (YUSUF et al., 2010; KRASENSKY; JONAK, 2012; CAMPOS-BERMUDEZ et al., 2013; DUAN et al., 2013).

Great advances in the understanding of the complex mechanisms concerning the transport of compounds released by roots began to be published, although they have not yet been fully clarified. Traditionally, root exudation was referred to as a passive process mediated by three distinct pathways: diffusion, ion channels and vesicular transport. During diffusion, the transport of low molecular weight compounds (eg., some sugars, amino acids, carboxylic acids and phenolics) is determined by membrane permeability. Furthermore, ion channels mediate the controlled release of some carboxylates (e.g., citrate, malate, oxalate), that in conditions of specific stress such nutritional deficiency or toxicity by some free metal ions, can not be transported by diffusion. The release of high molecular weight compounds (e.g., some proteins, polysaccharides) generally involves the vesicular transport (BADRI; VIVANCO, 2009; BERTIN et al., 2003). However, recent studies have elucidated a central role of primary and secondary active transport processes mediated by membrane-bound proteins in the rhizodeposition. These proteins include ABC transporters (ATP-binding cassette) which drive the transport of a wide range of compounds (metabolic products, ions, lipids and xenobiotics) using the energy from ATP hydrolysis; MATE transporters (multidrug and toxic compound extrusion) which drive a wide variety of compounds, generally using a ionic gradient (sodium or protons); MFS transporters (major facilitator superfamily) involved in the release of phytosiderophores; and ALMT proteins (aluminium-activate malate transporter) which facilitate the efflux of malate anion, involved in resistance to aluminum toxicity in many plant species (BAETZ; MARTINOIA, 2014; HUANG et al., 2014; MIMMO et al., 2014; WESTON et al., 2012).

Different studies on rhizodeposition have been carried out during the last years, noting that the plant roots can release a wide range of organic compounds (**Fig. 3.3**). These compounds have been divided into two classes: low molecular weight and high molecular weight. (BAIS et al., 2006; BADRI; VIVANCO, 2009). Following, some aspects of these classes of compounds will be presented.

Fig. 3.3. Classes and functional role of organic compounds released by plant roots into the rhizosphere (compiled from BERTIN; YANG; WESTON, 2003; UREN, 2007).



Organic acids

Stimulate or inhibit plant growth depending on concentration. Chelators of poorly soluble mineral nutrients. Detoxifiers of Al. Acidifiers of soil

acetic, aconitic, aldonic, ascorbic, benzoic, butyric, caffeic, citric, ferulic, formic, fumaric, glutaric, oxaloglutaric, glycolic, glyoxilic, lactic, malic, malonic, oxalacetic, oxalic, propionic, pyruvic, succinic, tartaric, tetronic, valeric, vanillic, chiquimic, ρ-hydroxybenzoic, ρ-coumaric

Amino acids and Amides

Metabolic intermediates. Chelators of poorly soluble mineral nutrients. Chemoattractant signals to microbes

α-alanine, β-alanine, leucine, lysine, arginine, asparagine, aspartic acid, citrulline, cystathionine, cysteine, cystine, glutamine, glutamic acid, glycine, homoserine, histidine, isoleucine, methionine, phenylalanine, proline, serine, threonine, Tryptophan, tyrosine, valine, ornithine, mugineic acid, deoxymugineic acid, 3-epihydroximuginic acid, α-aminoadipic acid, γ-aminobutyric acid, phytosiderophores

Phenolics and Flavonoids

Chemoattractant signals to microbes. Stimulation of symbiotic interactions between plants and microorganisms. Phytoalexins against soil pathogens.

isoflavonoids, flavonols, flavanones, flavones, anthocyanins, hydroxybenzoic acids, hydroxycinnamic acids, lignans, coumarins, terpenoids

Miscellaneous

Functions including: Chelators of poorly soluble mineral nutrients, chemoattractant signals, promoters of plant and microbial growth nutrient source

Indolic compounds, strigolactones, scopoletin, inositol, glycinebetaine, ρ-benzoquinone, hydrocyanic acid, ethanol, quercetin, 8-hydroxyquinoline, glucosides, hydroxamic acids, luteolin, quercitrin, sorgoleone, unidentified soluble proteins, reducing compounds

Sugars and Polysaccharides

Provide a favorable environment for the growth of microorganisms

glucose, fructose, maltose, galactose, mannose, xylose, raffinose, rhamnose, ribose, sucrose, arabinose, deoxyribose, oligosaccharides, mucilages of various compositions

3.2.2.1 High molecular weight compounds released by plant roots

3.2.2.1.1 Mucilage

Mucilage is a gelatinous material of high molecular weight that surrounds the root tips and represents one of the few clearly visible signs of organic carbon release from the roots. This substance is composed mainly of polysaccharides (i.e. arabinose, xylose, mannose, galactose, glucose, fucose, uronic acid), up to 6% proteins (i.e. arabinogalactan proteins), some phospholipids and small amounts of extracellular DNA (JONES; NGUYEN; FINLAY, 2009; CARMINATI; VETTERLEIN, 2013).

Mucilage has the potential to increase the ability of young roots to uptake water from soils, in this way, plants have the possibility to survive in drought periods (AHMED et al., 2014). Carminati et al. (2015) reported that through mucilage exudation, the plant roots can modify the physical properties of the rhizosphere triggering complex soil-plant interactions that regulate the acquisition of carbon through photosynthesis, since the mucilage acts as a hydraulic bridge between the roots and the soil, facilitating the uptake of water and keeping the sweat in dry soils. In opposition to the previous reports, mucilage can also reduce water holding capacity, since as it dry and ages, becomes hydrophobic. Carminati e Vetterlein (2013) explained this variability in water uptake as an expression of the plasticity of the rhizosphere, which can be a strategy for plants to control which part of the root system will or will not have easy access to water in soil. Mucilage can also contribute to the protection and stabilization of soil aggregates due to the ability to adhere very rapidly to soil particles (MOREL et al., 1991; DI MARSICO et al., 2018). Traoré et al. (2000) reported that the addition of corn mucilage in soil increased the stability of aggregates, and that the duration of this effect was dependent on the chemical composition of mucilage.

Several studies have shown that mucilage can stimulate the growth and development of microorganisms that colonize the rhizosphere. Knee et al. (2001) demonstrated that strains of *Rhizobium leguminosarum* can use the mucilage extracted from maize roots as the sole source of carbon. Benizri et al. (2007) investigated the effect of adding maize root mucilage on bacterial community structure of an agricultural soil and found that there was a 450% increase in the number of culturable bacteria and that the physiological and genetic profile of these bacteria was affected by the addition of mucilage. Other studies have reported that specific components of the mucilaginous matrix released by the roots, like arabinogalactan proteins and extracellular DNA (exDNA) have antimicrobial properties and may be important in preventing the attack of pathogens (WEN et al., 2009; CANNESAN et al., 2012; DRIOUICH et al., 2013), since provides a scaffold to trap, immobilize, and subsequently kill root-infecting pathogens in the mucilage matrix (BAETZ; MARTINOIA, 2014).

Mucilage can modify the flow of metal cations to the root. Due to complexation reactions, the diffusion of potentially toxic metals (such as Al, Pb, Zn, Cd and Cu) with high affinity for mucilage may decrease in the rhizosphere. Thus, the mucilage can act as a selective filter for metals protecting the root meristem (JONES; NGUYEN; FINLAY, 2009; KOO et al., 2013). Has been reported that mucilage of maize roots binds strongly to aluminum and this bounded aluminum is less toxic, suggesting the possible role of mucilage in the protection of the root tip against the toxicity of this metal (LI et al., 2000). Cai et al. (2013) reported that the root mucilage of two soybean cultivars decreased the absorption of aluminum by the roots and, consequently, the inhibition of root growth.

To date, the knowledge about the amount of mucilage released in the soil remains unknown. Some studies have reported that the amounts of mucilage synthesized *in vitro* range from 11 to 47 µg mg^{-1} of root. However, these quantities were determined from roots grown in water or in nutrient solution, which increases the diffusion of the mucilage out of the periplasmic region and probably stimulates the biosynthesis of this polymer (NGUYEN, 2003; HAICHAR et al., 2014).

3.2.2.1.2 Proteins

Proteins are synthesized and secreted into the rhizosphere from the root cap and/or border cells (WEN et al., 2007) and are involved in the maintenance of cell wall structure, communication between different cells in the plant, root-microbe communication and defense response to biotic and abiotic stresses (TANVEER et al., 2014). Multidimensional protein analysis confirmed that the proteins secreted by the plant cells, termed as plant secretome, includes a mixture of ~120 proteins including defense and signaling enzymes as well as structural proteins (WEN et al., 2007; ALEXANDERSSON et al., 2013)

Previous studies have indicated that plants have developed systems for monitoring the presence of microorganisms or microbial molecules through quanti/qualitative changes in protein secretion into the rhizosphere (DE-LA-PEÑA et al., 2008). Thurich et al., (2018) analyzed the secretomes of the mutualistic root-endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana* and more than one hundred proteins were identified as differentially secreted, including proteins associated with growth, development, abiotic and biotic stress response and mucilage. Using a proteomic approach, de la Peña et al., (2008) identified more than 100 secreted proteins, prevalently hydrolases and peptidases, during the interaction between *Medicago sativa* and *Arabidopsis thaliana* with the beneficial bacteria *Sinorhizobium meliloti* or the pathogenic bacteria *Pseudomonas syringae*. They reported that the presence of a specific plant induces the differential secretion of proteins by a given bacteria, that culminate in either a symbiotic or defense response.

During pathogenic interactions, protein composition in the rhizosphere is altered, leading to the accumulation of antimicrobial peptides (AMPs) (BAETZ; MARTINOIA, 2014). AMPs contain less than 100 amino acids that display broad spectrum antimicrobial activity against bacteria, fungi and viruses. AMPs are classified based on their structure and presence of cysteine disulfide bonds and the main classes secreted by plants are cyclotides, defensins, thionins, lipid transfer proteins and snakins (GOYAL; MATTOO, 2014). The antimicrobial activity of peptides results from the amphiphilic character and presence of high density of positively charged residues within their structure (SALAS et al., 2015). Weiller et al., (2017) reported the production of defensins peptides from *Heliophila coronopifolia,* considered to be part of the innate defense of plants which provide a protective environment even before pathogens are sensed.

Root-secreted proteins are also involved in the mechanisms used by plants to cope with adverse environmental conditions such as cold, drought, and salinity. By using LC-MS/MS, Song et al., (2011) identified 69 proteins, from which 37 were mainly involved in the processes of carbohydrate metabolism, oxido-reduction, and protein processing and degradation. Results of this study suggested an extracellular stress-responsive protein network which reflects the possible mechanisms involved in the initial perception of the stress for the plant. In a study conducted by Badri et al., (2012) they described that root secreted proteins are also involved in the early biochemical events of plant neighbor recognition, suggesting that plants can sense and respond to the presence of different plant neighbors by increasing the total amount of defense proteins in the exudates.

In addition, enzymes secreted by both roots and microorganisms catalyze the decomposition of root exudates and other rhizodeposits into absorbable forms and make nutrients available for plant/microbial uptake (MA et al., 2018). By using the zymography method, some researchers have determined the two-dimensional spatial distribution of plant and microbial-derived extracellular enzymes in the rhizosphere (SPOHN; CARMINATI; KUZYAKOV, 2013; RAZAVI et al., 2016). It has been described that roots of several agricultural plants are able to exude proteases and use them to create a pool of accessible N, and the level of proteolytic activity of root-secreted proteases is species-specific (ADAMCZYK et al., 2010). Root proteases may be an important aspect of root development, differentiation and stress signaling and can positively affect nutrient and water status more than any other class of proteins under drought stress (KOHLI et al., 2012). Secretion of phosphatases and phytases from roots is an important plant response to P deficiency (MARUYAMA et al., 2012; GERKE, 2015). Part of soil formation processes is the degradation of the recalcitrant aromatic structures of lignin and humic substances. Peroxidases and polyphenoloxidases comprise other classes of root-secreted enzymes which contribute to the degradation of soil organic matter in planted soil by increasing water solubility, aromaticity, and content in phenolic OH groups (GRAMSS, 2018).

3.2.2.2 Low molecular weight compounds released by plant roots: root exudates

Exudates can be defined as low molecular weight diffusible rhizodeposits that are released passively by the root and on which the root exerts little direct control (JONES; NGUYEN; FINLAY, 2009). Within the root, these compounds are often found at concentrations in the millimolar order while in the rhizosphere they are found in the micromolar order (NGUYEN, 2003). The rate of release of these compounds into the rhizosphere depends on three critical factors: the gradient of root-soil concentration, the permeability of the plasma membrane and the spatial location of the solutes in the root tissue (JONES; NGUYEN; FINLAY, 2009).

The release of root exudates occurs mainly in the apical region and at lateral branching sites (1-2 cm away from the root surface) where the microbial density is smaller than in the older parts of the roots (NEUMANN, 2007) and the amount released decreases as the distance from the root surface increases (GAO et al., 2011). This spatial variation in the concentration of root exudates in the rhizosphere depends mainly on two aspects: 1) radial diffusion of the exudates in soil layers and 2) degradation by chemical (sorption or desorption) or biological (e.g. microbial consumption) processes (GAO et al., 2011; ROHRBACHER and St-ARNAUD, 2016).

Neumann (2007) reported that the exudation of low molecular weight compounds mediated by diffusion in the apical regions of the root varies around 300-2000 μM accumulated over a period of 12-24 h. However, because of the microbial degradation of sugars, amino acids and organic acids, these exudates released have an average life time of 1-5 h. Therefore, the concentration of compounds frequently found in rhizospheric soil ranges from 1-50 μM.

The major low molecular weight compounds found in the rhizosphere include organic acids, amino acids, sugars, vitamins, phytosiderophores, phenolics, alcohols, purines, nucleosides, and various other secondary metabolites (DAKORA; PHILLIPS, 2002; WALKER et al., 2003a). These compounds are involved in important processes such as modulation of nutrient availability, protection against toxic metals or pathogenic organisms, attraction and/or repulsion of microorganisms, among others (MIMMO et al., 2014). The following section will describe some characteristics and functions of the main types of compounds exuded by plant roots:

3.2.2.2.1 Carbohydrates, amino acids and organic acids

Low molecular weight organic compounds (LMWOC) such as carbohydrates, amino acids and organic acids are primarily responsible for microbial growth in the rhizosphere (PARK et al., 2011) and the proportion of these compounds in exudates is important because it affects the relative amounts of C and N available for microbial growth (JAEGER III et al., 1999). Using the ^{14}C labeling technique, Fischer et al. (2010) studied the dynamics and distribution of carbon derived from LMWOC into the rhizosphere, using one representative from each group: carbohydrates (glucose), amino acids (alanine) and organic acids (acetate). They found that these three types of compounds were preferentially incorporated into microbial biomass. However, the transformation of released carbon into the rhizosphere, either by sorption processes, microbial incorporation or decomposition to CO_2, was strongly dependent on the concentration of organic compound released.

Under nutrient deficiency conditions, some plants exhibit adaptations that allow them to mobilize poorly available elements. Carvalhais et al. (2011) reported that maize plants grown under axenic and poor nutritional conditions such as N, P, K and Fe, showed a variation in the composition of exudates. Under Fe and P deficiency there was an increase in the release of organic acids and carbohydrates, whereas the deficiency of N and K decreased the release of amino acids and carbohydrates.

Carbohydrates have been cited as the most abundant molecules released by plants in the rhizosphere and comprise the most labile carbon source for microorganisms (JAEGER III et al., 1999; SCHULZE; POSCHEL, 2005). Derrien et al. (2004) evaluated the nature and dynamics of carbohydrates in wheat rhizosphere and identified seven monosaccharides: glucose, mannose, galactose, arabinose, xylose, rhamnose and fucose; however, glucose was the dominant molecule representing 37% of the sugars found. Kuzyakov and Jones (2006) predicted that glucose concentration in the rhizosphere in response to maize roots exudation, if there was no microbial degradation, would be in the range 100-500 μM, after a few hours of exudation.

Amino acids are generally considered the second class of most abundant compounds and being a source of carbon and nitrogen, can theoretically support a broad population of microorganisms (MOE, 2013). All twenty essential amino acids have been found in the rhizosphere of plants, although at different concentrations. Among the most abundant are alanine, asparagine, glutamate, glycine, proline, serine, threonine, lysine and leucine (SIMONS et al., 1997; BOBILLE et al., 2016). The amino acid tryptophan plays a significant role in synthesis and regulation of auxins, a class of phytohormones that regulate the root development and architecture processes (YUE; HU; HUANG, 2014). Jaeger III et al. (1999) reported that tryptophan concentrations in the rhizosphere increased along the root as the distance from the root tip and was highest in the region where lateral roots had begun to emerge, suggesting that the accumulation of tryptophan in the rhizosphere could be related to the role of indolacetic acid (IAA) in lateral root initiation. However, γ-aminobutyric acid (GABA), a non-essential amino acid that functions as a neurotransmitter in the mammalian central nervous system, is also abundant in plants and has been detected in root exudates where it acts as a signaling molecule mediating interactions with other organisms, including bacteria (CARVALHAIS et al., 2011; REYES-DARIAS et al., 2015).

Organic acids are characterized by the presence of one or more carboxylic groups and depending on the number of these groups and the dissociation properties, can carry varying negative charge, thereby allowing the complexation of metal cations in solution and the displacement of anions from the soil matrix (JONES, 1998). Organic acids represent a labile source of C in soil and, once released into the rhizosphere, can be rapidly absorbed and degraded by the microbial community. They have been implicated in many beneficial functions for the plant, due to their potential to stimulate microbial growth, mineralize and mobilize poorly soluble nutrients (e.g., P, Mn, Cu, Zn, and Fe) or detoxify potentially toxic metals (OBURGER et al., 2009). Several authors have reported that under low concentrations of P, the plants exhibit an increased exudation of organic acids like citric, malic, malonic, oxalic, succinic, tartaric and piscidic (JOHNSON; LOEPPERT, 2006; JAITZ et al., 2011) and the extraction efficiency of P by organic acids follows the series oxalate>citrate>malate (STRÖM et al., 2005). However, the rate of P extraction is highly dependent on the type of organic acid exuded, as well as the concentration, pH and contact time with soil. Furthermore, different mechanisms are responsible for assessing different forms of P and the organic acids production does not necessarily result in a higher absorption of P (PEARSE et al., 2007; PARK et al., 2011).

3.2.2.2.2 Flavonoids

Flavonoids are low molecular weight secondary metabolites produced by plants and are generally described as non-essential for plant survival (Weston and Mathesius, 2013). The flavonoids includes compounds like flavones, flavonols, flavanones, isoflavonoids, isoflavones, anthocyanins and pterocarpans and more than 10,000 structures have been reported (Hassan and Mathesius, 2012). Several factors may exert great influence on the quantities of exuded flavonoids. These include the species and age of the plant, the zone and developmental stage of roots, the cultivation conditions, as well as the extraction procedures and the sensitivity of the methods used in analysis of these compounds (CESCO et al., 2010). The mechanism of root exudation of flavonoids is not well understood. However, several studies have pointed the transport through of vesicles and ABC transporters as the main mechanisms of flavonoid release. Meanwhile, MATE transporters have also been detected (WESTON; MATHESIUS, 2014).

Flavonoids have significant multifunctional roles in plants (CESCO et al., 2012). They may act as chemoattractant and signaling molecules involved in establishment of symbiotic relationships between rhizobia, mycorrhizal fungi or *Frankia* with plant roots (ABDEL-LATEIF; BOGUSZ; HOCHER, 2012). Several studies have reported that the release of flavonoids by leguminous plants activate the expression of nodulation genes. For example, flavones like luteonin, apigenin, chrysoeryol, chrysin and galangin and flavanones like naringenin, eriodictiol, hesperetin and geraldone induce *nod* genes activation in *R. leguminosarium*; however, the isoflavones genistein and daidzeine have been identified in *nod* gene activation in *B. japonicum* (COOPER, 2004; JANCZAREK et al., 2014). Neverless, a flavonoid may simultaneously be an inducer for one species of rhizobia and one repressor for another. The isoflavone daidzein, for example, induces *nod* gene expression in *B. japonicum* but is an inhibitor in *R. trifolii* and *R. leguminosarum* (GOULD; LISTER, 2005). Flavonoids like daidzein, genistein and coumestrol have been reported to stimulate spore germination, hyphal branching and root colonization by mycorrhizal fungi in soybean (ANTUNES et al., 2006; JUGE et al., 2012).

In addition, flavonoids can act as phytoalexins, antimicrobial stress molecules synthesized and accumulated in plants in response to microbial infection, as well as phytoanticipins, molecules constitutively synthesized by the plant in anticipation to pathogen attack (CHEYNIER et al., 2013). Sakuranetin (7-O-methylnaringenin) and 3-deoxyanthocyanidins are the most important flavonoids described as phytoalexins in cereals, produced in response to elicitors from pathogens (EJIKE; GONG; UDENIGWE, 2013). Du et al. (2010) reported that after inoculation with anthracnose pathogen *Colletotrichum sublineolum*, sorghum seedlings showed a faster and higher accumulation of luteolin and apigenin. Soares et al. (2015) suggested that hesperidin and rutin identified in *Citrus limonia* plants infected with *Xylella fastidiosa* may play a role in the plant-pathogen interaction, probably as phytoanticipins.

Flavonoids have been reported as chemical mediators in allelopathic interactions in the rhizosphere, since they were identified in significant concentrations in many bioactive root exudates (Weston and Mathesius, 2013). Allelopathic interactions include competition for resources and inhibition of germination and growth of neighboring plants (ZHUANG et al., 2013). Some examples include flavonoids released by *Stellera chamaejasme* roots that inhibit the germination and growth of *Arabidopsis thaliana* (YAN et al., 2014). Flavones isolated from rice and identified by Kong et al. (2004) inhibited the growth of weeds *Echinochloa crus-galli, Cyperus difformis* and *Cyperus iris*, and the spore germination of the phytopathogens fungi *Pyricularia oryzae* and *Rhizoctonia solani*.

3.2.2.2.3 Strigolactones

Strigolactones, terpenoid lactones derived from carotenoids, were initially studied as inducer molecules of germination of parasitic plants (MATUSOVA et al., 2005). However, other roles have been suggested for these compounds that go beyond of their involvement in plant parasitism. The beneficial roles of strigolactones was unveiled through the discovery that these compounds act as branching factors for symbiotic AM fungi, from which the plants benefit (XIE; YONEYAMA; YONEYAMA, 2010; BONFANTE; GENRE, 2015; RASMANN; TURLINGS, 2016). To date, at least 20 naturally occurring strigolactones have been identified and characterized in root exudates of various plants species, and several structural analogues were synthesized (LOPEZ-OBANDO et al., 2015). The natural strigolactones 5-deoxy-strigol, sorgolactone and strigol (AKIYAMA; MATSUZAKI; HAYASHI, 2005) and the carlactone (MORI et al., 2016), induced extensive hyphal branching of the AM fungi *Gigaspora margarita* at very low concentrations.

There is also significant interest in examining strigolactones as putative regulators of responses to environmental stimuli, especially the response to nutrient availability (FOO et al., 2013). Some studies have reported that P and N deficiency may promote and increase the exudation of strigolactones (YONEYAMA et al., 2012). For example, P deficiency showed an increase in the production of the strigolactones orobanchol and solanacol in tomato (LÓPEZ-RÁEZ et al., 2008). In sorghum, P and N deficiency promoted the increase of 5-deoxy-strigol exudation (YONEYAMA et al., 2007) and in rice there was an increase in the production of 2-epi-5-deoxystrigol and orobanchol (JAMIL et al., 2011).

3.2.2.2.4 Phytosiderophores

Iron is an essential micronutrient for plants, since it serves as a cofactor for many enzymes with redox activity (PODILE; KISHORE, 2006; BEASLEY; HEINRICHS, 2010). Although the total amount of this element is high in most soils, their availability is usually very low because of the low solubility of iron oxides.

Plants evolved different strategies applied for iron absorption, in order to deal with the scarcity of this micronutrient (LEMANCEAUSOILS et al., 2009; PII et al., 2016). While dicotyledons (strategy I plants) may exude organic acids and promote acidification of the rhizosphere, some monocotyledons of the Poaceae family (strategy II plants) respond to the Fe deficiency condition by producing iron-chelating molecules known as phytosiderophores, that increase the availability of this element on the cell surface (NEUMANN; RÖMHELD, 2011; MIMMO et al., 2014).

Phytosiderophores are low molecular weight non-protein organic compounds (500-1000 Da), of structurally diverse nature, excreted mainly under iron deficiency conditions, although they may also form complexes with other micronutrients like Zn, Cu, Mn, Mo and Ni (Dotaniya et al., 2013). More than 500 types of phytosiderophores are known and depending on the characteristics of functional groups, they have been divided into three main families: hydroxamates, catecholates and carboxylates. Nicotinamide, mugineic acids, avenic acid and disticonic acid are the most common phytosiderophores released by grasses (Ahmed and Holmström, 2014). However, a recent study by Suzuki et al. (2016), showed that 2-deoxymugineic acid was synthesized by olive (*Olea europaea*) plants, a dicot plant, contributing to maintaining Fe homeostasis and representing a valid opportunity in Fe acquisition process by dicotyledons when intercropped with monocotyledons.

3.2.2.2.5 Benzoxazinoids

The benzoxazinoids are a group of cyclic hydroxamic acids that are found in a multitude of species of the family *Poaceae* of the monocot plants, including important agricultural crops as maize, wheat and rye. However, they have also been found in single species of two orders of the dicots, the Ranunculales and the Lamiales (within the families Ranunculaceae, *Consolida orientalis*; Lamiaceae, *Lamium galeobdolon*; and Plantaginaceae, *Scoparia dulcis*) (FREY et al., 2009). These compounds are heterogeneously accumulated within the plant at concentrations that vary considerably with tissue age and are exuded in relatively large quantities from cereal roots, being considered as the main secondary metabolites for defense, acting as allelochemicals against microorganisms, insects and weeds or competing plants (NIEMEYER, 2009). Benzoxazinoids 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) and its derivate 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) are the most predominant in plants (NIEMEYER, 1988). These compounds are usually stored in the vacuole as non-toxic glucosides linked to D-glucose and, upon tissue injury, are hydrolyzed in the respective aglucones, which are toxic to pathogens (FREY et al.,

2009; NIEMEYER, 2009).

Several studies involving benzoxazinoids have been carried. Macías et al. (2014) reported the characterization of a complete allelochemical pathway involving the benzoxazinones which participated in allelopathic interactions between rye and the weed *Avena fatua*. Yang et al. (2014) and Ding et al. (2015) carried a large-scale experiment using an intercropping system between pepper (*Capsicum annuum*) and maize. They reported that zoospores of *Phytophthora capsici*, an important pepper pathogen, were inhibited by the benzoxazinoids exuded by maize roots and that the defense related pathways or genes in maize roots were activated by elicitors from the *P. capsici* or pepper root exudates. Guo et al. (2016) reported that DIMBOA and its derivatives 2-benzoxazolinone (BOA), 6-chloro-2-benzoxazolinone (CDHB), and 2-mercaptobenzothiazole (MBT) exhibited potential as antimicrobial for controlling tobacco bacterial wilt caused by *R. solanacearum*. Benzoxazinoids have also been described as chemoattractants for beneficial bacteria. Neal et al. (2012) evidenced that root exudation of DIMBOA during the initial growth of maize, induced bacterial genes with putative functions in chemotactic responses promoting the colonization by the plant-beneficial bacteria *Pseudomonas putida* KT2440.

3.3 RHIZOSPHERE METABOLOMICS

The rhizosphere is a constantly changing microenvironment, where there is a flux of energy, nutrients and molecular signals between the plant roots and microbes that affects their mutual interactions (REUBEN; BHINU; SWARUP, 2008). Since qualitative and quantitative metabolic composition of the rhizosphere differs strongly from bulk soil and is affected by several biological and environmental factors, the study of root exudates is necessary to have a better understanding of this microenvironment.

Metabolomics is becoming as a tool accepted for the analysis of metabolites in root exudates (VAN DAM; BOUWMEESTER, 2016), since it allows comprehensive information on the composition of a metabolite pool that can explain several metabolic processes that occur in the rhizosphere (JORGE; MATA; ANTÓNIO, 2016), for example, to understand the effects of the metabolites exuded in the ecological relationships of the soil or in the interactions plant-microorganisms or other soil organisms (REUBEN; BHINU; SWARUP, 2008).

Without metabolomics, it would be necessary to use a large number of different conventional methods that cover all main groups of metabolites (GIKA; WILSON;

THEODORIDIS, 2014). However, the continual refinement of analytical technologies and the increasing of profiling-scale of metabolites have led to the evolution of metabolomics, which has matured as a valuable tool for advancing our understanding of the metabolic processes that occur in plants and rhizosphere (SUMNER et al., 2014). In this way, one of the major advantages of metabolomics compared with conventional methods of analysis is that all compounds are measured at once in only one step, rather than put through iterative purification steps (SARDANS; PEÑUELAS; RIVAS-UBACH, 2011).

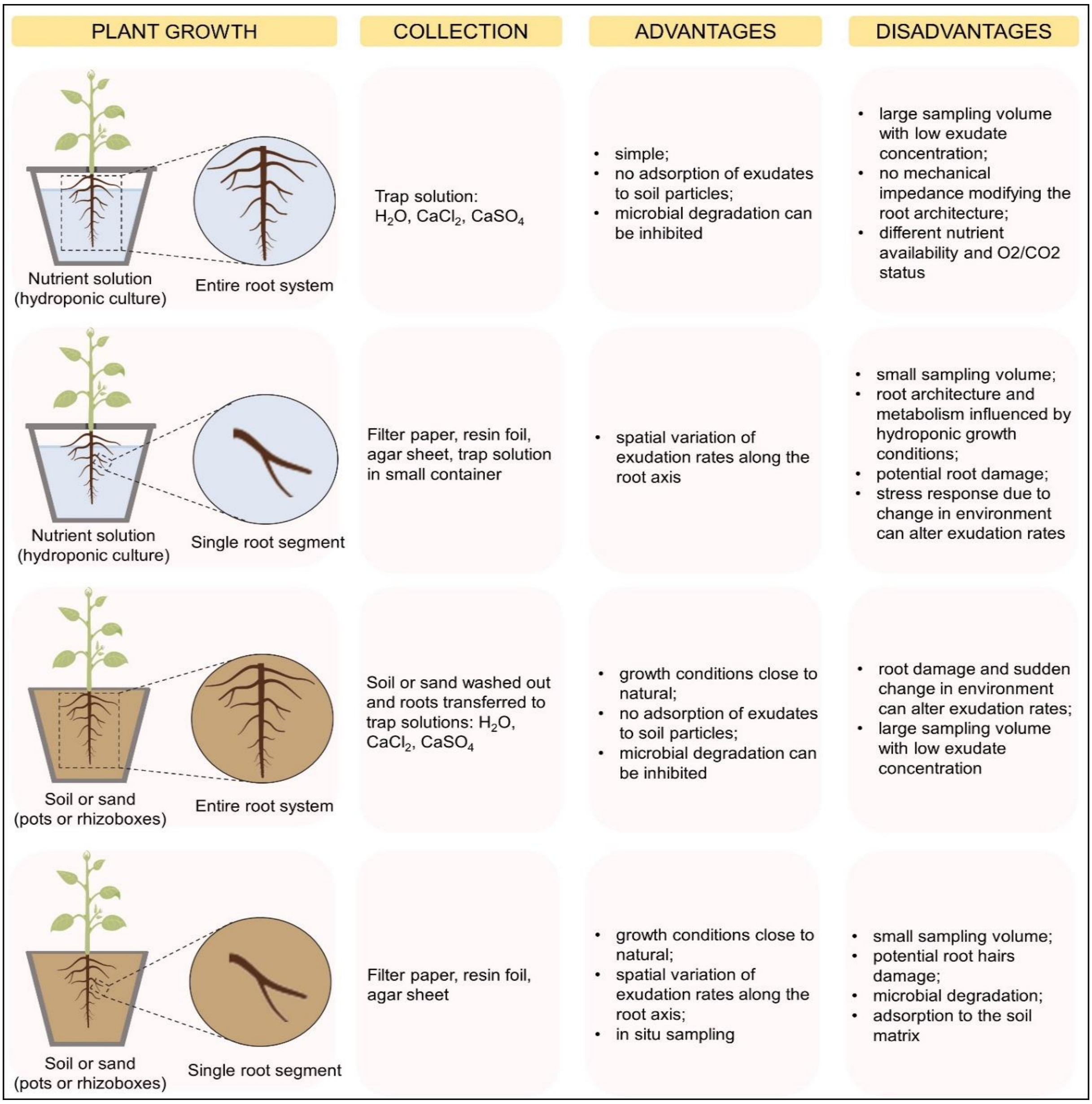
Currently, metabolomics is being used to address specific topics related to environmental changes or biotic interactions of plants (TENENBOIM; BROTMAN, 2016). Through metabolomics it is possible to monitor metabolic changes of plant root exudates in response to various abiotic stress conditions such as drought, salinity, temperature, nutrient availability, heavy metals (SHULAEV et al., 2008; RODZIEWICZ et al., 2014; JORGE et al., 2015), or biotic interactions, including systemic acquired resistance, induced resistance, allelopathy as well as the beneficial interactions with symbiotic microorganisms and PGPR (KUSHALAPPA; GUNNAIAH, 2013; TENENBOIM; BROTMAN, 2016). In this way, the identification and mapping of the metabolites that are differentially produced in a given stress condition can be carried out, thus making it possible to determine molecules with protective function.

Metabolomics can also be coupled to genomic studies for a faster determination of genes involved in adaptive responses (SARDANS; PEÑUELAS; RIVAS-UBACH, 2011) and to have a better understanding of the regulation and biological importance of root exudates (VAN DAM; BOUWMEESTER, 2016). Several studies have shown that there is genetic variation in the concentration and composition of the metabolites of root exudates. For example, Mönchgesang et al. (2016) used 19 *A. thaliana* accessions, with large degree of geographic and phenotypic diversity to find out if the root exudate composition is genetically determined. For this purpose, they analyzed which metabolites showed natural variation, if similar metabolic phenotypes shared a genetic base and if certain characteristics could directly link phenotype and genotype. They found that there was natural genetic variation of secondary metabolism in root exudates of *A. thaliana*.

3.3.1 Methods of Collection of Plant Root Exudates

In the root exudation and rhizosphere metabolomics studies, the selection of the collect method to be used is fundamental to determine the root exudates composition which will allow a better understanding of dynamic processes occurring in the rhizosphere. Several researches have been developed and various methodologies for the exudates collection have been proposed, each with its advantages and disadvantages (**Fig. 3.4**).

Fig. 3.4. Some of the most frequently techniques used for collecting root exudates according to the plant growth conditions and their advantages and disadvantages (adapted from OBURGER et al., 2013; OBURGER; JONES, 2018).



PLANT GROWTH	COLLECTION	ADVANTAGES	DISADVANTAGES
Nutrient solution (hydroponic culture) Entire root system	Trap solution: H_2O, $CaCl_2$, $CaSO_4$	• simple; • no adsorption of exudates to soil particles; • microbial degradation can be inhibited	• large sampling volume with low exudate concentration; • no mechanical impedance modifying the root architecture; • different nutrient availability and O2/CO2 status
Nutrient solution (hydroponic culture) Single root segment	Filter paper, resin foil, agar sheet, trap solution in small container	• spatial variation of exudation rates along the root axis	• small sampling volume; • root architecture and metabolism influenced by hydroponic growth conditions; • potential root damage; • stress response due to change in environment can alter exudation rates
Soil or sand (pots or rhizoboxes) Entire root system	Soil or sand washed out and roots transferred to trap solutions: H_2O, $CaCl_2$, $CaSO_4$	• growth conditions close to natural; • no adsorption of exudates to soil particles; • microbial degradation can be inhibited	• root damage and sudden change in environment can alter exudation rates; • large sampling volume with low exudate concentration
Soil or sand (pots or rhizoboxes) Single root segment	Filter paper, resin foil, agar sheet	• growth conditions close to natural; • spatial variation of exudation rates along the root axis; • in situ sampling	• small sampling volume; • potential root hairs damage; • microbial degradation; • adsorption to the soil matrix

Frequently, collection of exudates from the entire root system is assessed using plants grown in hydroponic culture by immersion of the root system in trap solution for a certain period (CESCO et al., 2010). The most commonly used trap solutions are nutrient solutions containing $CaSO_4$ or $CaCl_2$ to provide Ca^{2+} for membrane stabilization or simply distilled water. The exudates collection using this method, usually requires concentration by vacuum evaporation or lyophilization, due to the low concentration of exudate compounds. However, depending on the composition of the trap solution, the reduction of sample volume can lead to high salt concentrations, which may interfere with subsequent analysis. Therefore, prior to sample concentration, is recommended use of ion-exchange resins or solid-phase extraction techniques for enrichment of exudates contained into trap solution (NEUMANN; RÖMHELD, 2007; VRANOVA et al., 2013). The main disadvantage of this method is that growth conditions differ significantly from the natural soil environment (nutrient availability, O_2/CO_2 concentration, mechanical impedance, microbial community composition) and the exudation rates are not comparable to a plants exudation behaviour in soil (OBURGER et al., 2013).

On the other hand, there are localized sampling techniques, based on the use of nutrient solution traps or plants grown in rhizobox systems that comprise the use of sorption media such as paper filters, membrane filters, glass-fiber or solid agar sheets placed onto the surface of the respective root zones (NEUMANN; GEORGE; PLASSARD, 2009). This method allows a more realistic estimation of exudates concentrations in the rhizosphere, since adsorption of exudates to sorption media placed onto the root surface resembles the behavior of these compounds in the soil (CESCO et al., 2010) and usually is not necessary the metabolites concentration, being possible a direct analysis (NEUMANN, 2006). Nonetheless, one disadvantage of this collection method is that the adsorption of exudates to the soil matrix, microbial degradation as well as microbial exudation, can modify the concentration of exudates released (OBURGER et al., 2013). Furthermore, low amounts of exudates are frequently a problem, requiring sensitive analytical techniques such as HPLC-MS, GC-MS, capillary electrophoresis or NMR for quantification (NEUMANN; RÖMHELD, 2007; CESCO et al., 2010).

A major problem of all techniques used for collection of root exudates is the risk of microbial degradation during the collection period and the limited ability to differentiate between root exudates and microbial metabolites. In this sense, short collection periods (1-2 h) can minimize the risk of microbial degradation, and isotopic labeling techniques can help to differentiate between root exudates and microbial metabolites (NEUMANN, 2006).

Another important factor that should be considered in sampling of root exudates is the sterility condition of the system used. The microorganisms influence the root exudation by affecting the membrane permeability altering the carbon displacement to the roots and maintaining an exudation gradient in the soil solution (LAMBERS et al., 2009), besides stimulating consumption of O_2 and increase of CO_2 production, which consequently stimulates exudation of certain compounds (VRANOVA et al., 2013). Kuijken et al. (2014) reported that in non-sterile rhizospheres exogenous carbon disappeared quickly with a half-life of 2 to 3 h and root exudate concentrations remained below detection limit. In sterile rhizospheres, exogenous carbon levels were relatively stable or depleted slower than in non-sterile rhizospheres and organic acid build-up occurred.

Similarly, Bobille et al. (2016) found differences in exudation profile of amino acids between sterile and non-sterile soils. They suggested that these differences were not only due to uptake by microorganisms in the soil, but also because plants growing in sterilized substrate completely modify the metabolism and exudation of amino acids in the root compared with plants growing in non-sterilized soil, indicating an adaption to the rhizospheric environment.

It is possible to perceive that in spite of the methodological advances, sampling of exudates remains a major challenge. In geral, the exudates collection techniques by trap with nutrient solution serve as tools for basic model studies, while localized collection techniques with soil grown plants offer the opportunity to for real rhizosphere studies under more realistic conditions (NEUMANN, 2006). Thus, considering the factors previously described, the method adopted for their collection is one of the most crucial aspects in the root exudate determination. Caution should therefore be taken in the interpretation of data from exudation studies and comparisons using different collection times and procedures (VALENTINUZZI et al., 2015).

3.3.2 Analytical Techniques in Rhizosphere Metabolomics

There are two approaches to analyse small molecules, and they differ in the number of compounds analyzed, the level of structural information obtained, and their sensitivity (YVES et al., 2012). The *targeted* metabolomic analyzes a small number of known metabolites in specific compound classes (e.g. sugars, amino acids, phenolics). On the other hand, the *untargeted* metabolomics allows a more comprehensive information of the metabolic profiling, but the structures of the compounds are rarely identified (GORROCHATEGUI et al., 2016).

The analytical technologies commonly used in targeted and untargeted metabolomics studies include separation methods coupled with mass spectrometry (MS), such as gas chromatography (GC-MS), liquid chromatography (LC-MS) or capillary electrophoresis (CE-MS). Other technologies such as nuclear magnetic resonance (NMR), direct injection mass spectrometry (DIMS) and Fourier transform infrared spectroscopy (FT-IR) have also been used (ZHANG et al., 2012; TUGIZIMANA; PIATER; DUBERY, 2013; ERNST et al., 2014). Currently, metabolomics methods typically allow measuring hundreds of compounds, with a small number being definitively identified, a larger number being identified as belonging to particular compound classes, and many remaining unidentified (YVES et al., 2012).

Furthermore, the analytical platform chosen greatly determines the classes of molecules that can be detected. For example, GC-MS is the platform choice when studying rhizosphere interactions mediated by volatile compounds and compound appearing mainly in primary metabolism like amino acids, fatty acids and sugars (after derivatization to make them volatile). By contrast, a variety of semi-polar groups of secondary metabolites in exudates, like phenolics or flavonoids, need to be analyzed by LC-MS or NMR platforms (SARDANS; PEÑUELAS; RIVAS-UBACH, 2011). However, if there is no prior knowledge on the type of molecules present in the root exudates, a combination of platforms may be used (van Dam e Bouwmeester, 2016).

3.3.2.1 Gas chromatography–mass spectrometry (GC-MS)

GC-MS is a powerful analytical technique widely used in metabolomic analysis to provide a high efficiency and reproducibility in the separation of (un)known metabolites, even at low concentrations (JORGE et al., 2015). This technique is commonly applied for the separation of volatile and semivolatile compounds. However, derivatization reactions are also used additionally prior to analysis directly or indirectly making a large number of non-volatile and more polar compounds amenable to GC-MS separation and analysis (RUGOVA et al., 2017).

One reason why GC-MS is one of the most reproducible techniques is partly due to the electron impact ionization (EI) method, generally employed in combination with GC. In this method, gas-phase molecules interact with kinetically activated electrons at average standard energy of 70 eV, causing strong molecular fragmentation (ERNST et al., 2014; JORGE et al., 2015). After the ions are generated in the ionization source, they are separated in an analyzer for detection, and thus obtain a mass spectrum. The most commonly used mass analyzers for GC-MS metabolomics studies are the quadrupole (Q) and time-of-flight (TOF). Analysis by GC-TOF-MS provide higher mass accuracy, higher duty cycles and faster acquisition times than GC-Q-MS analysis. Those parameters are particularly helpful for the accurate deconvolution of overlapping peaks, typically found in plant extracts (JORGE et al., 2015; JORGE; MATA; ANTÓNIO, 2016).

Several applications of this technique in the rhizosphere of plants have been described. Lu et al. (2014) evaluated the root exudates of two aquatic plants species and, using the GC-MS technique, identified fatty acid methyl esters and fatty acid amides that stimulated nitrogen removal of denitrifying bacteria. Zhu et al. (2016) tested the effect of increasing levels of urea fertilization on maize root exudation, rhizosphere microbiome and nitrogen use efficiency. Using GC-MS they found that when maize was supplied with increasing amounts of N, roots secreted more sugars, sugar alcohols, and phenolics, which subsequently altered the soil microbial community structure and abundance. Badri et al. (2013) collected root exudates of *A. thaliana* and applied them repeatedly to a soil. They used GC-MS to characterize the chemical diversity present in the exudates and to get an initial understanding of how these metabolites can promote or inhibit the growth of specific groups within a natural soil microbiome in the absence of the plant.

GC-MS was also used along with NMR, by Lima et al. (2014) to analyze the root exudates of maize seedlings inoculated with *Herbaspirillum seropedicae* and thus to have improved understanding of the metabolic changes induced by the inoculation to create new approaches in the use of compounds used as additives in bioinoculant formulations. Similarly Saia et al. (2015) used GC-TOF-MS technique to evaluate the effects of AMF and PGPR inoculation on wheat root metabolome. Based on GC-MS technology, Luo et al. (2017) analyzed the metabolic profile of root exudates from the Pb-accumulating and non-accumulating plants, identifying 15 compounds assumed to be potential biomarkers associated with the accumulation or tolerance to this metal.

In another study using GC-TOF-MS, Brooks and Benson (2016) compared the metabolome of nodulated roots of two actinorrhizal plants species (*Alnus glutinosa* and *Casuarina cunninghamiana*) with roots from non-nodulated plants. They detected 729 metabolites from the nodules of two plant species, of which 441 were identified and classified as organic acids, sugars, alcohols and amino acids. The other unnamed metabolites were described having masses below 500 Da and above 85 Da after derivatization.

3.3.2.2 Liquid chromatography–mass spectrometry (LC-MS)

LC-MS is a most versatile and sensitive separation method than GC-MS since it allows compounds separation of a wide polarity range as well as thermolabile and high molecular weight metabolites, without the need for sample volatility (DUNN; ELLIS, 2005; ZHANG et al., 2012). LC-MS uses soft ionization methods such as electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), resulting in protonated (in positive mode) or deprotonated (in negative mode) molecular masses (DE VOS et al., 2007; ALLWOOD; GOODACRE, 2010). However, LC-ESI-MS is most commonly used in rhizosphere research since it allows the ionization of a wide range of polar and moderately polar compounds, offering high selectivity and mass sensitivity, as well as reasonable estimates of molecular formulas (PARK; SEO; HEGEMAN, 2014; RUGOVA et al., 2017).

There is a large variety of mass analyzers applied in plant metabolomics studies by LC-MS, such as quadrupole (Q), triple quadrupole (QqQ), ion trap (IT), Orbitrap, time of flight (TOF) and Fourier transform ion cyclotron mass spectrometers (FT-ICR-MS), and combination of some of these, such as Q-TOF (ERNST et al., 2014; PARK; SEO; HEGEMAN, 2014). However, in rhizosphere studies where are analyzed complex mixtures of metabolites, is recommend the use of high precision instruments with wide dynamic range that facilitates a finer distinction between closely-related signals that lead to the correct identification of these compounds (MOCO et al., 2007). Among the rhizosphere compounds frequently analyzed by LC-MS include the important group of secondary metabolites such as flavonoids, phenolics, saponins, phenylpropanoids, glucosinolates, polyamines, benzoxazines (DE VOS et al., 2007; VAN DAM; BOUWMEESTER, 2016), and also primary metabolites such as amino acids, organic acids, sugars and alcohols (ALLWOOD; GOODACRE, 2010).

In order to explore the chemical composition of root exudates of the model plant *A. thaliana*, Strehmel et al. (2014) developed a workflow using the UPLC-ESI-QTOF-MS technique. When evaluating the untargeted metabolic profiling of the semipolar fraction of root exudates, they detected 103 compounds, mainly secondary metabolites, of which more than 90 were structurally characterized or classified and among them, 42 compounds were rigorously identified using an authenticated standard. Marti et al. (2013) also developed UHPLC-TOF-MS-based metabolomics approach to evaluate local and systemic herbivore-induced changes in leaves, sap, roots and root exudates of maize. They identified 32 differentially regulated compounds and suggested that the presented metabolomic approach may serve as a template to study plant stress responses in an unbiased manner, from metabolic profiling to activity mapping. Nebbioso et al. (2016) used the UHPLC-ESI-IT-TOF-MS technique to assess changes in chemical profile of roots of tomato plants treated with different microbial inoculats. They reported that the use of this technique successfully represented the metabolic profile of tomato roots, allowing the identification of chemical structures for most compounds as well as the identification of marker compounds that represented the metabolic responses to different microorganisms. Similarly, Rivero et al. (2015) also reported that use of LC-ESI-QTOF-MS allowed successfully characterization of important changes in the metabolome of mycorrhizal roots of tomato. LC-MS is also a well-suited technique to perform a targeted approach in which specific compounds classes of the plant metabolome can be analyzed (JORGE; MATA; ANTÓNIO, 2016). For example, using LC-ESI-MS, McRae and Monreal (2011) described a quantitative method for the analysis of 15 free reducing carbohydrates in soil solutions and Weisskopf et al. (2006) studied the isoflavonoids composition in *Lupinus albus* roots, whereas Jaitz et al. (2011) developed a highly sensitive method using LC-ESI-TOF-MS to quantify low molecular weight organic acids present in samples related to rhizosphere studies. In this sense, results of the previously presented studies indicate that LC-MS based metabolomics really is a holistic and efficient method to monitor variations of the bioactive metabolic composition in roots and rhizosphere of plants.

3.3.2.3 Nuclear magnetic resonance (NMR)

NMR is a spectroscopic technique that takes advantage of the spin properties of the nucleus of hydrogen (^{1}H-NMR) or carbon (^{13}C-NMR) atoms present in a molecule and is considered one of the most selective techniques for elucidation of compounds, since it provides a unique spectrum and allows highly specific evidence for the identification of a particular molecule (MOCO et al., 2007). However, analyzes performed with plant extracts or root exudates, usually produce complex spectra with overlapping peaks that make it difficult to identify and quantify the metabolites (KIM; CHOI; VERPOORTE, 2011). In light of this, two-dimensional nuclear magnetic resonance (2D NMR) has been increasingly explored as a tool with a superior resolution compared with one-dimensional NMR (1D NMR) (GUENNEC; GIRAUDEAU; CALDARELLI, 2014).

Compared with MS-based techniques, NMR spectroscopy is less biased since the results of MS analyses greatly depend on choice of ionization conditions and the specific instrumentation used (FORSETH; SCHROEDER, 2011). Moreover, NMR is a non-destructive and high-throughput technique that allows for short analysis times without the need for calibration curves or reference standards for each single compound (KIM; CHOI; VERPOORTE, 2010; MAHROUS; FARAG, 2015). However, due to its low sensitivity and restricted dynamic range relative to MS, NMR-based analyzes can only detect and quantify the most abundant metabolites (in the micromolar range) and, therefore, is most widely used as a metabolic fingerprinting technique (KIM; CHOI; VERPOORTE, 2011; JORGE et al., 2015). In this sense, nuclear magnetic resonance (1D NMR and 2D NMR) is an appropriate method for metabolomic analysis of plants and rhizosphere because it allows to have a general view of all the metabolites present in an organism under certain conditions, allowing the simultaneous detection of diverse groups of secondary metabolites besides abundant primary metabolites (KIM; CHOI; VERPOORTE, 2010).

Recent studies have used NMR to help to elucidate the structures of the compounds present in root exudates of several plant species. Using ^{1}H-NMR and ^{13}C-NMR techniques along with 2D NMR experiments, Kim et al. (2014) unveiled the chemical structure of avenaol, a strigolactone extracted and purified from root exudates of *Avena strigosa*. Hooper et al. (2015) analyzed the root exudates of different plants species from the genus *Desmodium*, which in intercrops systems with cereals, are known to inhibit allelopathically the parasitism caused by species of *Striga* genus.

Using ^{1}H-NMR and ^{13}C-NMR they could identify various C-glycosylflavonoids as the major compounds in these exudates. In an earlier study by Walker et al. (2003b), the metabolic profile of *A. thaliana* root exudates was evaluated. Using HPLC it was possible to quantify 289 metabolites and using ^{1}H-NMR and ^{13}C-NMR techniques they could characterize the chemical structures of 10 of these compounds: butanoic acid, *E*-cinnamic acid, o-coumaric acid, p-coumaric acid, ferulic acid, phydroxybenzamide, methyl p-hydroxybenzoate, 3-indolepropanoic acid, syringic acid, and vanillic acid.

NMR has also been used to detect changes in plant metabolism when exposed to some stress factor. Escudero et al. (2014) used a combined metabolomic approach employing the techniques of ^{1}H-NMR, HPLC-MS and excitation–emission matrix (EEM) fluorescence spectroscopy to detect changes in the rhizodeposition of the tritrophic system formed between tomato, the nematode *Meloidogyne javanica* and the fungus *Pochonia chlamydosporia*. Using untargeted ^{1}H-NMR and GC-MS based metabolomics, Zhao et al. (2016) evaluated the metabolic changes induced by nano-Cu particles in cucumber plants. Similarly, Choudhury and Sharma (2014) evaluated the effect of Al^{3+} on growth responses, along with physiological and metabolic changes in chickpea. They reported that the ^{1}H-NMR spectrum showed variation in the abundance and metabolic profile in chickpea root during Al^{3+} stress.

3.4 ACKNOWLEDGEMENTS

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4 RESEARCH PAPER

INOCULATION WITH PGPB ALTERS THE METABOLIC PROFILING OF ROOT EXUDATES AND THE FUNCTIONAL DIVERSITY OF RHIZOSPHERE MICROBIAL COMMUNITIES IN TOMATO

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ABSTRACT

Inoculation with plant growth-promoting bacteria (PGPB) represent an efficient method in sustainable agriculture to improve nutrients availability and crop production in diverse environmental conditions. In the present work, untargeted metabolomics and community-level physiological profiles (CLPP) approaches were employed to investigate the shaping of tomato root exudation and potential metabolic activity imposed by rhizosphere-associated microbiome, following inoculation with two different PGPB (*Enterobacter* sp. 15S and *Pseudomonas* sp. 16S). Significant increases in root and shoot dry biomass were observed in both inoculated treatments, when compared to uninoculated plants ($p < 0.05$). The untargeted metabolomics allowed discriminating the metabolic profile of root exudates, with distinct modulations imposed by either *Enterobacter* 15S or *Pseudomonas* 16S. Phenolics and flavonoids were among the most frequently identified differential metabolites in exudates from both *Enterobacter* 15S and *Pseudomonas* 16S. Nevertheless, other metabolites like phytohormones and amino acids were also decisive to this specific modulation. Interesantly, with regard to the CLPP analysis, a differential distribution of the metabolic activity profile of tomato rhizosphere-associated microbiome modulated by the bacterial inoculation was also observed, the same distribution as that found in the metabolomic analysis. In this instance, the microbiome from treatments 16S and control showed a higher functional diversity than treatment 15S and carbohydrates, carboxylic acids, amino acids, and polymers were the main classes of substrates which contributed to such differences.

Key-words: beneficial bacteria; untargeted metabolomics; differential metabolites; community-level physiological profiles (CLPP); metabolic activity profile.

4.1 INTRODUCTION

The rhizosphere is considered the region of the soil that is influenced by root activity, having distinct physical, chemical and biological characteristics from bulk soil (HINSINGER et al., 2005). Important processes related to growth, nutrition and health of plants, as well those related to the biogeochemical cycles take place in the rhizosphere, in part due to the higher density and metabolic activity of the organisms than the bulk soil (MENDES; GARBEVA; RAAIJMAKERS, 2013). Inoculation with beneficial bacteria, also known as plant growth-promoting bacteria (PGPB) constitute a powerful tool in sustainable agriculture by maintaining a balanced nutrient cycling in the rhizosphere and improving crop productivity, soil fertility and plant tolerance to stress (PII et al., 2015a; GOUDA et al., 2018) These bacteria inhabit naturally the soil, roots or rhizosphere and their use positively affects the host plant through multiples mechanisms: they may solubilize and provide nutrients, fix atmosphere nitrogen, supress phytopathonenic microorganisms and secrete phytohormones and phytochelators (BULGARELLI et al., 2013). The most common bacteria which have been used in inoculation studies include in majority diverse genera of diazotrophic bacteria like *Rhizobium*, *Bradyrhizobium* and *Azospirillum*, or P-solubilizing bacteria like *Pseudomonas* and *Bacillus* (BASHAN et al., 2014). However, in the last years, the use of other genera with potential to improve the plant growth such as *Burkholderia*, *Herbaspirillum*, *Enterobacter*, *Streptomyces*, *Erwinia*, *Rhodococcus*, *Azotobacter*, *Gluconacetobacter*, *Klebsiella*, *Pantoea*, among others, has been also explored (SOUZA; AMBROSINI; PASSAGLIA, 2015; SANTOS et al., 2017).

Through roots, plants actively release a large variety of organic and inorganic compounds called rhizodeposits or root exudates (UREN, 2007), many of which have biological activity on other organisms. Root exudates consist mainly of low molecular weight compounds such as amino acids, organic acids, sugars, phenolic compounds and high molecular weight compounds such as polysaccharides and proteins and their quali/quantitative composition depends on plant species, developmental stage, soil type, roots traits and external environmental biotic and abiotic factors (BADRI; VIVANCO, 2009; ZHALNINA et al., 2018). The complexity of the root exudates composition is related to its functional multiplicity. Therefore, plants can modify soil and rhizosphere properties to adapt and ensure their survival by using diverse strategies involving the modulation of root exudates (VIVES-PERIS et al., 2019). Furthermore, root exudates represent an important nutrient source for rhizosphere microorganisms and variations in their concentration and composition can exert a positive, negative or neutral effect, thus playing a valuable role in the configuration of the rhizosphere microbiome (CANARINI et al., 2019).

According to this, two different life strategies have been described for the microbial communities inhabiting the rhizosphere, inferred from their relative response to root exudates utilization: copiotrophy and oligotrophy (HO; DI LONARDO; BODELIER, 2017). Copiotrophic microorganisms are described as generalists, being able to adapt to a wide range of habitats and are characterized for exhibiting high growth rates when carbon sources conditions are abundant; on the other hand, oligotrophics are assumed to be more specialized, exhibiting slower growth rates, but they are able to sustain growth under nutrient-scarce conditions (FIERER; JACKSON, 2007; KURM et al., 2017). However, it is known that microbial activity in the rhizosphere increases when the exudation rate is high, consequently favoring the growth of copiotrophic bacteria, which are able to metabolize the large amounts of simple substrates such as sugars, amino acids and organic acids released by the plants into rhizosphere; these conditions mostly occur in the period when plants are photosynthetically active (HAASE et al., 2007; PHILLIPS; FINZI; BERNHARDT, 2011). To better understand the processes that take place in the rhizosphere, a diversity of molecular and microbiological methods have been developed and implemented. For instance, untargeted metabolomics is described as the most suited approach for the analysis of complex interactions facilitating the insight of the reciprocal responses between plants and microorganisms within the rhizosphere (MHLONGO et al., 2018). On the other hand, since availability of carbon (C) sources is the key factor dominating microbial growth in soil, the community-level physiological profiling (CLPP) based on sole C source utilization patterns provides an effective, simple and fast method to measure the potential metabolic activity of soil and rhizosphere microbiome (LLADÓ; BALDRIAN, 2017).

In this context, the exploration of the biotic processes anchored in the rhizosphere, especially the relationships between the plants, the soil matrix and its associated microbiota, as well the dynamic role of plant exudation in the establishment of rhizosphere microbiota, could provide new management approaches for agriculture, by increasing plants nutrient use efficiency and the mitigation of environmental stresses (GUPTA et al., 2015; ZHALNINA et al., 2018). Studies comparing the response to inoculation with different PGPB on the variability in dicot root exudation and the modulation of the metabolic activity of rhizosphere microbial communities, are scarcely described. Thereby, in order to study the impact of the inoculation with *Enterobacter* 15S and *Pseudomonas* 16S (two species of beneficial Gammaprotebacteria) on the rhizosphere of tomato, two integrative approches were used in this study: (i) untargeted metabolomics by UHPLC-ESI/QTOF-MS for elucidating the metabolic profiling of tomato root exudates aiming the comprehesion of plant-microbe interaction in the rhizosphere and; (ii) Biolog EcoPlates to obtain and compare the community-level physiological profiles (CLPP), by characterizing the metabolic versatility of the tomato rhizosphere-associated microbial community shaped by the inoculation treatments.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strains

Enterobacter sp. 15S and *Pseudomonas* sp. 16S strains (belonging to the Gammaproteobacteria class) were originally isolated from horticultural soil under conventional and organic management, respectively, in the laboratory of molecular biochemistry of State University of Londrina (Paraná, Brazil) according to the data previously published by (ZULUAGA et al., 2020). The bacterial inoculants were individually prepared for each strain in DYGS liquid medium (2 g L^{-1} glucose, 2 g L^{-1} yeast extract, 1.5 g L^{-1} peptone, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, final pH = 6.8) and shaken at 180 rpm and 28 ± 2 ˚C for 24 h. Then, the bacterial population density was determined by direct counts in a Neubauer chamber and diluited to the final concentration of 1×10^6 cells mL^{-1}.

4.2.2 Rhizobox Experiment

An inoculation trial using rhizoboxes was set up under greenhouse conditions in order to investigate the effects on the rhizosphere microbiota activity and the metabolic profile of tomato root exudates after inoculation with the beneficial bacteria *Enterobacter* 15S or *Pseudomonas* 16S. Rhizoboxes with transparent observation windows (36 × 11.5 × 2.5 cm; Neumann et al. (2014) were filled with 1.5 kg of an unsterilized substrate, obtained by mixing sand and oxisol with high clay content (78%) in a proportion of 4:1 (w/w) with the following characteristics: pH_{H2O}, 5.1; H + Al ($cmol_c\ dm^{-3}$), 9.28; K ($cmol_c\ dm^{-3}$), 0.63; P ($mg\ dm^{-3}$), 13.0; Ca ($cmol_c\ dm^{-3}$), 4.1; Mg ($cmol_c\ dm^{-3}$), 1.5; Al ($cmol_c\ dm^{-3}$), 0.32; and organic matter (%), 2.23. Tomato seeds (Santa Cruz Kada Gigante, Topseed, Santo Antonio da Posse, Brazil) were surface desinfected and sown at a depth of 1-2 cm into each rhizobox. At seven days after germination, the seedlings were thinned to two plants per rhizobox, and the inoculation was carried out by applying 1 ml of bacterial suspension (1×10^6 cells mL^{-1}, prepared as described above) to the seedlings. In order to promote the root growth along the observation window, rhizoboxes were fixed at a 45˚ inclination (with the aid of a wood rhizobox holder) and covered with aluminiun foil to avoid light-derived effects on the roots and to prevent algae growth.

The experiment comprehended three treatments with six replicates: two treatments where plants were inoculated with either *Enterobacter* 15S or *Psedomonas* 16S plus a control treatment with uninoculated plants. During the experimental period, 100 mL of Hoagland's nutrient solution (with 50% ionic strength) was applied to each rhizobox every two days. Rhizoboxes were distributed randomly and the experiment was conducted for 40 days (counting from the germination day) under greenhouse with day-night temperature of 25–23 ˚C. Plant harvest and sampling were assessed as described below.

4.2.3 Exudates Collection, Plant Biomass and Rhizospheric Soil Sampling

At the end of the experiment, two independents rhizoboxes out of the six per treatment were used to create a composed sample, obtaining three biological replicates. The root observation windows of each rhizobox were opened and root exudates were collected from apical root zones as described by (HAASE et al., 2007). Briefly, 60 discs of moist filter paper (5 mm diameter; 3MM CHR, Whatman, Kent, UK; previously washed with methanol and distilled water) were placed onto the surface of apical roots per rhizobox. Root exudates were collected 2 h after the beginning of the light period (around 8:00 a.m. time of Brazil) and after 3 h of collection, the filter discs onto the root surface were transferred into 5 mL eppendorf tubes and stored at -20 °C until further analysis. Thereafter, plants were separated in roots and shoots to recorded the dry weight at 65 °C and samples of soil immediately adjacent to the roots (rhizospheric soil) were collected for the microbiological determinations.

4.2.4 Metabolomic Analysis of Root Exudates

Metabolites from the root exudates and rhizosphere soil solution contained into each pool of 120 filter discs per treatment (one biological replicate) were extracted by adding 3.5 ml of 80% methanol. Eppendorf tubes were shaken during 4 h and methanolic extracts were afterwards filtered through a 0.22 µm membrane filter. Then, the exudates were concentrated by evaporation at room temperature (18 ± 2 ˚C) to a volume of approx. 1 mL and stored at -20 ˚C until further analysis.

Root exudate chemical profiles were determined by using a quadrupole-time-of-flight mass spectrometer (G6550 IFunnel QTOF, Agilent technologies Santa Clara, CA, USA) coupled to an UHPLC liquid chromatograph (1290 series, Agilent technologies) via a JetStream Electrospray ionization system (UHPLC/QTOF-MS) according to (PAUL et al., 2019). Briefly, the QToF was run in SCAN mode (positive polarity, 100–1200 m/z range) and extended dynamic range mode. Chromatographic separation was achieved in reverse phase mode using an Agilent Zorbax Eclipse-plus column (75 x 2.1 mm i.d., 1.8 μm) through a 33-min binary gradient from 5% to 90% acetonitrile in water, with a flow rate of 200 μL min^{-1}. The injection volume was 6 μL. The isotopic profile (i.e., monoisotopic accurate mass, isotopes spacing and ratio) was used for compounds annotation purposes, following mass and retention time alignment, in Profinder B.04 (Agilent technologies). A mass accuracy of 5 ppm was adopted. The reference database was an in house root exudates databased previously prepared including the most common metabolites found in these matrix (LUCINI et al., 2019). According to the COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi), our identification could be assigned to Level 2 (putatively annotated compounds).

4.2.5 Functional Potential of Bacterial Communities

In order to investigate the effect of inoculation with the beneficial bacteria *Enterobater* sp. 15S or *Pseudomonas* sp. 16S on the functional diversity of microbial communities associated with tomato rhizosphere, the community-level physiological profiles (CLPP) using Biolog EcoPlates (96-well plates consisting of three replicates of 31 diverse carbon sources and a water control; Biolog Inc, Hayward, CA, USA) were analyzed. Briefly, microbial suspensions were prepared from 1 g fresh rhizospheric soil in 10 mL of sterile saline solution (0.85% w/v NaCl) and shaking at 180 rpm for 30 min. Then, the suspension was serially diluted to10^{-3} using sterile saline solution and 150 µL aliquots were added to each of the 93 wells per plate. The plates were incubated at 28°C, and the optical density at 590 nm was recorded every 24 h across 168 h.

The well optical density values were adjusted by subtracting the absorbance value of the control well (containing water) from the absorbance values of each well containing a carbon source. To estimate the general microbial activity, the average well color development (AWCD) was calculated as described by (PENG et al., 2016) using the equation $AWCD = \Sigma ODi / 31$ where ODi was the adjusted optical density value from each well. The most intensive utilization of carbon substrates was observed after 96 h of incubation and, as such, this time was chosen to analyze the results. Community functional diversity was expressed as Shannon's diversity (H) and Evenness (E), calculated as described by (GE et al., 2018) based on the Biolog EcoPlates data obtained at 96 h. Substrate richness (R) was estimated as the total number of C substrates ($ODi > 0.2$) oxidized at 96 h by the microbial communities.

In order to assess differences in CLPP, data at 96 h were normalized dividing each ODi value by the AWCD and a principal component analysis (PCA) was perfomed according to Ge et al. (2018). A heatmap analysis was performed to represent the consumption differences in each treatment for the same substrate. As previously described by (CHOI; DOBBS, 1999), the 31 substrates in the Biolog EcoPlate were divided in six groups of carbon sources (carbohydrates, amino acids, carboxylic acids, amines, phenolic compounds and polymers) and then the carbon utilization rates were determined for each of the substrates groups.

4.2.6 Data Analysis and Statistics

Biolog Ecoplates and biomass data were submitted to analysis of variance (one way-ANOVA) and means were separated by the Scott-Knott test at $p < 0.05$ using the SASM-

Agri statistical software (CANTERI et al., 2001). The absorbance means from Biolog EcoPlates data were used in principal component analysis (PCA) and a heatmap based on standardized data, which were performed by using R software (https://www.r-project.org, using “FactoMineR,” “pheatmap”, “ggplot2” and “RColorBrewer” packages). For the heatmap analysis, Ward’s clustering method (WARD, 1963) based on the Euclidean distance was used.

However, Mass Profiler Professional B.12.06 (agilent technologies) was used for the chemometric interpretation of the metabolomics dataset. Compounds abundance was Log2 transformed, normalized at the 75th percentile, and baselined against the median. An unsupervised hierarchical cluster analysis was formerly done to naïvely describe relatedness/distance of metabolomic signatures across treatments. With this aim, the heatmap based on fold-change values was used, similarity was set as ‘Euclidean’ and the ‘Wards’ linkage rule was chosen. Pareto-scaling and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) supervised analysis were then carried out using SIMCA 13 software (Umetrics, Malmo, Sweden). Outliers were preliminary investigated using Hotelling’s T2 (95% and 99% confidence limits for suspect and strong outliers, respectively). CV-ANOVA ($p < 0.01$) and permutation testing (N=300) were also applied to validate the model and to exclude overfitting. Goodness-of-fit R^2Y and goodness-of-prediction Q^2Y were calculated for the OPLS-DA model. Variable Importance in Projection (VIP) analysis allowed selecting the most discriminant metabolites in the OPLS-DA model. Afterward, VIP markers (VIP score > 1.1) were subjected to fold-change analysis.

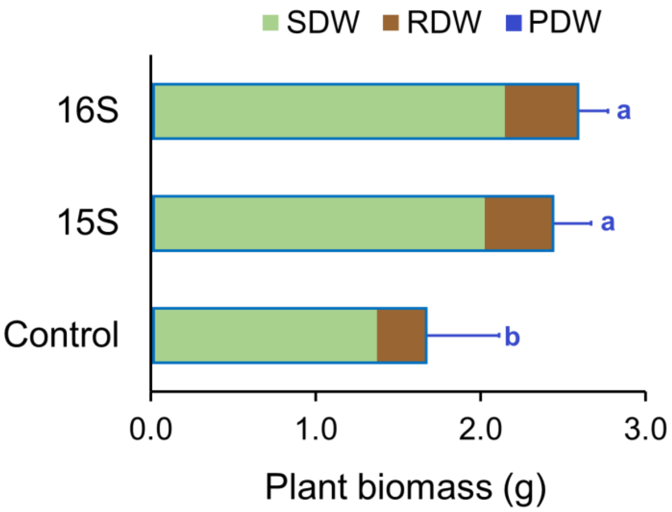
Chemical Similarity Enrichment Analysis (ChemRICH) was finally performed, as previously described (BARUPAL; FIEHN, 2017). The online web-app tool (http://chemrich.fiehnlab.ucdavis.edu) was used for this purpose. Therein, Tanimoto substructure chemical similarity coefficients were used to cluster metabolites into non-overlapping chemical groups.

4.3 RESULTS

4.3.1 Plant-Growth Response to Inoculation

The effects of bacterial inoculation on plant biomass responses after 40 days of growth in rhizoboxes system are shown in **Fig. 4.1**. Root and shoot biomass of tomato plants inoculated with *Enterobacter* 15S were significantly increased by more than 40% as compared to control plants, whereas inoculation with *Pseudomonas* 16S was significantly higher (over 50%) than the obtained in uninoculated plants. No significant differences across inoculated treatments were observed related to plant biomass accumulation.

Fig. 4.1. Effects of bacterial inoculation with *Enterobacter* 15S and *Pseudomonas* 16S on root dry weight (RDW), shoot dry weight (SDW) and plant dry weight (PDW) of tomato plants. Bars on top of columns correspond to standard error (n = 3) for PDW. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments for the PDW (the original data available in **Supplementary Table 4.3**).

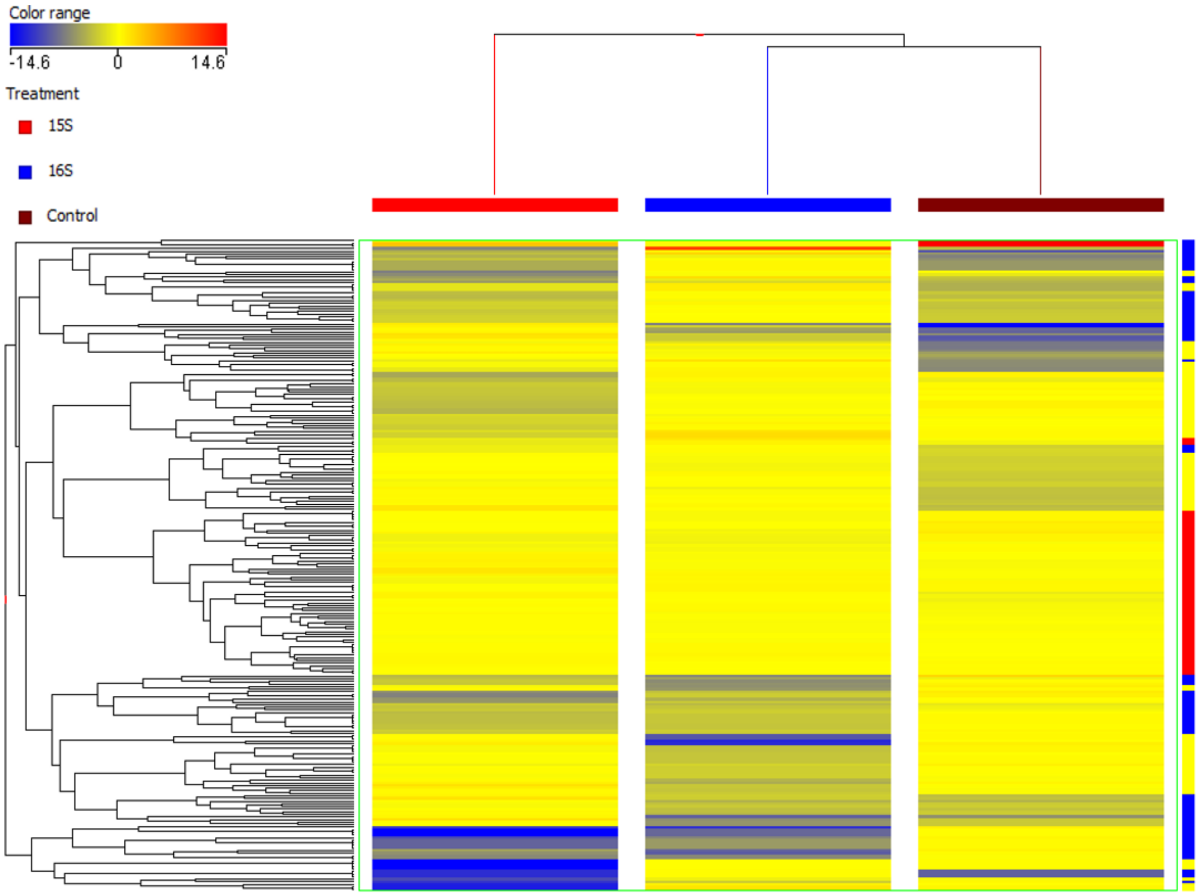


4.3.2 Metabolic Profile of Tomato Root Exudates by UHPLC-ESI/QTOF-MS

In order to investigate differences in the metabolic profile of the tomato root exudates related to the inoculation treatments (15S: inoculated with *Enterobacter* 15S, 16S: inoculated with *Pseudomonas* 16S, and control: uninoculated plants), a metabolomic approach based on UHPLC-ESI-QTOF mass spectrometry was performed. The untargeted approach allowed to putatively annotate 417 compounds in the tomato root exudates that belong to different classes such as flavonoids (35.5%), phenolic acids (20.4%), other polyphenols (12.9%, such as alkylphenols, hydroxycoumarins, phenolic terpenes), lignans (6.7%), amino acids (5.8%), carboxylic acids (2.9%), glycosides (3.6%), carotenoids (2.4%), fatty acids (2.2%), stilbenes (1.2%) and others compounds (6.5%, including indoles, phytohormones, phytosiderophores and sugars) (**Suppplementary Spreadsheet 4.1**).

With the aim of grouping and discriminating the compounds involved on the tomato response to the bacterial inoculation, a multivariated statistical analysis was carried out. For this purpose, an unsupervised hierarchical cluster analysis (HCA) followed by a supervised OPLS discriminant analysis (OPLS-DA), were performed. The HCA showed two main clusters as observed in the fold-change-based heatmap (**Fig. 4.2**). Root exudates obtained from tomato plants inoculated with *Enterobacter* 15S were separately clustered from those obtained from uninoculated plants and inoculated with *Pseudomonas* 16S.

Fig. 4.2. Unsupervised hierarchical cluster analysis of 419 metabolites identified in the root exudates of tomato plants inoculated with *Enterobacter* 15S or *Pseudomonas* 16S. Euclidean distance and Ward's linkage rule were applied and the clustering was generated on the basis of fold change based heatmap.

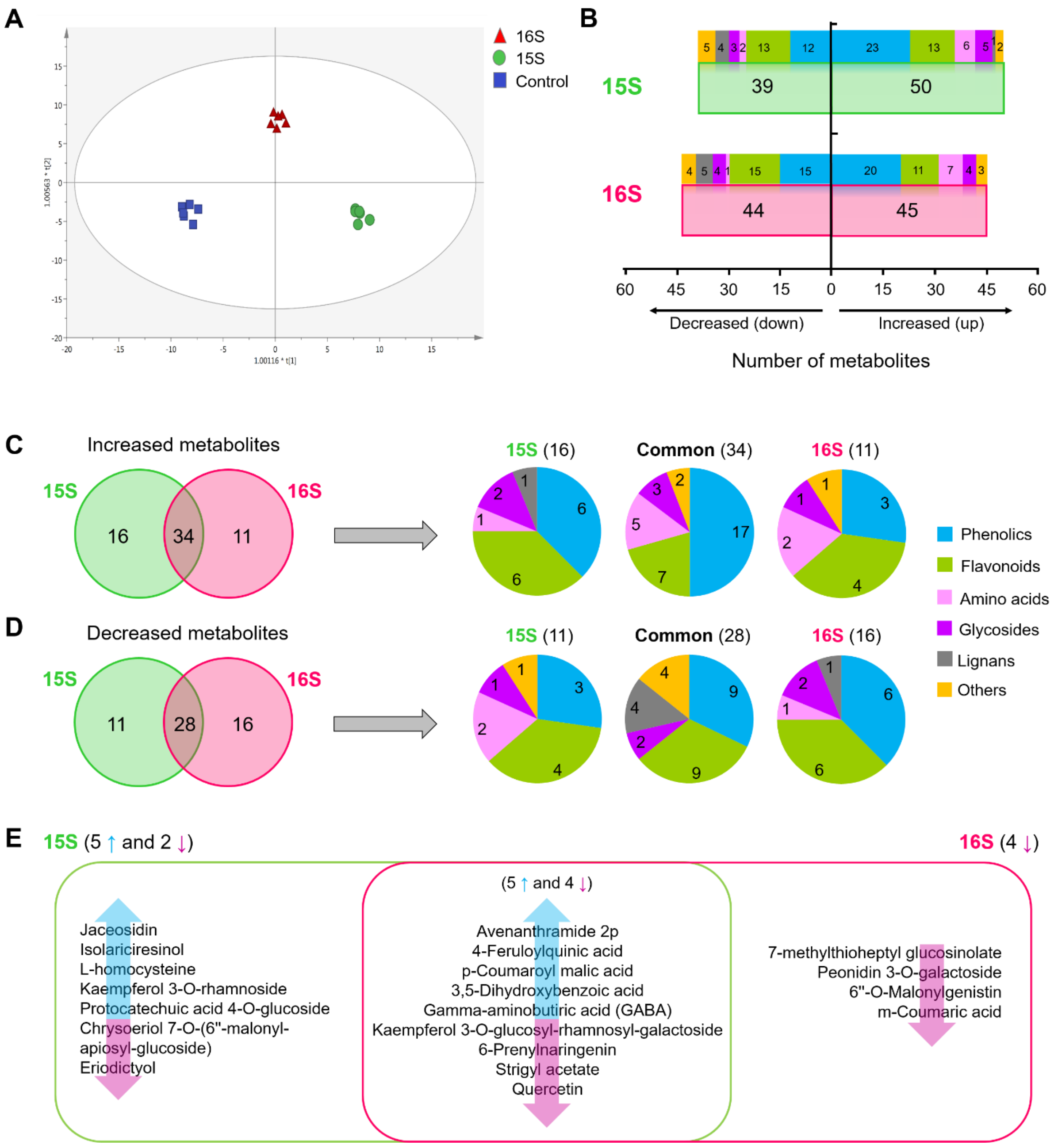


Thereupon, the supervised OPLS-DA was performed in order to better understand the differences in metabolic profiling of tomato plants root exudates depending on the bacterial inoculation and thus to remark the separation between treatments (**Fig. 4.3A**). In this regard, the OPLS-DA score plot obtained showed a clear and distinctive distribution in which the root exudates from each treatment were completely separated between them. The OPLS-DA model parameters were highly predictive as indicated by the goodness-of-fit ($R^2Y= 0.99$) and the goodness-of-prediction ($Q^2Y = 0.68$) and adequate cross-validation parameters were confirmed by the CV-ANOVA coefficient ($p = 0.0048$) and permutation test (N=100), showing the robustness of in the supervised model.

Afterwards, the variable importance in projection (VIP) was used as variable selection method, to identify the compounds with the highest discrimination potential by ranking them according to their contribution to the OPLS model. The metabolites possessing VIP scores > 1.1 were selected and subjected to a fold-change analysis to evaluate their distribution between the treatments (**Supplementary Table 4.1**). Overall, a total of 89 compounds were found to retain the highest discrimination potential; they could be ascribed to phenolics (35), flavonoids (26), amino acids (8), glycosides (8), lignans (5) and other classes (7). Nonetheless, these compounds exhibited distinctive modulations across treatment (**Fig. 4.3B-D**), hence supporting the separation of the groups obtained into the OPLS-DA score plot (**Fig. 4.3A**). A high number of VIP metabolites were up-accumulated in the treatment with *Enterobacter* 15S, while an equitable number of compounds were up/down regulated in the treatment with *Pseudomonas* 16S (**Fig. 4.3B**). Regarding to the metabolites up-accumulated (**Fig. 4.3C**), 34 were common to both treatments comprising a high abundance of phenolics, whereas 16 compounds were specifically accumulated in tomato root exudates from treatment 15S and 11 only in treatment 16S. Conversely, considering the metabolites down-accumulated (**Fig. 4.3D**), 28 were common in both treatments with a high abundance of phenolics, flavonoids and other compounds related to the phytohormones cluster, while 11 and 16 compounds were characteristically decreased in treatment 15S and 16S, respectively.

Fig. 4.3. Impact of bacterial inoculation on tomato root exudates metabolome. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) on the root exudates profiles of tomato plants inoculated with *Enterobacter* 15S or *Pseudomonas* 16S (**A**). Relative abundance and number of discriminant metabolites increased or decreased in root exudates of tomato plants identified by VIP method combined with fold-change analysis as function of the inoculation with *Enterobacter* 15S (green bar) or *Pseudomonas* 16S (pink bar) (**B**). Venn

diagrams exhibit the overlap of the number of discriminant metabolites that increased (**C**) or decreased (**D**) in tomato root exudates, according to the fold-change analysis on the VIP compounds. Pie charts display the chemical classes of the metabolites exclusively altered in the treatments 15S (left), 16S (right) and common to 15S and 16S (middle). Venn diagram showing the most increased (blue arrow) and decreased (purple arrow) metabolites in both inoculation treatments, according to the fold-change analysis on the VIP compounds presented in **Supplementary Table 4.1** (**E**).



Overall, 20 out of 89 VIP metabolites were found to be significantly modulated by the inoculation treatments (**Fig. 4.3E**) characterized by a strong up or down regulation according to their fold-change values (**Supplementary Table 4.1**). In this regard, it was found that seven compounds were specifically altered in root exudates from tomato inoculated with *Enterobacter* 15S, two of which being down-accumulated flavonoids [chrysoeriol 7-*O*-(6"-malonyl-apiosyl-glucoside) and eriodictyol] and five compounds were up-accumulated [L-homocysteine (amino acid); jaceosidin and kaempferol 3-*O*-rhamnoside (flavonoids); isolariciresinol (lignan); protocatechuic acid 4-*O*-glucoside (phenolic)]. On the other hand, four metabolites resulted to be decreased in root exudates from tomato inoculated with *Pseudomonas* 16S [peonidin 3-*O*-galactoside (anthocyanin); 6"-*O*-malonylgenistin (isoflavone); 7-methylthioheptyl glucosinolate (glucossinolate); m-coumaric acid (phenolic)]. In addition, nine compounds were common in both treatments, where phenolics were found to be the most increased [the hydroxycinnamics 4-feruloylquinic acid and p-coumaroyl malic acid; avenanthramide 2p; 3,5-dihydroxybenzoic acid] together with the gamma-aminobutiric acid (amino acid), whereas flavonoids were the most decreased [6-prenylnaringenin; quercetin; kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside] jointly with the strigolactone strigyl acetate (**Fig. 4.3E**). The higher number of discriminant metabolites and their fold-change values indicate that tomato root exudates underwent a more profound modulation following inoculation with *Enterobacter* 15S. Although phenolics and flavonoids were the most represented among differential metabolites, other metabolites like phytohormones and amino acids were also decisive to the specific modulation.

4.3.3 Effect of Bacterial Inoculation on Chemical Classes in Tomato Root Exudates

In view of the complexity of the data obtained in our metabolomic study, the use of adequate statistical approaches are crucial for understanding the biochemical processes induced as result of the bacterial inoculation. As multivariate methods oversimplify and do not consider biological relatedness, it becomes necessary to define the groups of metabolites that are biologically related to each other (Barupal et al., 2018). For this purpose, chemical enrichment techniques (e.g. ChemRICH) are key tools since reduce the complexity of the data and correlate the metabolic groups with biological functions (Barupal and Fiehn, 2017).

The ChemRICH analysis was performed to detect differential accumulation of chemical classes in tomato plants inoculated with beneficial bacteria (*Enterobacter* 15S or *Pseudomonas* 16S) compared to uninoculated plants (control). This analysis identified diverse compounds clusters (34 clusters) being significantly altered by the inoculation treatment (**Table 4.1 and Supplementary Fig. 4.1**). A generalized depletion of some sets of metabolites was observed in tomato root exudates of both inoculated treatments, although in different proportions (26 classes of compounds, including flavonoids, flavones, flavanones, lignans, fatty acids, glucosides, hydroxybenzoates, coumaric acids and some others polyphenols) compared to uninoculated plants, whereas flavonols were the only compounds resulting up-accumulated in both treatments. Nonetheless, it was possible to observe a differential alteration of some chemical clusters in each inoculation treatment. For instance, root exudates from tomato plants inoculated with *Enterobacter* 15S presented a particular up-accumulation of phenols, catechols, amino acids and ortho-aminobenzoates while plants inoculated with *Pseudomonas* 16S solely showed an increase of anthocyanins.

Table 4.1 Chemical similarity enrichment analysis (ChemRICH). The results show the most significant altered clusters as given by the Kolmogorov-Smirnov test. up: high proportion of increased metabolites for each cluster, down: high proportion of decreased metabolites for each cluster, equal: same proportion of increased and decreased metabolites. The colored circles give the proportion of increased (red) or decreased (blue) metabolites in the inoculated treatments (15S or 16S) compared to uninoculated treatment (control). Purple/pink circles have both increased and decreased metabolites (see **Supplementary Fig. 4.1**).

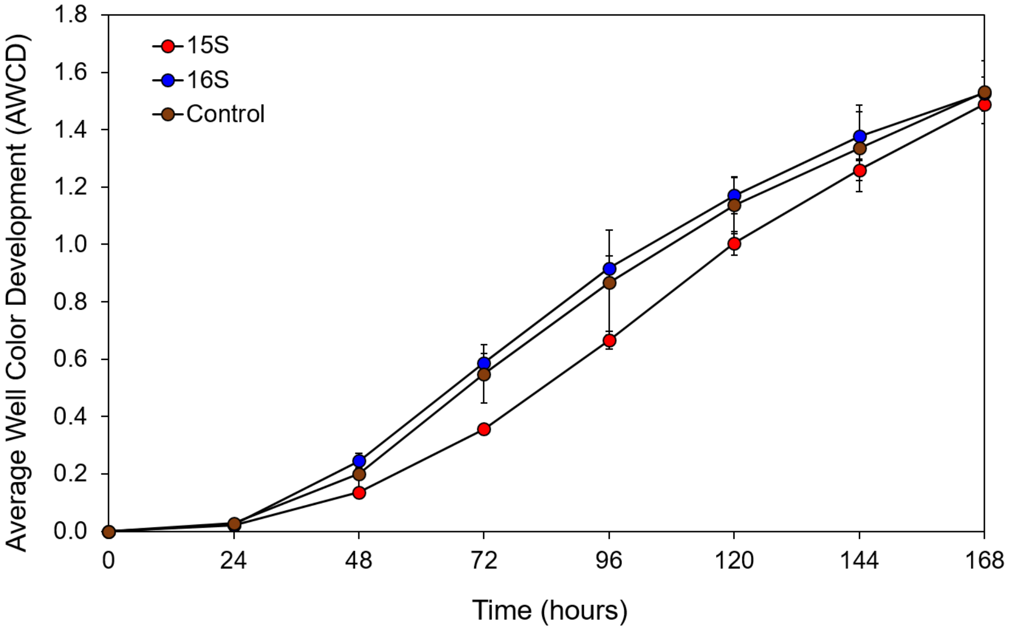
Cluster name	**Cluster size**		**Increased**		**Decreased**		**Accumulation**	
	15S	**16S**	**15S**	**16S**	**15S**	**16S**	**15S**	**16S**
Flavonoids	17	16	4	1	13	15	down ●	down ●
Lignans	12	12	2	1	10	11	down ●	down ●
Isoflavones	11	11	2	1	9	10	down ●	down ●
Phenols	11	11	7	3	4	8	up ●	down ●
Flavones	10	10	0	1	10	9	down ●	down ●
Catechols	9	9	5	2	4	7	up ●	down ●
Coumaric acids	9	9	1	3	8	6	down ●	down ●
Saturated FA	9	9	1	0	8	9	down ●	down ●
Unsaturated FA	8	8	1	0	7	8	down ●	down ●
Anthocyanins	7	7	2	4	5	3	down ●	up ●
Caffeic acids	7	7	2	1	5	6	down ●	down ●
Glucosides	6	6	1	1	5	5	down ●	down ●

Hydroxybenzoates	6	6	1	1	5	5	down	●	down	●
Amino acids	5	5	3	2	2	3	up	●	down	●
Amino acids, basic	5	5	1	0	4	5	down	●	down	●
Cinnamates	5	5	0	0	5	5	down	●	down	●
Flavanones	5	5	1	1	4	4	down	●	down	●
Glucosinolates	5	5	1	0	4	5	down	●	down	●
Glycosides	5	5	0	1	5	4	down	●	down	●
Kaempferols	5	4	2	1	3	3	down	●	down	●
Dicarboxylic acids	4	4	1	0	3	4	down	●	down	●
Naphthalenes	4	3	1	0	3	3	down	●	down	●
Stillbenes	4	4	2	0	2	4	equal	●	down	●
Xanthophylls	4	4	1	1	3	3	down	●	down	●
Acids, carbocyclic	3	3	1	0	2	3	down	●	down	●
Amino acids, aromatic	3	3	0	0	3	3	down	●	down	●
Amino acids, cyclic	3	3	1	1	2	2	down	●	down	●
Amino acids, sulfur	3	-	1	-	2	-	down	●	-	-
Diterpenes, abietane	3	3	0	0	3	3	down	●	down	●
Flavonols	3	3	2	2	1	1	up	●	up	●
Ortho-aminobenzoates	3	3	2	1	1	2	up	●	down	●
Sugar acids	3	3	1	0	2	3	down	●	down	●
Terpenes	3	3	0	1	3	2	down	●	down	●
Umbelliferones	3	3	0	0	3	3	down	●	down	●

4.3.4 Effects of Inoculation on the Metabolic Activity of Rhizosphere-Associated Microbial Community

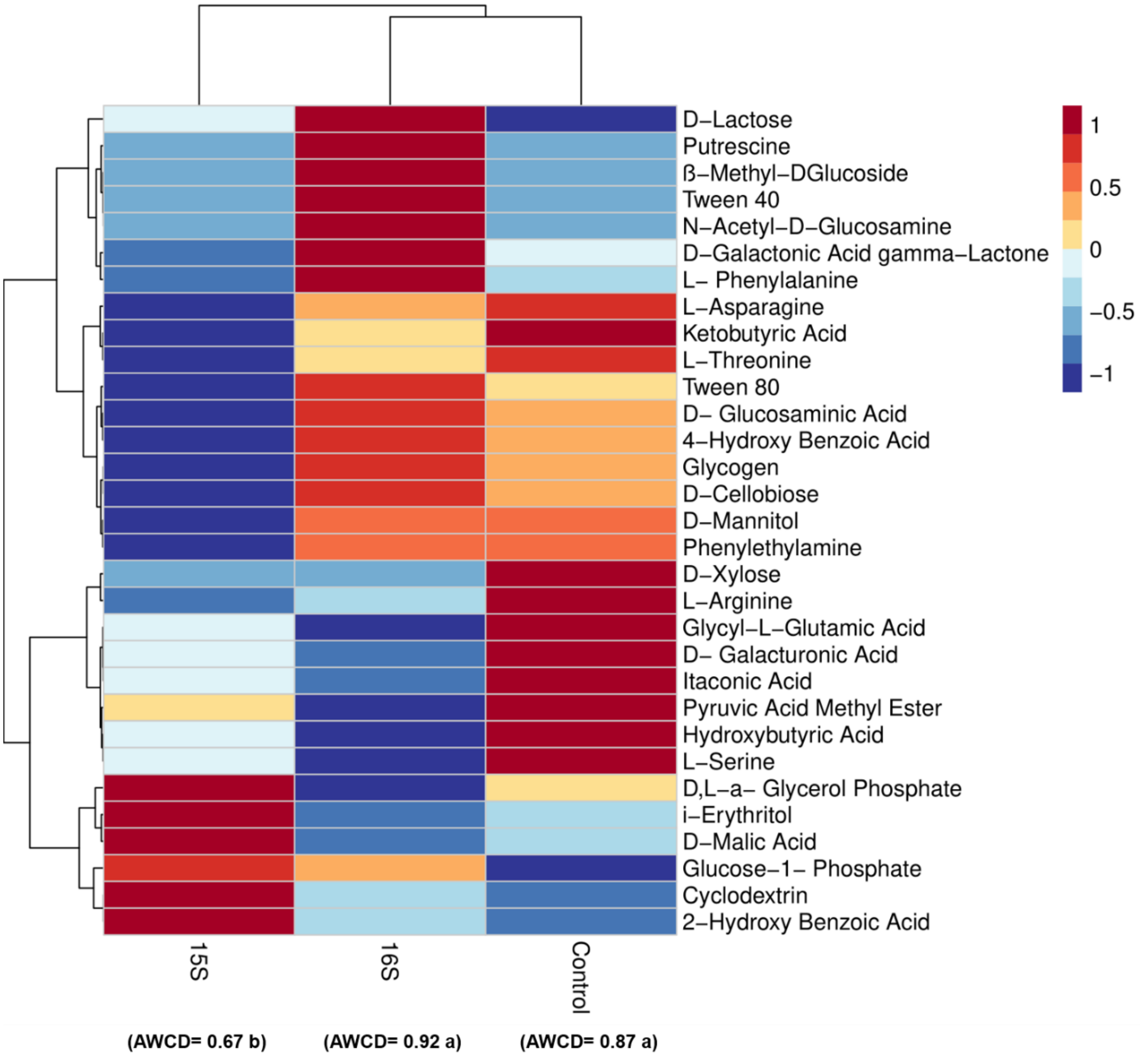
To estimate the general microbial activity in each treatment, the ability to metabolize all 31 carbon substrates in the Biolog EcoPlates by calculating the AWCD was assessed (**Fig. 4.4**).

Fig. 4.4. The AWCD of utilized carbon substrates with incubation time in Biolog EcoPlates in the different treatments (mean ± SE, n = 3). 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, Control: uninoculated plants (the original data available in **Supplementary Table 4.4**).



The results showed that the microbial communities in all treatments were able to metabolize the substrates in Biolog EcoPlates, as the AWCD increased along the evaluated time, augmenting from 0 to around 1.5 after 169 h. Nonetheless, the higher consumption of the substrates was observed after 72-120 h of incubation. Moreover, significant differences in the metabolic profile after incubation at 96 h between treatments were observed; hereof, the profile of substrates metabolized for the microbial communities in the rhizosphere of tomato plants inoculated with *Enterobacter* 15S was different than the obtained for the microbiome in uninoculated plants and inoculated with *Pseudomonas* 16S (**Fig. 4.5 and Supplementary Table 4.2**).

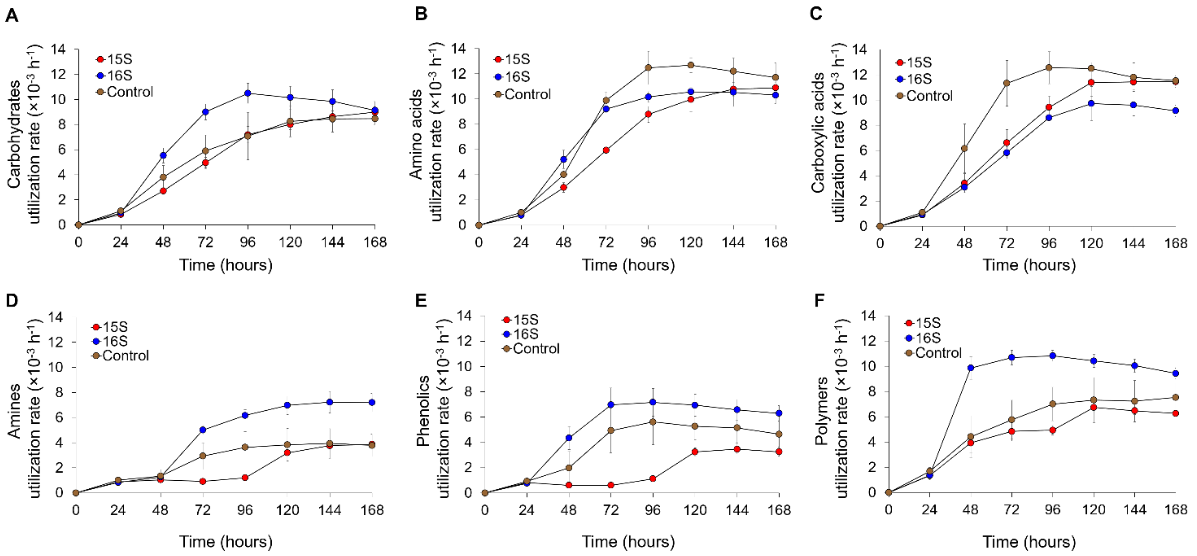
Fig. 4.5. Metabolic profile showing the utilization of the 31 carbon substrates for the microbial communities assessed by the Biolog EcoPlates (at 96 h incubation) in tomato plants inoculated with *Enterobacter* (15S), *Pseudomonas* (16S) or uninoculated (Control) after growing in a rhizobox system for 40 days. The highest consumption is represented by a dark red color and the lowest consumption by a dark blue color. AWCD for each treatment is given in parentheses (lower-case letters following the AWCD values indicate a significant difference across treatments ($p < 0.05$) according to Skott-Knott’s test). The original data available in **Supplementary Table 4.2**.



For instance, substrates like mannitol, phenylethylamine, tween-80, glucosaminic and 4-hydroxybenzoic acid were better metabolized by the microbial communities in treatments 16S and control (inoculated with *Pseudomonas* and uninoculated, respectively) than 15S (inoculated with *Enterobacter*), where malic acid and glucose-1-phosphate were higher consumed. On the other hand, α-cyclodextrin and 2-hydroxybenzoic acid were poorly metabolized in the three treatments. In addition, rhizospheric samples from treatments 16S and control showed the highest activity in carbon substrates metabolization (reflected by the AWCD) when compared to the treatment 15S.

The efficiency in the metabolism of the six groups of carbon sources was different (**Fig. 4.6**). Overall, amino acids, carboxylic acids and carbohydrates were the carbon sources with the highest rate of metabolization by microorganisms, whereas amines, phenolics and polymers showed a less efficient rate of metabolization. Besides, significant differences in the utilization rate of each group of substrates were found among treatments. The metabolization of carbohydrates, amines, polymers and phenolic compounds by microbial communities followed the order 16S > control > 15S, whilst carboxylic acids were better consumed by microorganisms in the control treatment. However, the utilization rates of amino acids exhibited no significant difference among the three treatments ($p < 0.05$).

Fig. 4.6. Effect of bacterial inoculation on carbon source utilization rates by the tomato rhizosphere microbial community based on specific carbon groups. (**A**) carbohydrates; (**B**) amino acids; (**C**) carboxylic acids; (**D**) amines; (**E**) phenolics; and (**F**) polymers. Bars represent the standard error of the mean (n = 3). 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, Control: uninoculated plants (the original data available in **Supplementary Table 4.5**).



4.3.5 Functional Diversity of Microbial Communities Associated with Tomato Rhizosphere Assessed by CLPP

Table 4.2 shows the diversity indices of tomato rhizosphere microbial communities in the Biolog EcoPlates in terms of the inoculation effects. The results clearly indicated that substrate richness and Shannon index were significantly higher ($p < 0.05$) in treatments 16S and control than that in the treatment 15S, whereas the evenness had no significant differences between the three treatments.

Table 4.2. Substrate richness and diversity indices calculated from Biolog EcoPlates at 96 h incubation time for the tomato rhizosphere microbial communities. 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, Control: uninoculated plants. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Treatment	**Richness (R)**	**Shannon index (H)**	**Evenness (E)**
15S	23.67 ± 0.58 b	2.93 ± 0.05 b	0.93 ± 0.01 a
16S	26.00 ± 0.00 a	3.07 ± 0.01 a	0.94 ± 0.00 a
Control	25.67 ± 1.15 a	3.07 ± 0.07 a	0.95 ± 0.01 a

The principal component analysis explained 66.2% of the total variability by the first two components (**Supplementary Fig. 4.2**) and the loading scores resulting from the analysis are shown in **Table 4.3** The more positives or negatives scores strongly contributed to the distribution of the treatments in the PC axis and are related to the ability of substrates metabolization by the rhizosphere microorganisms. Thereby, considering the higher scores (loadings ≥ |0.18|), it could be seen that PC1 was mainly determined by 17 kinds of substrates (5 carboxylic acids, 3 amino acids, 3 carbohydrates, 3 polymers, 2 amines and 1 phenolic). Besides, mainly 15 kinds of substrates had effects on the PC2 (5 carbohydrates, 4 carboxylic acids, 2 polymers, 2 phenolics, 1 amino acid and 1 amine). From the results above, it was clear that the main kinds of substrates which contributed to the functional diversity of the microbiome in the evaluated treatments were carbohydrates, carboxylic acids, amino acids, and polymers.

Table 4.3. Loadings of the first (PC1) and second (PC2) principal components based on the 31 carbon sources in Biolog EcoPlates (factor loadings ≥|0.18| are shown in bold). The original data to perform PCA are available in **Supplementary Table 4.6**.

Substrate group	**Substrates**	**PC1 (44.6%)**	**PC2 (21.6%)**
Amines	Phenylethylamine	**0.19**	**-0.24**
	Putrescine	**0.25**	0.06
Amino acids	L-Arginine	**-0.19**	**-0.21**
	L-Asparagine	-0.13	-0.09
	L- Phenylalanine	0.15	0.09
	L-Serine	**-0.22**	-0.16
	L-Threonine	-0.17	0.04
	Glycyl-L-Glutamic Acid	**-0.18**	-0.17
Carbohydrates	D-Cellobiose	0.09	-0.05

	α-D-Lactose	0.15	**0.28**
	β-Methyl-D-Glucoside	**0.25**	0.12
	D-Xylose	-0.10	**-0.22**
	i-Erythritol	**-0.19**	**0.21**
	D-Mannitol	0.03	**-0.23**
	N-Acetyl-D-Glucosamine	0.13	0.11
	Glucose-1- Phosphate	-0.04	**0.36**
	D,L-α- Glycerol Phosphate	-0.04	0.15
	D-Galactonic Acid γ-Lactone	**0.26**	-0.02
Carboxylic acids	Pyruvic Acid Methyl Ester	**-0.25**	-0.04
	D- Glucosaminic Acid	**0.22**	**-0.19**
	D- Galacturonic Acid	**-0.25**	-0.09
	Hydroxybutyric Acid	-0.17	-0.06
	Itaconic Acid	**-0.21**	**-0.18**
	α-Ketobutyric Acid	-0.04	**-0.24**
	D-Malic Acid	**-0.18**	**0.21**
Phenolic compounds	2-Hydroxy Benzoic Acid	-0.08	**0.20**
	4-Hydroxy Benzoic Acid	**0.20**	**-0.22**
Polymers	Tween 40	**0.26**	0.10
	Tween 80	**0.22**	-0.11
	α- Cyclodextrin	-0.12	**0.27**
	Glycogen	**0.21**	**-0.22**

4.4 DISCUSSION

With the constant increase in the world population, the implementation of agroecological practices aimed at increasing crop productivity and preservation of agro-ecosystems becomes mandatory. Hence, utilization of PGPB as inoculants turned into a potential strategy to reduce chemical inputs and enhance plant nutrient availability. Nonetheless, an integrative understanding of the dynamics and functions in the interrelationships comprising the soil-plant-microorganism system is needed (WEZEL et al., 2014). Here, we studied the changes in tomato root exudation and the variation in the functional diversity of the rhizosphere-associated microbiome in response to inoculation with two different PGPB. *Enterobacter* 15S and *Pseudomona*s 16S strains used in this study significantly enhanced the tomato root and shoot biomass compared to uninoculated plants. Previous studies have also reported the positive effects of PGPB on tomato growth (LI et al., 2016; CORDERO et al., 2018; TAKISHITA; CHARRON; SMITH, 2018). It has been described that through the production of phytohormones and other signals, PGPB may modify root functioning and architecture, consequently enhancing the uptake of minerals and water, leading to the increasing of plant biomass (VACHERON et al., 2013; PII et al., 2015a). Despite the biostimulant effect of PGPB is known, comparatively less information is available on the molecular mechanisms underlying such positive effects. Nonetheless, shedding light onto such mechanisms can help the definition of effective and sustainable agricultural applications.

Every plant releases root exudates which are diverse, dynamic and represents carbon and nitrogen substrates for microbial growth (SASSE; MARTINOIA; NORTHEN, 2018). Additionally, these exuded bioactive molecules may act as signaling, attractants, stimulants, inhibitors or repellents, thus shaping the configuration of rhizosphere-associated microbial communities (BAETZ; MARTINOIA, 2014). In this framework, considering the importance of root exudates for the formation of the rhizospheric environment and its highly diverse chemical nature, the development of untargeted metabolomics approaches has granted the implementation of strategies aiming at analyzing in detail the chemical changes which are associated with plant-microbe interactions and the biochemical mechanisms behind them (MHLONGO et al., 2018). However, the method used to collect root exudates is crucial and can determine the ecological relevance of the exudation profiles obtained. An ideal sampling method should guarantee realistic growth conditions and no root damage. Nonetheless, in spite of the efforts made, a perfect and widespread technique applicable to sampling root exudates does not exist (OBURGER; JONES, 2018). Herein, we used the soil-based filter paper sampling technique and, although restricted exudation rates are obtained using this method (NEUMANN, 2006), an adequate diversity of compounds (419) could be annotated through metabolomics, including a fraction of primary metabolites such as amino acids, carboxylic acids and sugars (around 10%) and a wide set of secondary metabolites, mostly flavonoids and phenolic acids. Diverse studies have suggested that a greater part of root exudates, mainly primary metabolites, are passively lost from the root at the meristematic root apex and used by rhizosphere-dwelling microbes (CANARINI et al., 2019), whereas secondary metabolites are less quickly metabolized by the rhizosphere microbiome, they are often inducible by external factors and have antimicrobial and/or signalling activities, thus influencing root-microbe relations (ROLFE; GRIFFITHS; TON, 2019).

Our untargeted analysis showed a differential tomato root exudation profile as a function of the bacterial inoculation (**Fig 4.2**). Previous studies have also described that the quali/quantitative composition, i.e. the metabolic profile of root exudates, is inoculation-dependent (CHAMAM et al., 2013; DA SILVA LIMA et al., 2014; PII et al., 2015b; KUZMICHEVA et al., 2017). In this regard, the use of PGPB as inoculants is able to trigger deep changes in plant metabolism, signaling, exudation of secondary metabolites and hormone homeostasis, and the molecular mechanisms used by these bacteria are different for a particular PGPR specie/strain (TSUKANOVA et al., 2017). Nebbioso et al. (2016) reported that inoculation with PGPB revealed a relevant change in secondary metabolism of tomato roots with respect to control, thus strengthening the hypothesis that the inoculation of plant roots modifies their metabolism. Although related to different microorganisms, previous work reported that the modulation of root exudation patterns is not only species-specific, but also affected by the way of inoculation (LUCINI et al, 2019). On the other hand, the presence of dedicated pattern recognition receptors (PRRs) used by plant to recognize the associated rhizomicrobiome is known (VENTURI; KEEL, 2016).

Phenolics, flavonoids, amino acids, glycosides, lignans and other classes of metabolites like phytohormones, exhibiting distinctives accumulations in their magnitude and direction, were identified among the most discriminant compounds responsible for the differences between the treatments (**Table 4.1**) and are probably related to the biostimulant effects of both PGPB at molecular level. Therefore, the VIP selection method followed by the fold-change analysis allowed to identify that three hydroxycinnamic acids and a hydroxybenzoic acid presented an increased accumulation induced by both PGPB, whereas a reduction in the accumulation of three flavonoids was observed (**Fig 4.3E**). Flavonoids and phenolics are known to be the major secondary metabolites exuded by plant roots and they are involved in diverse biological processes (MANDAL; CHAKRABORTY; DEY, 2010; CESCO et al., 2012). Considering that phenolics include a wide diversity of chemical compounds, it becomes essential to depict the actual phenolic profile. For instance, phenolics acids participate in plant-microbe symbiosis, allelopathic activities and cross-linking with lignin to act as components of cell wall thus causing blockage to pathogen invasion (DENG; LU, 2017), whereas flavonoids comprise roles in plant nutrient acquisition, chemoattractans for plant–bacteria interactions, nod gene inducers for rhizobia-legume symbiosis, antioxidants and metal chelators and protect plants from different biotic and abiotic stresses (CESCO et al., 2010; WESTON; MATHESIUS, 2013). Different studies have reported the effect of PGPB inoculation on the phenolic profile of root exudates. For instance, inoculation of rice roots with selected PGPB produced a common metabolomic signature characterized by the increased accumulation of several hydroxycinnamic acids (VALETTE et al., 2019) or by the differential accumulation of flavonoids and hydroxycinnamic derivatives (CHAMAM et al., 2013). Conversely, Dardanelli et al. (2010) suggested that the absence of the flavonoids quercetin and naringenin in soybean root exudates inoculated with a PGPB, could be due to a blocking in the flavonoids biosynthesis by the PGPB or the use of these compound as C source. However, it was reported that inoculation with the PGPB *Streptomyces lydicus* caused a decrease in the mRNA levels of flavonoid-synthesis-related genes, which was associated with the positive effect on tomato plant growth (WU et al., 2018). In this sense, inoculation with PGPB may modify the phenolic profiles of plant root exudates in a way (up or down) to improve plant growth, attract more of the beneficial rhizospheric microbiota as well as self-fortification against phytopathogens (RAY et al., 2018). Noteworthy, the fact that a unique accumulation trend could not be observed for phenolics in root exudates is in agreement with previous literature, where a species-specific modulation could be observed (STEINKELLNER et al., 2007).

Diverse other metabolites associated with microbial colonization processes were detected in plants which were inoculated with *Enterobacter* and *Pseudomonas* in this study. For instance, strigolactones such as strigyl acetate, GR24 and strigol-derivates were reduced in all of the inoculated plants. Strigolactones (SLs) are a class of plant hormones exuded by roots regulating several different processes in plants (FOO et al., 2013). It has been reported that different levels of SLs produced by plants contribute to shaping the abundances of certain bacterial groups in the rhizosphere (SCHLEMPER et al., 2017) and plays a role in the effectiveness of PGPB use in promoting the plant growth (SCHLEMPER et al., 2018). Peláez-vico et al. (2016) suggested that the reduction in SLs might be a general phenomenon in plant mutualistic associations to avoid an excessive colonization and speculated that plant SLs might act as rhizospheric signals altering transcriptional activity and microbial traits, that could impact the interaction with the plant host. Interestingly, a strong up-accumulation of gamma-aminobutyric acid (GABA) was observed in both inoculated treatments. It has been reported that this non-protein amino acid, rapidly accumulates in plant tissues in response to biotic and abiotic stress, and regulates plant growth (RAMESH et al., 2015). Using tomato as a model system, Reyes-Darias et al. (2015) showed that significant amounts of GABA are released by the roots and the detection of a GABA receptor in non-pathogenic species of *Pseudomonas* indicated that GABA taxis may be tightly related to colonization and/or infection of plant roots by rhizosphere-dwelling microbes. Consistently, GABA has been also linked to anions efflux from root cells and to alteration in cell membrane potential (RAMESH et al., 2015).

Additionally, based on the chemical similarity analysis, the compounds found in this study were correlated to different metabolic clusters, mostly linked to the shikimate, phenylpropanoid, flavonoid, anthocyanin, and lignin pathways, confirming the effect of both PGPB on the regulation of phenolic compounds. The plasticity of these pathways and their functional properties depend on the demands of the local habitat to redirect the flows of intermediate molecules towards biosynthesis of diverse biologically active compounds, crucial in the plant adaptive strength (MOURADOV; SPANGENBERG, 2014). Considering the belowground habitat, phenolics and flavonoids (normally accumulated in the root tip and root cap cells) are released into the rhizosphere where they are involved in numerous plant-plant, plant-microbe and plant-pathogen interactions (WESTON; MATHESIUS, 2013). Accumulation of flavonols were increased in both inoculated treatments, suggesting a modulation in the auxin transport, possibly due to the PGPB inoculation. Flavonols have been described as the most effective class of compounds able to reduce or inhibit the auxin transport, consequently enhancing a localized auxin accumulation, which in turn, is involved in plant growth and development processes (PEER et al., 2004; PEER; MURPHY, 2007). Besides the generalized induction of phenylpropanoid compounds by both PGPB, the inoculation with *Enterobacter* 15S also elicited the increased accumulation of amino acids, indicating a more dynamic participation of this bacteria in the N availability in the rhizosphere than *Pseudomonas* 16S under the conditions evaluated in this study. Amino acids represent important triggers of plant responses to changing N availability in soil (MOE, 2013) and it has been also reported that diverse compounds produced by microorganisms, can enhance the efflux of amino acids from plant roots (PHILLIPS et al., 2004). Additionally, it was previously reported that when cultivated in a medium supplemented with L-tryptophan, *Enterobacter* 15S was able to produce high amounts of IAA (ZULUAGA et al., 2020), a phytohormone which regulates a wide repertoire of plant developmental processes, including phytostimulation, signaling in plant-microbe interactions, as well as the impact on plant root development and architecture (SPAEPEN; VANDERLEYDEN, 2011).

Monitoring the catabolic activity of the rhizosphere microbial community can be a powerful tool for understanding diverse agroecological contexts. Thus, community-level physiological profiling (CLPP) constitutes a valuable tool to estimate differences in the functional diversity of both soil or rhizosphere-associated microbiome in different treatments

(KOLTON et al., 2017; SI et al., 2018). To date, few studies using the CLPP analysis have described the changes in the metabolic activity of soil microbial communities in response to inoculation with PGPB, and such studies have been performed in monocot plants like wheat, rice and maize (NAIMAN; LATRONICO; SALAMONE, 2009; SALAMONE et al., 2010; SALVO et al., 2018a, 2018b). In this study, the rhizosphere microbial communities of tomato, a dicot plant, were analyzed using Biolog EcoPlates to infer the effect of the inoculation with two different PGPB on the CLPP. The communities present in the rhizosphere of tomato inoculated with *Enterobacter* 15S had a reduced ability or a smaller number of microorganisms with potential to use the substrates in the Biolog EcoPlates, whereas the microbiome in the rhizosphere of plants inoculated with *Pseudomonas* 16S exhibited a higher catabolic activity no significantly different from uninoculated plants. Thereby, a differential metabolic activity profile in the tomato rhizosphere communities was obtained as a function of the bacterial inoculation (**Fig 4.5**), indicating that the use of specific PGPB species as inoculants can alter the root exudation pattern leading to the selection of microbial communities with different physiological levels.

Notably, carbohydrates, carboxylic acids, amino acids, and polymers were the main kinds of substrates that contributed to the differences in the functional diversity of the rhizosphere microbiome between treatments. The majority of primary metabolites in root exudates are believed to be used by microorganisms for their growth (CANARINI et al., 2019). Previous reports have described that organic acid, sugar and amino acid fractions represents the major classes of compounds in tomato root exudates functioning as the main nutrients for the rhizosphere microbial communities (SIMONS et al., 1997; LUGTENBERG; DEKKERS; BLOEMBERG, 2001; KAMILOVA et al., 2006). However, despite the carbon sources tested in Biolog EcoPlates are compounds commonly found in plant root exudates (CAMPBELL; GRAYSTON; HIRST, 1997), the CLPP does not represent the functional potential of the entire microbial community, favoring copiotrophic microorganisms such as Beta- and Gammaproteobacteria, Actinobacteria and Bacteroidetes, characterized for exhibiting high growth rates when carbon sources conditions are abundant (LLADÓ; BALDRIAN, 2015, 2017).

In conclusion, the aforementioned results provide insights about the tomato-PGBP interactions taking place in the rhizosphere which can be explored to develop useful strategies for a sustainable agriculture. Two integrative approaches were used to investigate the ability of

PGPB in modulating tomato root exudation and shaping the functional activity of the rhizosphere microbiome. Inoculation with both PGPB promoted the regulation of a high amount of phenylpropanoid compounds involved in the processes of PGPB colonization and plant growth promotion. Furthermore, the tomato rhizosphere-associated microbiome exhibited a differential catabolism in response to the inoculation. Overall, inoculation with *Enterobacter* 15S promoted tomato growth and strongly modified the functionality of the rhizosphere as revealed by the root exudation profile and the metabolic activity of the associated microbiome. Despite the effect of *Pseudomonas* 16S was less pronounced in the experimental conditions performed in this study, the potential beneficial use of this bacteria can not be underestimated. In this regard, further studies aiming to investigate the ability of these bacteria to enhance biotic or abiotic stress tolerance might be performed.

4.5 ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Supplementay Table 4.1. Discriminant metabolites in root exudates of tomato plants identified by VIP (variable importance in projection) selection method as function of the inoculation with *Enterobacter* 15S or *Pseudomonas* 16S. Metabolites were thereafter subjected to fold-change analysis (each inoculation treatment vs Control).

Class	**Cluster**	**Compound**	**VIP score**	**Inoculation with *Enterobacter* 15S**		**Inoculation with *Pseudomonas* 16S**	
				fold-change	**modulation**	**fold-change**	**modulation**
Amino acids	Amino acids	L-aspartate	1.27 ± 1.17	1.89	up	1.75	up
		L-homoserine	1.27 ± 1.22	-0.66	down	0.05	up
		L-valine	1.16 ± 1.32	3.17	up	2.02	up
		L-citrulline	1.14 ± 0.74	1.08	up	0.64	up
		Gamma-aminobutiric acid	1.10 ± 0.96	18.16	up	17.06	up
	Amino acids,	L-asparagine	1.36 ± 1.29	0.60	up	0.62	up
	Amino acids, cyclic	Aminocyclopropane-1-carboxilic	1.19 ± 1.36	-0.23	down	0.87	up
	Amino acids,	L-homocysteine	1.12 ± 1.68	18.26	up	0.00	down
Flavonoids	Anthocyanins	Quercetin 3-O-	1.37 ± 1.60	-0.57	down	2.00	up
		Quercetin 3-O-galactoside 7-O-	1.33 ± 1.21	1.42	up	2.40	up
		Delphinidin 3,5-O-	1.32 ± 1.50	-0.67	down	1.33	up
		Pigment A	1.32 ± 1.56	1.65	up	3.60	up
		Vitisin A	1.19 ± 0.94	-0.39	down	-0.44	down
		Peonidin 3-O-galactoside	1.18 ± 1.04	0.64	up	-19.58	down
		Cyanidin 3-O-(6"-malonyl-3"-glucosyl-	1.36 ± 1.24	0.84	up	1.76	up
	Flavanones	Eriodictyol	1.49 ± 1.39	-18.12	down	1.44	up
		Pinocembrin	1.25 ± 1.33	-1.10	down	-1.27	down
		6-Prenylnaringenin	1.38 ± 1.16	-22.01	down	-22.01	down
	Flavones	Hispidulin	1.31 ± 0.99	-0.86	down	-0.85	down
		Cirsilineol	1.22 ± 0.78	-0.18	down	-0.24	down
		Rhamnetin	1.19 ± 1.18	0.50	up	-0.17	down
		Neodiosmin	1.16 ± 1.92	0.88	up	1.40	up
		Apigenin	1.10 ± 0.43	0.76	up	-0.81	down
		Jaceosidin	1.24 ± 1.15	23.27	up	0.00	down
		Chrysoeriol 7-O-(6"-malonyl-apiosyl-	1.14 ± 0.74	-19.67	down	-0.26	down
	Flavonols	Quercetin	1.44 ± 0.96	-19.63	down	-19.63	down
		Quercetin 3-O-rutinoside	1.41 ± 0.96	1.98	up	2.96	up
		Kaempferol 3-O-glucosyl-rhamnosyl-galactoside	1.72 ± 0.75	-20.97	down	-20.97	down
		(+)-Catechin 3-O-glucose	1.58 ± 0.57	-3.26	down	0.98	up
		Dihydroquercetin 3-O-rhamnoside	1.19 ± 0.79	1.27	up	1.28	up
	Isoflavones	Biochanin A	1.12 ± 0.75	-1.05	down	-0.48	down
		6"-O-Malonylgenistin	1.11 ± 1.53	4.65	up	-20.19	down
	Kaempferols	Kaempferol 3-O-	1.36 ± 1.12	18.37	up	0.00	down
		Pelargonidin 3-O-	1.14 ± 0.85	0.35	up	0.55	up
Glycosides	Glucosides	Isorhamnetin 3-O-	1.49 ± 0.56	0.34	up	0.14	up
		Arbutin	1.31 ± 0.85	-0.77	down	-0.17	down
	Glucosinolates	4-methylthiobutyl glucosinolate	1.51 ± 1.01	0.29	up	0.61	up

		6-methylsulfinylhexyl glucosinolate	1.33	±	0.68	0.78	up	0.45	up
		7-methylthioheptyl glucosinolate	1.12	±	1.93	1.57	up	-18.72	down
		6-methylthiohexyldesulfo glucosinolate	1.24	±	1.05	-1.04	down	-1.63	down
	Glycosides	avenacin A2	1.13	±	1.15	0.57	up	-0.13	down
		soyasaponin I	1.46	±	1.38	-1.36	down	3.76	up
Lignans	Lignans	Demethoxycurcumin	1.58	±	1.02	-1.16	down	-0.87	down
		Arctigenin	1.42	±	0.69	-1.27	down	-1.40	down
		Pinoresinol	1.10	±	1.35	-1.15	down	-0.38	down
		Episesamin	1.10	±	1.33	-0.08	down	-0.05	down
		Isolariciresinol	1.66	±	0.74	21.03	up	0.00	down
Phenolics	Anisoles	Anethole	1.48	±	0.55	0.75	up	0.53	up
	Caffeic Acids	4-Feruloylquinic acid	1.23	±	1.71	20.87	up	17.03	up
	Catechols	3-Methylcatechol	1.34	±	0.85	2.51	up	1.11	up
		Curcumin	1.33	±	0.88	-0.97	down	-1.55	down
		4-Methylcatechol	1.30	±	0.95	2.05	up	0.66	up
		Guaiacol	1.29	±	0.98	2.05	up	0.63	up
		4-Ethylcatechol	1.11	±	0.83	1.70	up	0.44	up
	Cinnamates	5-Caffeoylquinic acid	1.82	±	0.42	-0.16	down	-0.10	down
		5-O-Galloylquinic acid	1.20	±	0.84	-0.60	down	-0.01	down
	Coumaric Acids	m-Coumaric acid	1.39	±	0.32	0.96	up	-19.16	down
		Sitostanyl ferulate	1.25	±	1.14	-2.95	down	-1.89	down
		Stigmastanol ferulate	1.22	±	0.89	2.81	up	1.30	up
		p-Coumaric acid	1.22	±	0.71	-0.07	down	-0.47	down
		24-Methylcholesterol	1.21	±	1.58	0.22	up	1.77	up
		24-Methylcholestanol	1.18	±	1.37	-0.89	down	1.52	up
	Diterpenes,	Carnosol	1.28	±	1.08	-0.81	down	-0.98	down
	Hydroxybenzoates	2,4-Dihydroxybenzoic	1.10	±	0.87	3.81	up	3.01	up
	Naphthalenes	1,4-Naphtoquinone	1.22	±	0.92	-0.89	down	-0.60	down
	Ochratoxins	Mellein	1.11	±	1.21	2.24	up	1.12	up
	Ortho-aminobenzoates	Avenanthramide 2p	1.65	±	0.53	23.55	up	21.61	up
	Phenols	p-Coumaric acid ethyl	1.12	±	0.76	1.25	up	0.09	up
		p-Coumaroyl malic acid	1.12	±	0.88	18.43	up	18.14	up
		3,5-Dihydroxybenzoic	1.12	±	0.87	18.41	up	17.62	up
	Terpenes	Rosmanol	1.46	±	0.69	0.14	up	-2.38	down
		Epirosmanol	1.22	±	0.92	0.14	up	-1.74	down
		Thymol	1.11	±	1.14	0.42	up	1.62	up
	Umbelliferones	scopoletin	1.38	±	0.57	-0.13	down	-0.55	down
	Unclassified	Feruloyl tartaric acid	1.51	±	0.85	0.49	up	-0.12	down
		24-Methylenecholestanol ferulate	1.48	±	1.27	2.88	up	4.81	up
		p-Coumaroyl tyrosine	1.45	±	1.07	-0.24	down	0.81	up
		5-Tricosenylresorcinol	1.38	±	0.94	0.89	up	0.11	up
		24-Methyllathosterol	1.34	±	1.46	-2.12	down	0.88	up
		Gallic acid 3-O-gallate	1.27	±	0.84	3.19	up	-0.15	down
		Protocatechuic acid 4-O-glucoside	1.17	±	1.34	18.71	up	0.00	down
		Caffeoyl aspartic acid	1.13	±	1.13	-1.40	down	-1.16	down
Others	Pentanoic Acids	pentanoic acid	1.14	±	0.77	-0.19	down	-0.55	down
	Phytohormones	strigol-derivatives (strigol, orobanchol)	1.55	±	0.86	-0.92	down	-1.56	down
		Strigyl acetate	1.22	±	1.20	-17.65	down	-17.65	down
		GR24	1.16	±	1.32	-0.94	down	-0.94	down
		3-indolepropanoic acid	1.14	±	1.22	-1.55	down	0.23	up
	Polycyclic	Pallidol	1.18	±	1.71	0.56	up	0.84	up
	Saturated FA	Arachidic acid	1.23	±	0.88	1.79	up	0.05	up

Supplementary Table 4.2. The original data of **Fig. 4.5**. Metabolic profile assessed by the Biolog EcoPlates at 96 h incubation time for the tomato rhizosphere microbial communities. 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, Control: uninoculated plants. Capital letters compare each substrate across treatments and lower-case letters compare all substrates in each treatment. Equal letters do not differ with Skott-Knott's test ($p \leq 0.05$).

Average Well Color Development (AWCD)				
Carbon group	**Substrate**	**Treatments**		
		15S	**16S**	**Control**
Amines	Phenylethylamine	0.173 Bg	0.691 Ac	0.643 Ac
	Putrescine	0.059 Bg	0.494 Ac	0.054 Bd
Amino acids	L-Arginine	0.733 Be	0.806 Bc	1.121 Ab
	L-Asparagine	1.705 Ab	2.138 Aa	2.276 Aa
	L- Phenylalanine	0.576 Ae	1.265 Ab	0.742 Ac
	L-Serine	0.659 Be	0.306 Bd	1.085 Ab
	L-Threonine	1.048 Ad	1.207 Ab	1.298 Ab
	Glycyl-L-Glutamic Acid	0.340 Bf	0.131 Bd	0.657 Ac
Carbohydrates	D-Cellobiose	0.623 Ae	1.095 Ab	1.014 Ab
	α-D-Lactose	0.583 Be	1.113 Ab	0.124 Bd
	β-Methyl-D-Glucoside	0.118 Bg	0.282 Ad	0.134 Bd
	D-Xylose	0.030 Bg	0.032 Bd	0.233 Ad
	i-Erythritol	0.753 Ae	0.536 Ac	0.566 Ac
	D-Mannitol	1.294 Bc	1.899 Aa	1.873 Aa
	N-Acetyl-D-Glucosamine	1.079 Bd	1.859 Aa	1.174 Bb
	Glucose-1- Phosphate	1.492 Ab	1.168 Bb	0.285 Cd
	D,L-α- Glycerol Phosphate	0.386 Af	0.320 Ad	0.356 Ad
	D-Galactonic Acid γ-Lactone	0.547 Be	1.770 Aa	1.029 Bb
Carboxylic acids	Pyruvic Acid Methyl Ester	1.242 Ac	0.910 Bc	1.546 Bb
	D- Glucosaminic Acid	0.243 Bf	1.191 Ab	0.906 Ab
	D- Galacturonic Acid	1.595 Bb	1.215 Bb	2.230 Aa
	γ- Hydroxybutyric Acid	0.399 Af	0.297 Ab	0.537 Ac
	Itaconic Acid	0.586 Be	0.302 Bd	1.095 Ab
	α-Ketobutyric Acid	0.367 Af	0.537 Ac	0.654 Ac
	D-Malic Acid	1.900 Aa	1.327 Bb	1.474 Bb
Phenolic compounds	2-Hydroxy Benzoic Acid	0.031 Ag	0.029 Ad	0.028 Ad
	4-Hydroxy Benzoic Acid	0.183 Bg	1.347 Ad	1.049 Ab
Polymers	Tween 40	0.893 Bd	2.216 Aa	1.049 Bb
	Tween 80	0.734 Be	1.306 Ab	1.088 Bb
	α- Cyclodextrin	0.091 Ag	0.072 Ad	0.063 Ad
	Glycogen	0.179 Bg	0.564 Ac	0.492 Ab

Supplementary Table 4.3. The original data of **Fig. 4.1**. Effects of bacterial inoculation with *Enterobacter* 15S and *Pseudomonas* 16S on root dry weight (RDW), shoot dry weight (SDW) and plant dry weight (PDW) of tomato plants. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Treatment	**SDW (g)**	**RDW (g)**	**PDW (g)**
15S	2.0 ± 0.2 a	0.4 ± 0.1 a	2.4 ± 0.2 a
16S	2.1 ± 0.2 a	0.4 ± 0.0 a	2.5 ± 0.2 a
Control	1.4 ± 0.5 b	0.3 ± 0.1 b	1.7 ± 0.4 b

Supplementary Table 4.4. The original data of **Fig. 4.4**. The AWCD of utilized carbon substrates with incubation time in Biolog EcoPlates in the different treatments (mean ± SE, n = 3). 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, Control: uninoculated plants. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Average Well Color Development (AWCD)			
Incubation time (h)	**Treatments**		
	15S	**16S**	**Control**
0	0.000 ± 0.00 a	0.000 ± 0.00 a	0.000 ± 0.00 a
24	0.022 ± 0.00 a	0.023 ± 0.00 a	0.028 ± 0.00 a
48	0.136 ± 0.01 b	0.246 ± 0.03 a	0.200 ± 0.05 a
72	0.357 ± 0.01 b	0.587 ± 0.03 a	0.549 ± 0.10 a
96	0.666 ± 0.03 b	0.917 ± 0.04 a	0.867 ± 0.18 a
120	1.004 ± 0.04 b	1.171 ± 0.06 a	1.137 ± 0.10 a
144	1.260 ± 0.05 a	1.377 ± 0.09 a	1.335 ± 0.15 a
168	1.488 ± 0.02 a	1.527 ± 0.06 a	1.531 ± 0.11 a

Supplementary Table 4.5. The original data of **Fig. 4.6**. Effect of bacterial inoculation on carbon source utilization rates by the tomato rhizosphere microbial community based on specific carbon groups (amines, amino acids, carbohydrates, carboxylic acids, phenolics and polymers). 15S: tomato plants inoculated with *Enterobacter* 15S, 16S: tomato plants inoculated with *Pseudomonas* 16S, control: uninoculated plants. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

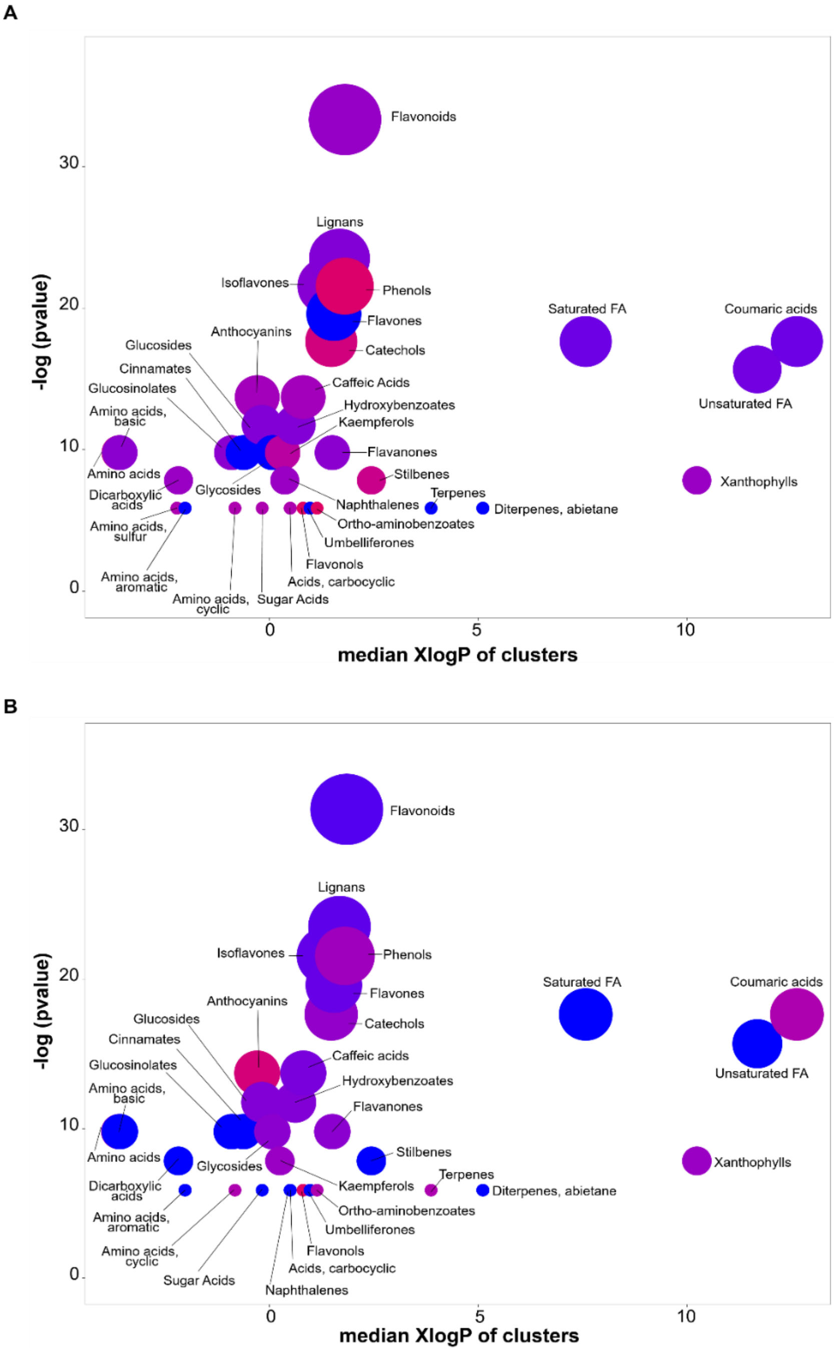
C utilization rate ($\times 10^{-3}$ h^{-1})				
Carbon group	**Incubation time (h)**	**Treatment (mean ± SD)**		
		15S	**16S**	**Control**
Amines	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	0.9 ± 0.2 a	0.8 ± 0.2 a	1.0 ± 0.2 a
	48	1.0 ± 0.0 a	1.3 ± 0.1 a	1.3 ± 0.5 a
	72	0.9 ± 0.1 c	5.0 ± 0.4 a	2.9 ± 1.1 b
	96	1.2 ± 0.0 b	6.2 ± 0.5 a	3.6 ± 1.3 b
	120	3.2 ± 0.1 b	7.0 ± 0.7 a	3.8 ± 1.3 b
	144	3.8 ± 0.0 b	7.2 ± 0.9 a	3.9 ± 1.2 b
	168	3.9 ± 0.2 b	7.2 ± 0.7 a	3.8 ± 0.9 b
Amino acids	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	0.8 ± 0.1 a	0.8 ± 0.2 a	1.0 ± 0.1 a
	48	3.0 ± 0.4 c	5.2 ± 0.7 a	4.0 ± 0.2 b
	72	5.9 ± 0.2 b	9.2 ± 0.2 a	9.9 ± 0.6 a
	96	8.8 ± 0.6 b	10.2 ± 0.4 b	12.5 ± 1.3 a
	120	10.0 ± 1.0 b	10.6 ± 0.6 b	12.7 ± 0.6 a
	144	10.8 ± 0.5 a	10.5 ± 1.1 a	12.2 ± 1.0 a
	168	10.9 ± 0.4 a	10.3 ± 0.7 a	11.7 ± 1.2 a
Carbohydrates	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	0.8 ± 0.1 a	1.0 ± 0.2 a	1.1 ± 0.2 a
	48	2.7 ± 0.3 b	5.5 ± 0.6 a	3.8 ± 0.9 b
	72	4.9 ± 0.5 b	9.0 ± 0.6 a	5.9 ± 1.3 b
	96	7.2 ± 0.7 b	10.5 ± 0.8 a	7.1 ± 1.9 b
	120	8.0 ± 0.4 b	10.2 ± 0.9 a	8.3 ± 1.2 b
	144	8.6 ± 0.5 b	9.8 ± 0.9 a	8.5 ± 1.1 b
	168	9.0 ± 0.4 a	9.1 ± 0.7 a	8.5 ± 0.5 a
Carboxylic acids	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	0.9 ± 0.1 a	0.9 ± 0.3 a	1.1 ± 0.2 a
	48	3.4 ± 0.8 b	3.1 ± 0.4 b	6.2 ± 1.9 a
	72	6.6 ± 1.0 b	5.8 ± 0.5 b	11.3 ± 1.8 a

	96	9.4 ± 0.9 b	8.6 ± 0.1 b	12.6 ± 1.3 a
	120	11.4 ± 1.1 a	9.7 ± 1.3 b	12.5 ± 0.5 a
	144	11.4 ± 0.6 a	9.6 ± 0.9 b	11.8 ± 1.1 a
	168	11.5 ± 0.5 a	9.1 ± 0.4 b	11.5 ± 0.4 a
Phenolics	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	0.8 ± 0.1 a	0.8 ± 0.1 a	0.9 ± 0.1 a
	48	0.6 ± 0.1 b	4.3 ± 0.9 a	2.0 ± 1.3 b
	72	0.6 ± 0.1 b	7.0 ± 1.3 a	4.9 ± 1.8 a
	96	1.1 ± 0.2 b	7.2 ± 1.1 a	5.6 ± 1.8 a
	120	3.2 ± 0.3 c	6.9 ± 0.9 a	5.3 ± 1.1 b
	144	3.5 ± 0.4 b	6.6 ± 0.8 a	5.1 ± 1.2 a
	168	3.3 ± 0.4 b	6.3 ± 0.6 a	4.6 ± 1.0 b
Polymers	0	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
	24	1.3 ± 0.4 a	1.4 ± 0.3 a	1.7 ± 0.3 a
	48	3.9 ± 0.8 b	9.9 ± 0.9 a	4.4 ± 1.7 b
	72	4.8 ± 0.8 b	10.7 ± 0.6 a	5.8 ± 1.5 b
	96	4.9 ± 0.4 b	10.8 ± 0.4 a	7.0 ± 1.3 b
	120	6.7 ± 0.3 b	10.4 ± 0.5 a	7.3 ± 1.8 b
	144	6.5 ± 0.3 b	10.0 ± 0.5 a	7.2 ± 1.7 b
	168	6.3 ± 0.2 b	9.4 ± 0.4 a	7.5 ± 1.1 b

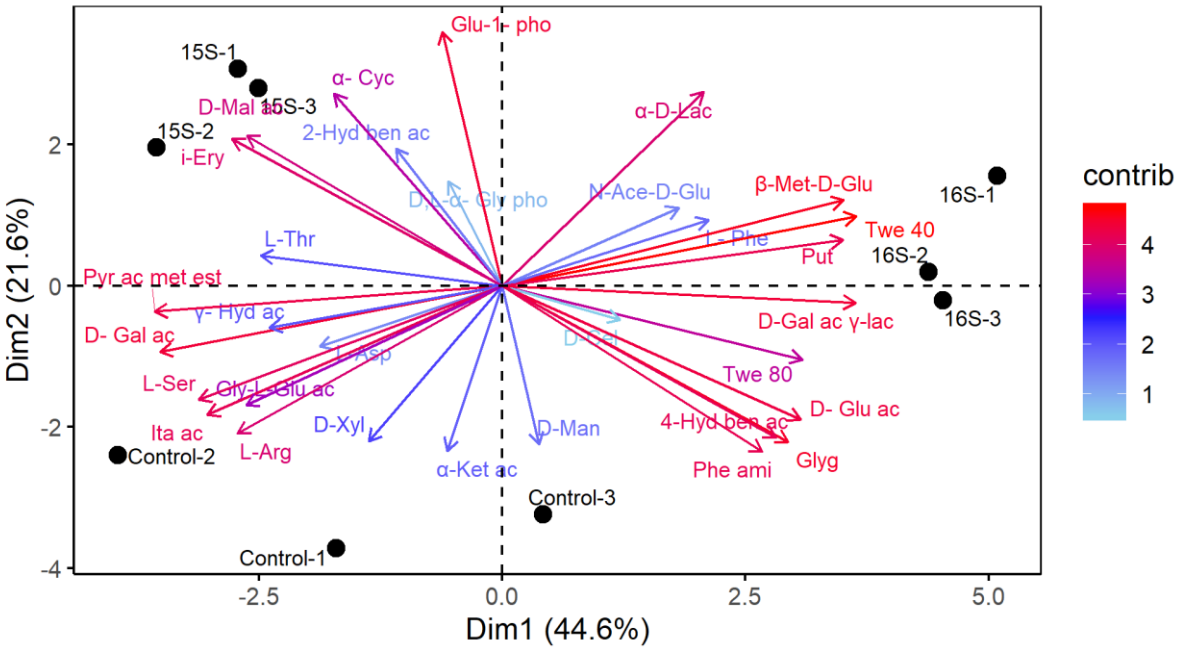
Supplementary Table 4.6. The original data to perform Principal Component Analysis. Biolog EcoPlates data at 96 h were normalized dividing each ODi value by the AWCD and the PCA was perfomed according to (GE et al., 2018).

Rsi values (Rsi = ODi/AWCD; Ge et al. 2018)									
Substrate	**Treatments**								
	15S-1	**15S-2**	**15S-3**	**16S-1**	**16S-2**	**16S-3**	**Control-1**	**Control-2**	**Control-3**
Pyruvic Acid Methyl Ester	1.932	1.949	1.724	1.020	0.944	1.009	2.067	1.744	1.583
Tween 40	1.476	1.192	1.361	2.407	2.419	2.425	0.986	0.745	1.694
Tween 80	0.939	1.199	1.156	1.345	1.488	1.446	1.138	1.130	1.431
α- Cyclodextrin	0.113	0.116	0.177	0.083	0.073	0.078	0.071	0.079	0.069
Glycogen	0.319	0.212	0.278	0.634	0.558	0.651	0.586	0.377	0.678
D-Cellobiose	0.432	0.552	1.767	1.651	0.933	0.965	1.420	0.750	1.245
α-D-Lactose	0.906	0.758	0.963	1.784	0.668	1.137	0.275	0.085	0.076
β-Methyl-D-Glucoside	0.201	0.150	0.183	0.309	0.298	0.313	0.116	0.148	0.189
D-Xylose	0.047	0.043	0.047	0.038	0.038	0.031	0.046	0.469	0.316
i-Erythritol	1.284	1.223	0.901	0.582	0.599	0.575	0.804	0.874	0.385
D-Mannitol	1.931	2.004	1.896	1.676	2.341	2.227	2.165	2.127	2.179
N-Acetyl-D-Glucosamine	1.902	1.305	1.669	1.669	2.309	2.135	1.763	1.482	0.945
D- Glucosaminic Acid	0.346	0.353	0.394	1.231	1.354	1.319	1.334	0.320	1.292
Glucose-1- Phosphate	2.308	2.230	2.189	1.061	1.326	1.448	0.077	0.546	0.387
D,L-α- Glycerol Phosphate	0.623	0.557	0.562	0.407	0.313	0.321	0.362	0.075	0.670
D-Galactonic Acid γ-Lactone	0.954	0.690	0.827	2.100	1.794	1.880	0.830	0.722	1.776
D- Galacturonic Acid	2.255	2.691	2.239	1.294	1.491	1.198	2.263	3.237	2.381
2-Hydroxy Benzoic Acid	0.051	0.026	0.064	0.029	0.040	0.027	0.023	0.036	0.038
4-Hydroxy Benzoic Acid	0.252	0.256	0.314	1.625	1.297	1.470	0.989	1.257	1.357
γ- Hydroxybutyric Acid	0.589	0.297	0.903	0.206	0.310	0.461	0.820	0.868	0.295
Itaconic Acid	0.882	1.115	0.653	0.431	0.430	0.128	1.570	1.456	0.891
α-Ketobutyric Acid	0.549	0.601	0.505	0.422	0.586	0.758	1.115	0.628	0.550
D-Malic Acid	2.982	3.453	2.157	1.482	1.501	1.359	1.429	2.023	1.704
L-Arginine	0.875	1.250	1.161	0.911	0.825	0.897	1.340	1.347	1.220
L-Asparagine	2.554	2.461	2.662	1.657	2.565	2.818	2.713	3.430	2.028
L- Phenylalanine	0.865	0.841	0.887	2.042	1.403	0.663	0.733	1.007	0.853
L-Serine	0.874	1.012	1.072	0.246	0.188	0.566	1.208	1.198	1.320
L-Threonine	1.473	1.695	1.549	1.331	1.431	1.190	1.200	1.834	1.514
Glycyl-L-Glutamic Acid	0.715	0.427	0.404	0.190	0.130	0.105	0.762	0.608	0.854
Phenylethylamine	0.269	0.246	0.263	0.648	0.843	0.778	0.717	0.336	1.028
Putrescine	0.100	0.092	0.073	0.489	0.506	0.621	0.078	0.062	0.050

Supplementary Fig. 4.1. Chemical similarity enrichment analysis (ChemRICH) of the identified metabolites in the inoculated treatment 15S (**A**) and 16S (**B**) compared to the uninoculated treatment (Control). X-axis indicates the XlogP values of metabolite clusters, and the y-axis presents the level of statistical significance as given by the Kolmogorov-Smirnov test. The colored nodes give the proportion of increased (red) or decreased (blue) metabolites in each cluster. Purple/pink nodes have both increased and decreased metabolites (BARUPAL; FIEHN, 2017).



Supplementary Fig. 4.2. Principal component analysis of the carbon substrate utilization by the microbial communities assessed using the Biolog EcoPlates (at 96 h incubation) in tomato plants inoculated with *Enterobacter* (15S), *Pseudomonas* (16S) or uninoculated (Control) after growing in a rhizobox system for 40 days. AWCD data were normalized dividing each ODi value by the AWCD (Rsi = ODi / AWCD, according to GE et al. (2018).



5 RESEARCH PAPER

A distinctive functional activity of the rhizosphere-associated microbiome and root exudation profile is revealed upon inoculation of tomato roots with PGPB and salt stress

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ABSTRACT

Salt-tolerant plant growth-promoting bacteria (PGPB) represent a potential strategy in remediating and enhancing the productivity of agroecosystems suffering from problems of salinity. Here, we investigated the effects of inoculation with two different PGPB (*Pseudomonas* 16S or *Enterobacter* 15S) on growth, metabolic exudation pattern and functional activity of the rhizosphere microbiome of tomato plants subjected to saline stress. An untreated control was also included. Exposition of inoculated plants with *Pseudomonas* 16S to salt stress showed higher tolerance since a significative biomass accumulation was obtained ($p < 0.05$). The microbial metabolic activities assessed with the Biolog EcoPlates method were significantly increased in the rhizosphere of all salt-treated plants. Moreover, functional activity differences between the treatments were also observed with a significantly varied consumption of substrates. Especially, carbohydrates, carboxylic acids, and amino acids played a decisive role in dominating the differences. The UHPLC/QTOF-MS metabolomic approach allowed the identification of distinct metabolic pattern of tomato root exudates, with inflected accumulations imposed by either *Pseudomonas* 16S, *Enterobacter* 15S or saline stress. Crucial compounds from primary and secondary metabolism including low molecular weight osmolytes (e.g., amino acids), as well as anthocyanins, hydroxycinnamics, coumarins and saponins, related to salinity stress tolerance and PGPB colonization, were detected. However, tomato plants inoculated with *Pseudomonas* 16S exhibited a higher accumulation of metabolites and improved maintenance of the homeostatic balance. Thus, the enhanced capability of this bacteria to increasing salt tolerance might have resulted from a coordinated action of mechanisms comprising Fe acquisition, through the accumulation of compounds such as coumarins and siderophores.

Key-words: Salt-tolerant plant growth-promoting bacteria; Biolog EcoPlates; UHPLC/QTOF-MS metabolomic approach; osmolytes; differential metabolites

5.1 INTRODUCTION

From the last decades, the increase in the World population has enhanced the demand for food production through sustainable approaches. Concomitantly, global climate change and inappropriate land and water resources management have increased the gravity of environmental stressors, thus threatening crop production (BARDGETT; GIBSON, 2017). Soil salinity is one of the main stress that negatively affects plant growth, crop productivity and soil microbial activity by inducing ions-mediated toxicity, osmotic stress and reactive oxygen species (ROS) production, which finally lead to the reduction of nutrient availability, inhibition of seed germination and plant growth (SALWAN; SHARMA; SHARMA, 2019). In order to increase crop yields in salt-affected soils and to eradicate free radicals produced during salinity stress, two main strategies have been proposed: engineering crops with enhanced salt stress tolerance traits and the use of halotolerant beneficial microorganisms (HANIN et al., 2016; ILANGUMARAN; SMITH, 2017).

Within soil microbial communities, some microorganisms known as salt-tolerant plant growth-promoting bacteria (PGPB) have been described for their inherent ability to cope with osmotic stress and to promote plant growth by producing osmoprotectants, compatible solutes, specialized transporters, phytohormones, ACC deaminase enzymes, exopolysaccharides and antioxidant enzymes (EGAMBERDIEVA et al., 2019). PGPB-mediated salinity tolerance is a complex phenomenon that involves modulation of abscisic acid (ABA) signalling, expression of genes related to salt overly sensitive (SOS) pathway, ion transporters and antioxidant machinery (BHARTI et al., 2016). Salt-stressed plants inoculated with differents PGPB strains exhibited significantly greater plant height, fresh weight, dry weight, and total chlorophyll content than uninoculated plants (HAHM et al., 2017; CORDERO et al., 2018; KEARL et al., 2019). In addition, an increased content of total soluble sugar, proline, phytohormones as well as an enhanced activity of several antioxidant enzymes (e.g., ascorbate peroxidase, guaiacol peroxidase, and catalase) have also been reported (VIVES-PERIS; GÓMEZ-CADENAS; PÉREZ-CLEMENTE, 2018; KHAN et al., 2019).

Plant-microbe-stress interactions are connected with complexes ecological processes within the rhizosphere. Therefore, understanding the structure and function of this habitat is a crucial step to explore such interactions and other rhizosphere activities as a possible tool to increase or restore plant productivity and improve plant response to stress (AHKAMI et al., 2017). Plants have the capability to recruits, shapes, activates and sustains its rhizomicrobiome by releasing root exudates and signaling molecules (VENTURI; KEEL, 2016). However, it is well known that salinity induces water deficit, which limits both the diffusion of substrates and the uptake of nutrients by the rhizosphere microbiota; consequently, this condition might have an impact on the structure of microbiam communities and on the increase in the competition for substrates between functionally different microbial groups (KARLOWSKY et al., 2018). In addition, considering that salt stress-induced root exudation is controlled by complex processes, the total amount of plant-derived metabolites released and accumulated into the rhizosphere is variable and can be dependent on both salt levels and microbial carbon mineralization (DE LEÓN-LORENZANA et al., 2018). Thus, whilst in some cases plants exudation represents as an adaptive response to the stress and to the rhizomicrobiome, in other cases the release of metabolites may just be due to leaking phenomena as a consequence of stress-induced impairment in the root cells membrane integrity (CESCO et al., 2010).

Since responses to salinity are usually accompanied by notable changes in the plant transcriptome, proteome and metabolome (AHUJA et al., 2010), the use of multidisciplinary approaches for the understanding of physiological and molecular mechanisms triggered in plants will be essential to unravel the dynamics arising from the plant-microbe-stress interactions. Indeed, significant advances in metabolomic techniques have enabled deciphering the complexity of root exudation patterns as affected by the plant-microbes interaction, as well as the underlying biochemical mechanisms (MHLONGO et al., 2018). Furthermore, different studies on metabolic mechanisms of plants under salt stress have indicated that metabolomics provides a wealth of data, which allows a better understanding of the changes in cell metabolism of salt-stressed plants (RUAN et al., 2010; GUPTA; DE, 2017; SKLIROS et al., 2018). On the other hand, considering that salinization induces changes in the pool of carbon sources available in the rhizosphere that directly affect the structure and functionality of the microbial communities inhabiting this habitat, understanding how salinity can affect such communities and their metabolic capacities is necessary. For this purpose, the use of Biolog EcoPlates has been described as an effective, simple and fast method to measure the functional ability of the culturable soil microbial community in disturbed rhizospheric samples (LLADÓ; BALDRIAN, 2017). On the base of these premises, the objectives of the present work are i) to assess the putative alleviating effect of two PGPB (*Pseudomonas* 16S or *Enterobacter* 15S) in salt-stressed tomato plants and ii) to investigate the changes in the metabolomic profile of salt-stressed tomato plants, either non-inoculated or inoculated with PGPBs. Collectively, the data collected will help improving the understanding of some mechanisms involved in plant-microbe-stress interactions.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial Isolates

Pseudomonas 16S and *Enterobacter* 15S were isolated from horticultural soils, in the laboratory of molecular biochemistry of the State University of Londrina (Paraná, Brazil) (ZULUAGA et al., 2020). Each strain was grown in DYGS liquid medium (2 g L^{-1} glucose, 2 g L^{-1} yeast extract, 1.5 g L^{-1} peptone, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgSO_4$, final pH = 6.8) at 28 ± 2 ˚C and shaken at 180 rpm for 24 h. The bacteria concentrations were determined with a Neubauer cell counting chamber and the bacterial inoculants were individually prepared for each strain by diluting to the final concentration of 1×10^6 cells mL^{-1}.

5.2.2 Growth Conditions and Sampling

Tomato seeds (Santa Cruz Kada Gigante, Topseed, Brazil) were surface sterilized by washing with 70% ethanol for 1 min, 2.5% sodium hypochlorite solution for 3 min and finally rinsed six times in sterile distilled water (VAIKUNTAPU et al., 2014). Thereafter, two seeds were sown at a depth of 1-2 cm into rhizoboxes (36×11.5×2.5 cm) filled with 1.5 kg of an unsterilized mixture containing red latosol and sand in a proportion of 1:4 (w/w) as described by Zuluaga et al. (2020). Each rhizobox was irrigated every two days with 100 mL of 1/2 strength Hoagland solution.

The experimental design consisted of four treatments with six replicates: two treatments were plants inoculated with either *Enterobacter* 15S or *Psedomonas* 16S followed by 100 mM NaCl application (denominated 15S+NaCl and 16S+NaCl treatments, respectively) and the other two treatments were uninoculated plants with and without NaCl application (denominated NaCl and Control treatments, respectively). In detail, seven days after germination, the specific group of plants were treated with 1 mL of the bacterial inoculant (1×10^6 cells mL^{-1}, prepared as described above) and the uninoculated plants were treated with the same volume of sterile DYGS medium. After 14 days of growing under the described conditions, salt stress was imposed via the 1/2 strength Hoagland solution by adding NaCl in 25 mM increments every two days until the 100 mM final concentration was reached (after seven days interval) and the salt stress was maintained at this concentration until the end of the assay. Rhizoboxes were distributed randomly and the experiment was conducted for 40 days (counting from the germination day) in a greenhouse with day-night temperature of 25–23 ˚C and natural photoperiod.

Tomato plants were grown for the collection of root exudates, biomass and rhizospheric soil samples as described by Zuluaga et al. (2020). Briefly, root exudates were collected by placing paper discs onto the surface of apical root zones. After 3 h of collection, the discs were transferred into 5 mL eppendorf tubes and stored at -20 °C until further analysis. For the microbiome metabolic activity analysis, samples of rhizospheric soil were collected and, tomato shoots and roots were separated and dried at 65 °C for biomass determinations.

5.2.3 Untargeted Profiling of Root Exudates by UHPLC-QTOF Mass Spectrometry

Root exudates retained into each pool of filter discs (120 filters per treatment) were extracted in 3.5 mL of 80% methanol by shaking for 4 h. Then, the extracts were filtered through

0.22 µm filter and concentrated to a final volume of 1 mL. An UHPLC liquid chromatograph (1290 series, Agilent technologies) coupled to a quadrupole-time-of-flight mass spectrometer (G6550 IFunnel QTOF, Agilent technologies Santa Clara, CA, USA) via a JetStream Electrospray ionization system (UHPLC/QTOF-MS) was used to determine root exudate chemical profiles according to Zuluaga et al. (2020). In brief, the QToF was run in SCAN mode (positive polarity, 100–1200 m/z range) and extended dynamic range mode. Chromatographic separation was achieved in reverse phase mode through a binary gradient from 5% to 90% acetonitrile within 33 minutes, an injection volume of 6 µl and a flow rate of 200 µL min^{-1} using an Agilent Zorbax Eclipse-plus column (75 x 2.1 mm i.d., 1.8 µm). Compounds annotation and post-acquisition processing, following mass and retention time alignment were donne in Profinder B.04 (Agilent technologies) considering a mass accuracy tolerance of <5 ppm. Identification was carried out according to Level 2 (putatively annotated compounds) of the COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi).

5.2.4 Physiological Profiling of Rhizosphere Bacterial Communities

The community-level physiological profiles (CLPP) of the tomato rhizosphere-associated microbiome was assessed by using Biolog EcoPlates (Biolog Inc, Hayward, CA, USA). Every 96-well plate consists of 31 different carbon sources and a water blank, in three replications. Freshly collected samples of rhizospheric soil (1 g) were shaken in 10 ml of 0.85% sterile NaCl physiological solution at 180 rpm for 30 min. The microbial suspensions were diluted 1:1000 and 150 µL aliquots were added into each of the 93 wells in the EcoPlate and incubated at 28°C in the dark. The optical density was measured at 595 nm at regular 24 h intervals over a 168 h incubation period. The optical density values of each well was corrected by the substraction of the absorbance (OD_{590}) of the well containing water.

For the subsequent statistical analyses, the average well color development (AWCD) method was applied as decribed by (PENG et al., 2016) and the readings at 72 h were choosen to evaluate the results, since it was the time point where the higher utilization rates of the carbon sources by the microbial communities in this experiement were observed. In order to assess differences in CLPP, a heatmap analysis and principal component analysis (PCA) were performed and the microbial diversity (R, substrate richness; H, Shannon index; E, evenness; and D, Simpson index) was also calculated according to Zuluaga et al. (2020).

5.2.5 Data Analysis

Biomass and Biolog Ecoplates data were subjected to analysis of variance (one way-ANOVA) and the means values were compared by the Scott-Knott test at $p \leq 0.05$ using the SASM-Agri statistical software (CANTERI et al., 2001). The principal component analysis (PCA) and the heatmap based on normalized data (z-score), were generated by using R software (https://www.r-project.org, using “FactoMineR,” “pheatmap”, “ggplot2” and “RColorBrewer” packages). Chemometric interpretation of the metabolomics dataset was performed using the Mass Profiler Professional B.12.06 (agilent technologies). Post-acquisition processing included compounds abundance transformation (Log2), normalization at the 75th percentile and baselining to the median of the control. An unsupervised hierarchical cluster analysis was formerly done to naïvely describe relatedness/distance of metabolomic signatures across treatments on the basis of a fold-change-based heatmap using the Ward’s clustering method and the Euclidean distance as the linkage rule. Thereafter, Pareto-scaling and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) supervised analysis were carried out using SIMCA 13 software (Umetrics, Malmo, Sweden). Outliers were preliminary investigated using Hotelling’s T2 (95% and 99% confidence limits for suspect and strong outliers, respectively). The model was validated through CV-ANOVA ($p < 0.01$) and permutation testing (N=300) was also applied to exclude overfitting. Goodness-of-fit R^2Y and goodness-of-prediction Q^2Y were calculated for the OPLS-DA model. Variable Importance in Projection (VIP) analysis was used to identify the most discriminant metabolites in the OPLS-DA model. Afterward, VIP markers (VIP score > 1.1) were subjected to fold-change analysis to determine the direction and extent of accumulation across treatments.

5.3 RESULTS

5.3.1 Plant Biomass Accumulation

In this study we assessed the effect of PGPB inoculation on the growth of tomato plants subjected to saline conditions (**Table 5.1**). No significant differences were observed for root dry weight (RDW) between the four treatments. However, inoculation with PGPB produced significant differences in shoot dry weight (SDW): *Pseudomonas* 16S increased the biomass accumulation under saline conditions, while *Enterobacter* 15S caused a reduction when compared to the uninoculated plants (**Table 5.1**). A clear difference in salinity tolerance behavior between the two tested bacteria was observed for the total biomass acumulation (PDW), where clear positive effects of *Pseudomonas* 16S in alleviating the salt-stressed plants could be observed.

Table 5.1. Effects of bacterial inoculation with *Enterobacter* 15S and *Pseudomonas* 16S on root dry weight (RDW), shoot dry weight (SDW) and plant dry weight (PDW) of tomato plants under saline conditions. Significant differences ($p<0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Treatment	**RDW (g)**	**SDW (g)**	**PDW (g)**
15S+NaCl	0.5 ± 0.1 a	1.7 ± 0.2 c	2.2 ± 0.1 b
16S+NaCl	0.6 ± 0.1 a	2.1 ± 0.2 a	2.7 ± 0.2 a
NaCl	0.4 ± 0.0 a	1.8 ± 0.1 b	2.2 ± 0.1 b
Control	0.4 ± 0.1 a	1.9 ± 0.2 b	2.3 ± 0.4 b

5.3.2 Metabolic Functional Activity of Tomato Rhizosphere-Associated Microbiome

The AWCD method was performed to estimate the ability to metabolize the 31 carbon sources in the Biolog EcoPlates by the rhizosphere-associated microbial communities. AWCD values increased across the incubation time (**Supplementary Fig. 5.1**). The higher utilization rates of the carbon sources were observed at 72 h incubation for all the treatments except for the Control, which showed a significantly lower value than the all NaCl treated plants (**Supplementary Table 5.1**). A principal component analysis (PCA) was performed to verify the functional activity differences in the rhizosphere microbial communities between the treatments (**Fig. 5.1**)

Fig 5.1. Principal component analysis of the carbon source utilization by the microbial communities assessed using the Biolog EcoPlates (at 72 h incubation) in tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application (15S+NaCl), *Pseudomonas* 16S and 100 mM NaCl application (16S+NaCl) or uninoculated plants with 100 mM NaCl application (NaCl) and without NaCl application (Control) after growing in a rhizobox system for 40 days. Variables with a high red color intensity contributed more to the principal components. AWCD data were normalized dividing each OD_{590} value by the AWCD according to (GE et al., 2018).

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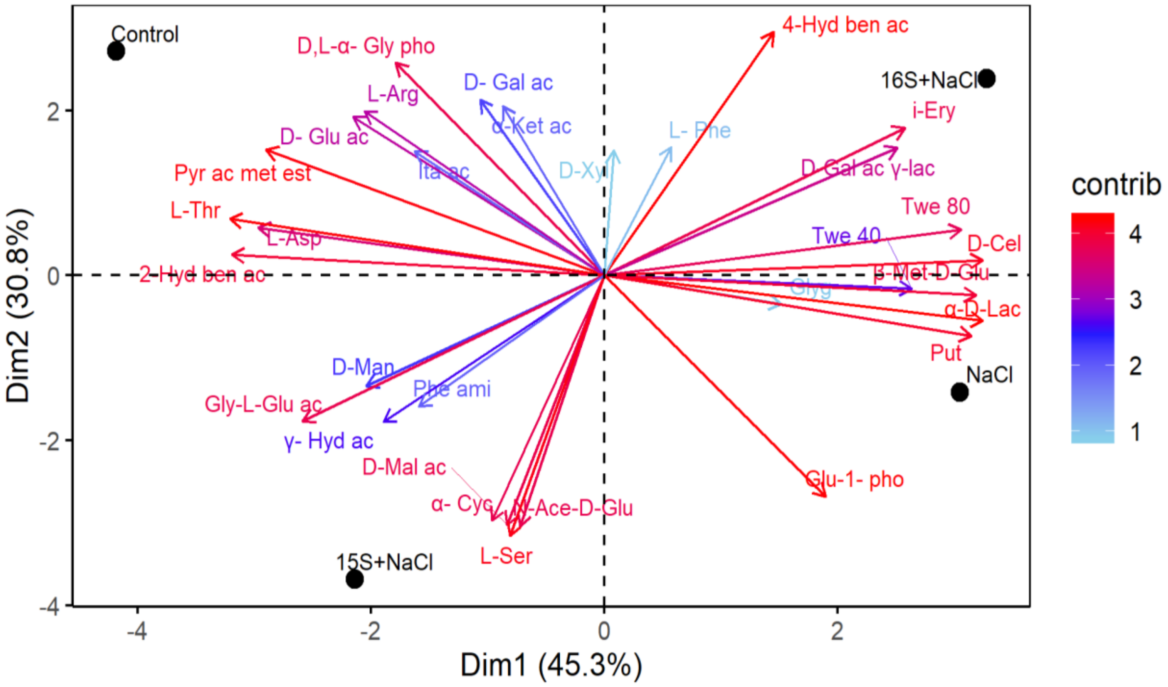
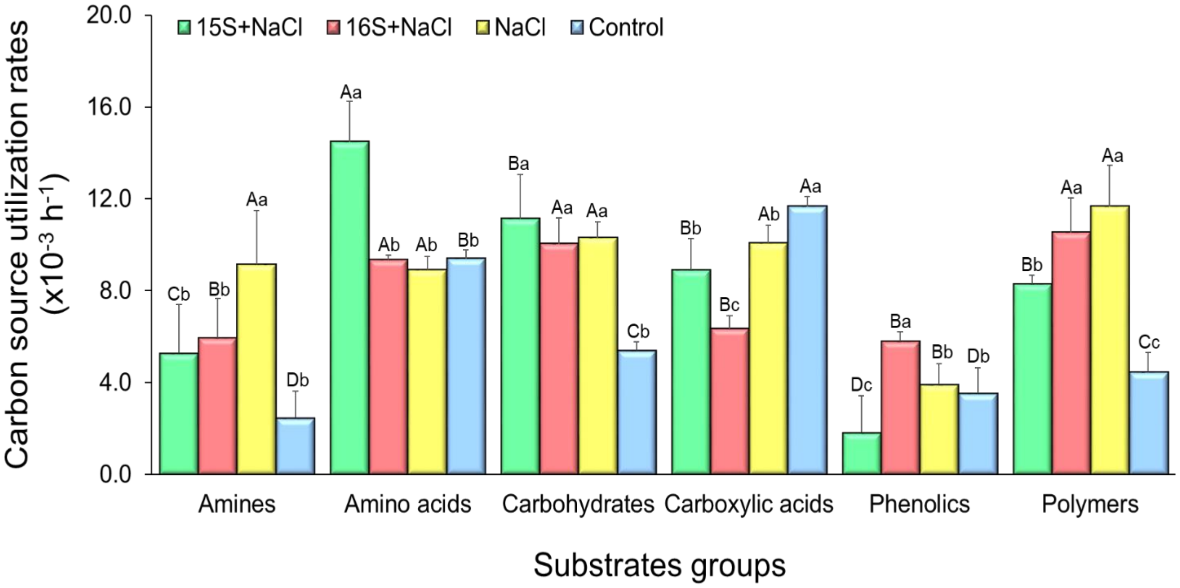


The PCA allowed a suitable separation among the different treatments in their use of specific carbon sources, where the first component (PC1) explained 45.3% of total variability, and the second component (PC2) described 30.8%. Treatments NaCl and 16S+NaCl were clearly separated from the treatments 15S+NaCl and control along the PC1, whilst the treatment 15S+NaCl was evidently separated from the Control along the PC2. **Supplementary Table 5.2** shows that the factor loading scores of the 31 substrates for the principal components and the higher loading scores (positives or negatives loadings ≥ |0.20|) were relevant to the treatments distribution in the PC axes. The main factors with effects on the PC1 were 14 differentind of substrates (five carbohydrates, three amino acids, and two types of carboxylic acids, amines and polymers). Additionally, there were mainly nine substrates which determined the PC2 (three carbohydrates, two carboxylic acids, and one kind of amine, amino acid, polymer and phenolic). These results were also reinforced by the metabolic activity profile of the tomato rhizosphere-dwelling microorganisms accessed by a heatmap using Euclidean distance with Ward grouping algorithm which allowed the clusterization in three groups (**Supplementary**

Fig 5.2). One cluster was formed by the treatments NaCl and 16S+NaCl, and the other two clusters comprised the treatments 15S+NaCl and Control, respectively, demonstrating that bacterial communities in these treatments have a differential ability to preferentially metabolize some of the carbon sources in the Biolog EcoPlates. For instance, the rhizosphere microbial communities from the treatments NaCl and 16S+NaCl presented a significantly higher capability to metabolize cellobiose, lactose, β-methyl-D-glucoside, putrescine and tween 80, whereas microbial communities from the treatment 15S+NaCl showed a significant consume of asparagine, serine, mannitol, N-acetyl-D-glucosamine, malic acid and cyclodextrin (**Supplementary Table 5.3**). Conversely, xylose and 2-hydroxy benzoic acid were the less metabolized substrates in all the treatments.

Additionally, in order to better appraise the carbon sources utilization efficiency, the 31 substrates in the Biolog EcoPlates were divided into six groups of carbon sources (**Supplementary Table 5.3**), as previously described by (CHOI; DOBBS, 1999). At 72 h incubation, the ability to metabolize six different groups of carbon sources was different between treatments (**Fig. 5.2**). Amino acids were significantly better metabolized by the microbial communities present in the rhizosphere from treatment 15S+NaCl, while carboxylic acids were distinctively better consumed by the microorganisms in Control plants. Furthermore, carbohydrates, amines and polymers were better metabolized by the microbiome present in the three treatments under saline conditions. On the other hand, the microbiome dwelling the rhizosphere in the treatments NaCl and 16S+NaCl showed a better metabolic versatility being able to consume most of the substrates groups.

Fig 5.2. Effect of bacterial inoculation and NaCl application on carbon source utilization rates by the tomato rhizosphere microbial community at 72 h incubation based on specific carbon groups in the Biolog EcoPlates (amines, amino acids, carbohydrates, carboxylic acids, phenolics and polymers). Bars represent the standard error of the mean (n = 3). Capital letters compare all group of substrates for each treatment and lower-case letters compare each group of substrates across treatments. Equal letters do not differ with Skott-Knott's test ($p \leq 0.05$). 15S+NaCl: tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application, 16S+NaCl: tomato plants inoculated with *Pseudomonas* 16S and 100 mM NaCl application, NaCl: uninoculated plants with 100 mM NaCl application, Control: uninoculated plants without NaCl application (the original data are available in **Supplementary Table 5.6**).



These findings were supported by the metabolic diversity reflected by the Shannon index (H), Simpson index (D), evenness index (E) and substrate richness (R) values (**Table 5.2**). All the indices had significant differences among the treatments ($p < 0.05$). The NaCl and 16S+NaCl treatments exhibited a high microbial diversity (higher Shannon index), a more evenly distribution of the rhizosphere microbial communities (higher evenness index), as well as a high diversity of the most common species (higher Simpson index) than those obtained for 15S+NaCl and Control treatments. Besides, the most quantity of C sources utilized (higher substrate richness) by the rhizosphre-associated microbiome followed the order NaCl > 15S+NaCl > 16S+NaCl > Control.

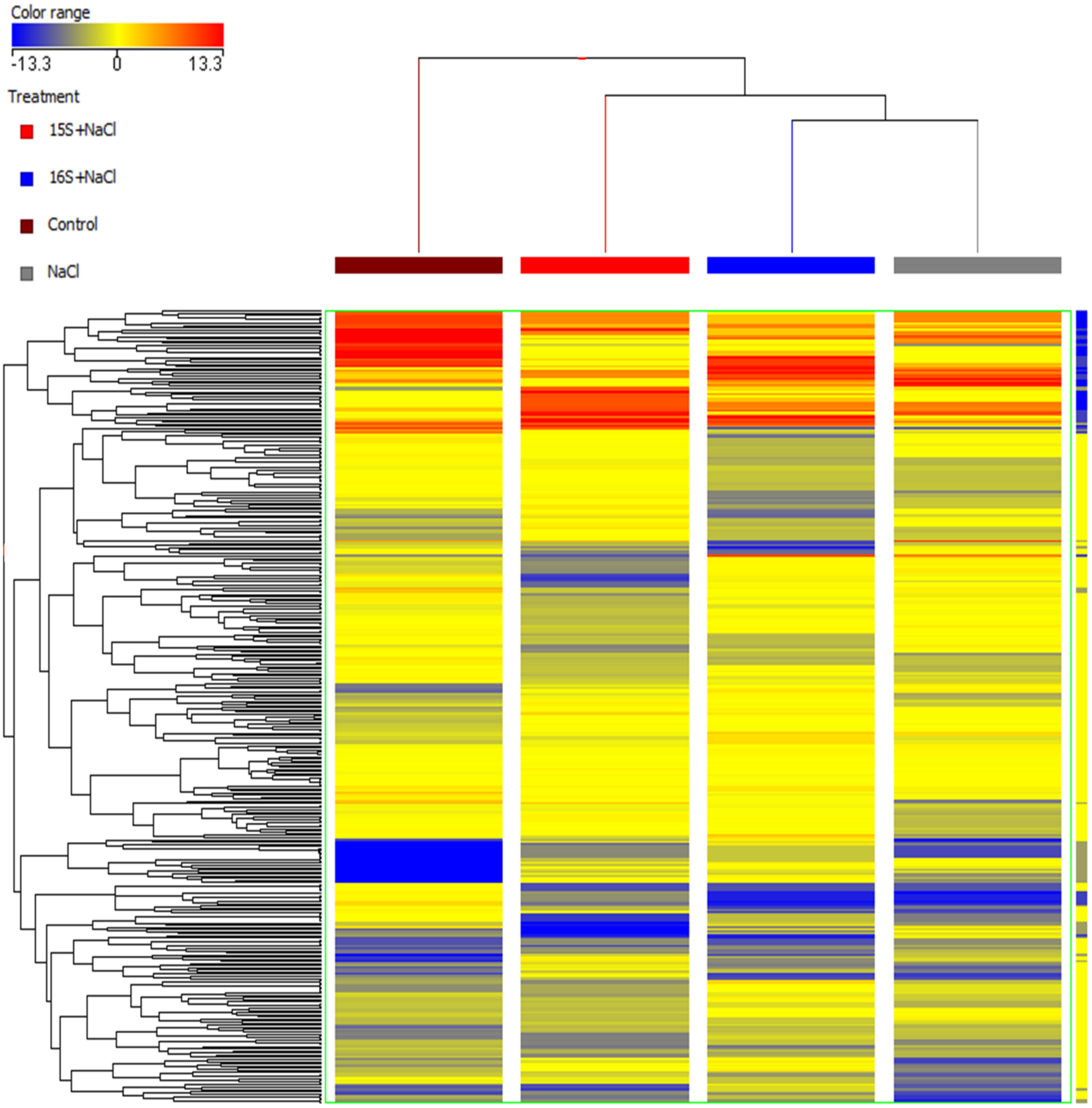
Table 5.2. Diversity indices and substrate richness calculated from Biolog Ecoplates at 72 h incubation time for the rhizosphere microbial communities of tomato plants inoculated with PGPB and subjected to saline stress. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Treatment	Shannon index (H)	Simpson index (D)	Evenness index (E)	Richness (R)
15S+NaCl	3.00 ± 0.05 c	0.94 ± 0.00 b	0.90 ± 0.01 b	27.67 ± 0.58 b
16S+NaCl	3.05 ± 0.03 b	0.95 ± 0.00 a	0.94 ± 0.00 a	25.67 ± 0.58 c
NaCl	3.16 ± 0.01 a	0.95 ± 0.00 a	0.94 ± 0.01 a	28.67 ± 0.58 a
Control	2.89 ± 0.03 d	0.94 ± 0.00 b	0.91 ± 0.01 b	24.00 ± 0.00 d

5.3.3 Untargeted Metabolomics Detected Tomato Rhizosphere-increased or -decreased Metabolites in Response to PGPB and Salt Treatments

UHPLC-QTOF mass spectrometry approach allowed the deconvolution of 411 compounds belonging to different chemical classes such as flavonoids (143), phenolic acids (83), other polyphenols (54, including alkylphenols, phenolic terpenes, hydroxycoumarins), lignans (26), amino acids (24), glycosides (17), carboxylic acids (13), saturated fatty acids (9), carotenoids (8), stilbenes (7) and others compounds (27, such as phytohormones, phytosiderophores, sugars) (**Suppplementary Spreadsheet 5.1**). Thereupon, multivariated statistical analyses were used to better denote differences across the treatments. In particular, an unsupervised hierarchical cluster analysis (HCA) was performed to group the identified compounds according to the relatedness/distances between the metabolic profiles of each treatment. The fold-change based HCA heatmap grouped the treatments in three clusters (**Fig. 5.3**). In the first cluster, tomato root exudates from control plants were separated from the treated plants. The second cluster was composed by the root exudates from treatment 15S+NaCl, whereas root exudates obtained from 16S+NaCl and NaCl treatments constituded together the third cluster. After the unsupervised HC analysis, a supervised OPLS-DA was conduted to better identify the separation between treatments as a function of the PGPB inoculation and the NaCl application.

Fig 5.3. Unsupervised hierarchical cluster analysis of 411 metabolites identified in the root exudates of tomato plants inoculated with *Enterobacter* and 100 mM NaCl application (15S+NaCl), *Pseudomonas* and 100 mM NaCl application (16S+NaCl) or uninoculated plants with 100 mM NaCl application (NaCl), and without NaCl application (Control). Euclidean distance and Ward’s linkage rule were applied and the clustering was generated on the basis of fold change based heatmap.

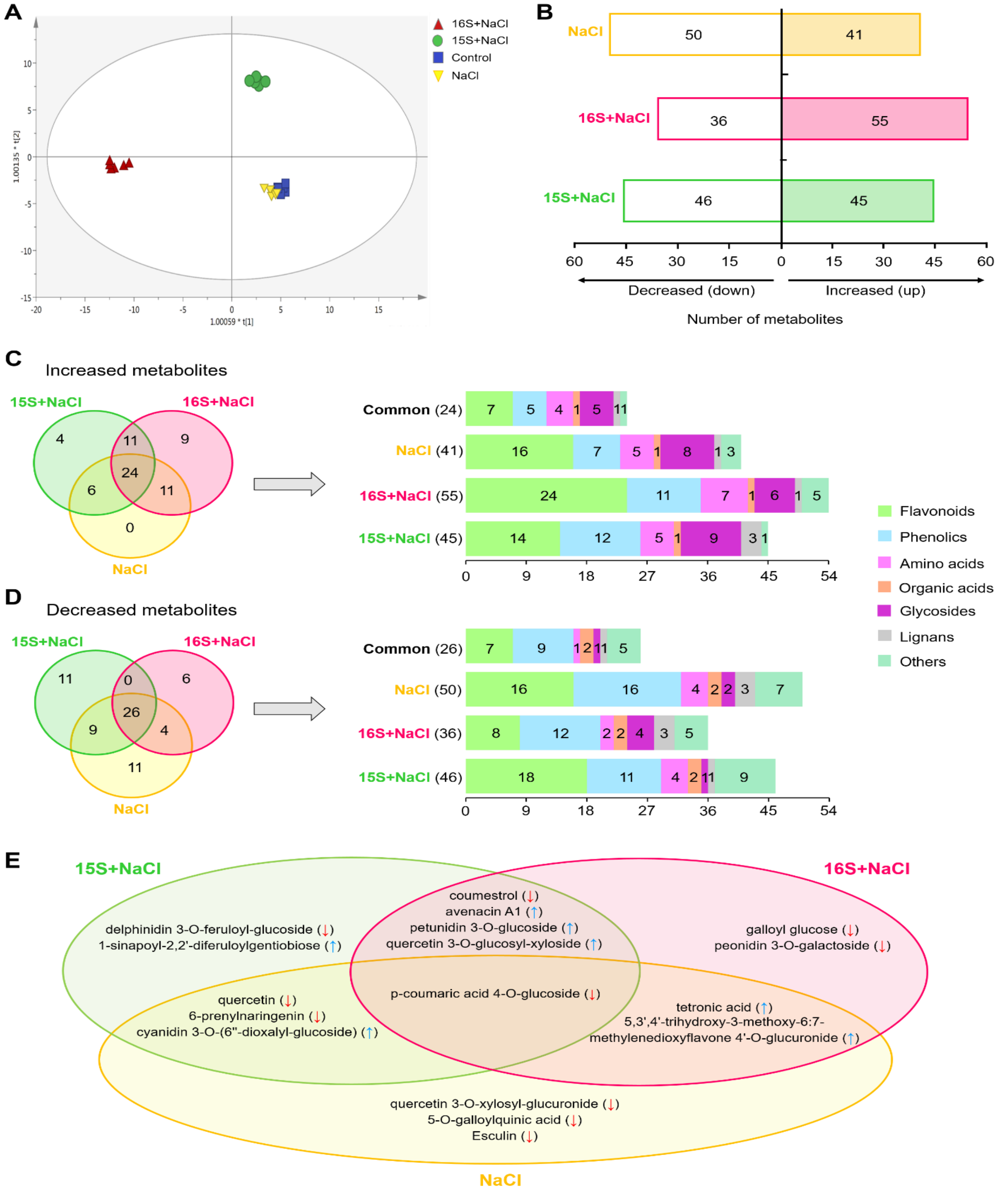


The resulting OPLS-DA score plot (**Fig. 5.4A**) clearly divided the treatments according to the PGPB species used to inoculate the tomato plants. On the other hand, both uninoculated treatments (NaCl and Control) were very similar indicating a marked effect of the bacterial inoculation on the exudation profile suggesting a contrasting behavior of both PGPBs in the amelioration of salt stress. Besides, the model showed a good prediction ability as detected by the discriminant parameters which were characterized by acceptable goodness-of-fit (R^2Y= 0.99) and goodness-of-prediction (Q^2Y = 0.68) values.

Subsequently, in order to list the compounds with the highest ability in maximize the differences observed in the OPLS-DA model built, the VIP selection method was applied. The most discriminant metabolites with a VIP score > 1.1 were selected and then subjected to a fold-change analysis by comparing each saline treatment versus the control. In this regard, 91 metabolites were recorded as discriminants highlighting the presence of 32 flavonoids, 23 phenolics, 9 amino acids, 10 glycosides, 4 lignans, 3 organic acids and 10 from other classes (**Supplementary Table 5.4**), although differentially altered in each treatment (**Fig 5.4B-D**). A high number of VIP metabolites were down-accumulated (50) in root exudates from plants treated solely with NaCl, whereas root exudates from inoculated plants (15S+NaCl and 16S+NaCl) showed an increased number of up-accumulated metabolites (**Fig 5.4B**). Regarding increased metabolites (**Fig 5.4C**), the fold-change analysis allowed to determine 24 VIP metabolites which were common to all treatments, while four compounds were exclusively accumulated in root exudates from treatment 15S+NaCl and nine in the treatment 16S+NaCl. None metabolites were explicitly up-accumulated in the treatment NaCl. Additionally, flavonoids, phenolics and glycosides were the most abundant increased metabolites (**Fig 5.4C**). Conversely, concerning to the decreased metabolites (**Fig 5.4D**), 26 were common to all treatments, whereas 11 were specifically down-accumulated in the treatments 15S+NaCl and NaCl, respectively. Interestingly, in addition to flavonoids and phenolics, other compounds such as phytohormones and phytosiderophores were the most decreased metabolites (**Fig 5.4D**).

Notably, among the 91 VIP compounds identified, 17 were strongly up- or down-accumulated (**Fig 5.4E**), possessing high fold-changes values (FC > 12) when compared to the control (**Supplementary Table 5.4**). Hereof, it was found that two phenolics (5-*O*-galloylquinic acid and esculin) and one flavonoid (quercetin 3-*O*-xylosyl-glucuronide) were specifically down-regulated in root exudates from tomato plants merely treated with NaCl, suggesting a possible relation of these compounds in the saline stress processes in tomato plants. On the other hand, four compounds were common and explicitly altered in the treatments using the beneficial bacteria (15S+NaCl and 16S+NaCl), three of which were up-accumulated [petunidin 3-*O*-glucoside and quercetin 3-*O*-glucosyl-xyloside (flavonoids); avenacin A1 (glycoside)] and one was a down-accumulated flavonoid (coumestrol), entailing that such metabolites were likely elicited by both PGP bacteria as mechanisms for salinity tolerance. In addition, only one phenolic compound (p-coumaric acid 4-*O*-glucoside) was found to be common and down-regulated in all treatments (**Fig 5.4E**). On the whole, it could be noted that flavonoids, phenolics and glycosides were found to be the most modulated metabolites in the rhizosphere of tomato plants under salt stress.

Fig 5.4. Impact of PGPB inoculation and NaCl stress on tomato root exudates metabolome. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) on the root exudates profiles of tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application (15S+NaCl), *Pseudomonas* 16S and 100 mM NaCl application (16S+NaCl) and plants with 100 mM NaCl application (NaCl) (**A**). Number of discriminant metabolites increased or decreased in root exudates of tomato plants identified by VIP method combined with fold-change analysis (B). Venn diagrams exhibit the overlap of the number of discriminant metabolites that increased (**C**) or decreased (**D**) in tomato root exudates, according to the fold-change analysis on the VIP compounds. Bars present the chemical classes of the metabolites exclusively altered in each treatment or common to the three treatments. Venn diagram showing the most increased (blue arrow) and decreased (red arrow) metabolites in the three evaluated treatments, according to the fold-change analysis on the VIP compounds presented in **Supplementary Table 5.4** (**E**).



5.4 DISCUSSION

Inoculation of crop plants with plant growth-promoting bacteria (PGPB) is gaining agronomic importance depicting an alternative strategy to improve plant stress tolerance. Soil salinity is one of the major abiotic stresses limiting plant growth and productivity; thus, the use of beneficial bacteria is growing worldwide as an environment friendly technique for regulating plant adaptation and tolerance to salinity stress (PAUL; LADE, 2014; ISAYENKOV; MAATHUIS, 2019). However, the use of multidisciplinary approaches are essential for the comprehension of the physiological and molecular mechanisms that drive the dynamics arising within the plant-microbe-stress interactions (JONES et al., 2019). In the attempt to better investigate the dynamics of such interactions, herein, we evaluated the metabolic functions of the tomato rhizosphere microbial communities, as well as the changes in the root exudation profile in response to inoculation with two PGPB and saline stress. It was evident that tomato plant biomass exhibited a contrasting tolerance to salinity dependent on the PGPB strain used (*Pseudomonas* 16S or *Enterobacter* 15S) with a more pronounced effect in the shoot dry weight when compared to uninoculated plants under salinity stress. It is well reported that inoculation with PGPB can modulate abiotic stress regulation through mechanisms that induce systemic tolerance resulting in an increase of some growth parameters (e.g. plant weight, leaf area, and root morphology), which help to improve nutrient content, thus strengthening plant health under salt stress (SHAHID et al., 2018; ABBAS et al., 2019; KHAN; BANO, 2019). Previous studies have documented that several species of *Pseudomonas* can express 1-aminocyclopropane-1-carboxylate (ACC) deaminase which confers plant tolerance to abiotic stresses such as salinity by lowering stress-related ethylene levels (WIN et al., 2018; DEL CARMEN OROZCO-MOSQUEDA et al., 2019). Other mechanisms comprising the release of exogenous hormones (e.g., auxins, gibberellins, abscisic acid) and extracellular molecules (e.g. exopolysaccharides, bacteriocins, polyamines, volatile compounds) may elicit stress-responsive pathways which contribute to increasing salt tolerance and stimulate plant growth (ILANGUMARAN; SMITH, 2017).

The native microorganisms inhabiting the rhizosphere of plants play a significant role in stress amelioration in crops grown in saline soils due to their unique properties of maintaining the balance of this ecosystem (PAUL; LADE, 2014). Assessing the metabolic activity of the microbial community in the rhizosphere represents an important indicator to monitor the ecological status of this habitat in a given environmental or experimental condition (MOSCATELLI et al., 2018). Differently from other studies reporting that salinization decreases the substrate utilization efficiency by the soil microbial communities (YUAN et al., 2007; ZHANG et al., 2015; CHEN et al., 2017a), we found that microorganisms dwelling the rhizosphere of tomato plants exposed to NaCl, regardless the inoculated PGPB strain, showed an increased metabolic activity and a differential ability to metabolize some of the carbon sources in the Biolog EcoPlates. Microbial communities and their metabolic functions may respond differently to abiotic stresses. Some studies have indicated a lower diversity in stressed environments (MCTEE et al., 2018; XU et al., 2019), whilst other authors have reported a higher microbial and functional diversity in stressed treatments when compared to the control (LIU et al., 2019). The competitive attributes and acclimation of microbes in response to abiotic stress are crucial to their ability to induce specific tolerance mechanisms that allow the colonization and proliferation in a stressed rhizosphere (SCHIMEL; BALSER; WALLENSTEIN, 2007; WOOD; TANG; FRANKS, 2018). Considering the large resource availability in the rhizosphere, in the form of root exudates, it has been suggested that the rhizosphere may exert a protective effect upon the structure and functions of microbial communities in which the plant would select for competitive and stress-tolerator microbes in the presence of abiotic stresses (WOOD; TANG; FRANKS, 2018).

Carbohydrates, amino acids, amines and polymers were better metabolized by the microbial communities present in the saline treatments and contributed to the differences in the functional diversity of the rhizosphere microbiome between treatments; however, a preferential metabolic pattern was observed as a function of the bacterial inoculation. In a previous study (ZULUAGA et al., 2020), we suggested that the use of specific PGPB species as inoculants can alter the root exudation pattern leading to the selection of microbial communities with different physiological levels. Furthermore, CHEN et al., (2017b) reported that carbohydrates, amino acids, carboxylic acids, and polymers can be regarded as sensitive carbon sources for distinguishing carbon utilization of soil microbial communities under different saline conditions. Nonetheless, Biolog technique does not represent the functional potential of the whole rhizomicrobiome, facilitating the selection of certain taxa within the cultivable microbial community (LLADÓ; BALDRIAN, 2017). Previous studies have described that with increasing salinity, the relative abundance of the Betaproteobacteria decreased, whereas Alpha- and Gammaproteobacteria were found to be the dominant phyla (VALENZUELA-ENCINAS et al., 2009; ZHANG et al., 2015; CHEN et al., 2017a). Thus, based on the above reports, since both PGPB used in this work belong to Gammaproteobacteria phylum and considering that bacterial inoculation build up high populations in the rhizosphere of the bacterial strain carried by the inoculant to efficiently colonize the root system (OLIVEIRA et al., 2017), it could be hypothesized that copiotrophic bacteria (i.e. from Proteobacteria phylum) characterized by faster growth rates (LEFF et al., 2015; LLADÓ; BALDRIAN, 2015; YAO et al., 2017) were the most abundant microorganisms in this study. However, since the Biolog technique can only indicate functionals aspects of the culturable fraction of the community inoculated to the wells (CHEN et al., 2017a), a future molecular analysis to determine the microbial community population structure would be needed in kind to obtain more clear evidence.

Root exudation pattern can be affected quali/quantitatively by different biotic and abiotic factors (VIVES-PERIS et al., 2019). One of the most studied abiotic factors responsible for changes in exudates is salinity, which induces an overall effect on primary metabolism, that in turn provide precursors to secondary metabolism pathways in order to alleviate osmotic and ionic toxicity stress (ACOSTA-MOTOS et al., 2017; ISAH, 2019). The use of untargeted metabolomics allowed the deconvolution of 411 compounds from primary and secondary metabolism such as amino acids, sugars, carboxylic acids, flavonoids and phenolics, with inflections in their accumulations, which were probably released as a natural process related to the putative functions of tomato metabolism but also elicited as a physiological adaptive response to the stress, as well as by the effect of PGPB inoculation in facilitating plant adaptations to counter the saline conditions. In fact, it has been highlighted that both plant and microbes have evolved numerous mechanisms as an adaptative response to reduce the negative effects of soil salinity (ISAH, 2019; SALWAN; SHARMA; SHARMA, 2019). Thereby, primary metabolites are essentially involved as direct markers of photosynthetic dysfunction and as effectors of osmotic readjustment, whilst secondary metabolites show a plant species-specificity and respond to salinity conditions as reactive oxygen species (ROS) quenchers, antioxidants and as regulatory molecules (ARBONA et al., 2013).

The root exudation profile was diverse and distinctively modulated in tomato with a marked effect of the PGPB inoculation. In a previous work conducted with the same PGPB (*Pseudomonas* 16S and *Enterobacter* 15S) without salt imposition, we reported that the metabolic profile of tomato root exudates was inoculation-dependent and PGPB strain-specific (ZULUAGA et al., 2020). Since this same dependence/specificity was observed with the exposition to salt stress, the hypothesis that each bacteria possess a distinctive machinery in developing tomato plant adaptation strategies to salinity stress is reinforced. The detection of discriminant compounds by the VIP selection method followed the fold-change analysis may provide useful insights into the effects of bacterial inoculation and saline stress in tomato plants at the molecular level. A discriminant up-accumulation of the amino acid proline was consistently observed across all NaCl treated plants when compared to the control

(**Supplementary Table 5.4**). In order to protect cells and tissues from oxidative damage, salt-stressed plants tend to accumulate low molecular weight (LMW) osmolytes that decrease the cytoplasmic osmotic potential, enabling water absorption (ABDELGAWAD et al., 2016). Some studies have reported that proline is the most important LMW solute accumulated in response to the imposition of salt stress in many plant species (PENG et al., 2008; QURESHI et al., 2013; VIVES-PERIS; GÓMEZ-CADENAS; PÉREZ-CLEMENTE, 2017; CHUN; PARAMASIVAN; CHANDRASEKARAN, 2018), contributing to the protection of cellular functions by the scavenging of reactive oxygen species (ROS) and the stabilization of proteins and membranes (KAUR; ASTHIR, 2015). Moreover, it has also been suggested its importance in certain regulatory functions, control of plant development and acting as a signal molecule (SZABADOS; SAVOURÉ, 2010). In addition to proline accumulation, other eight amino acids were found to be discriminants in this study. In a given abiotic stress condition, synthesis rates of amino acids can be up- or down-regulated in order to help the rapid recovery of plant metabolism following stress (BATISTA-SILVA et al., 2019). Hildebrandt (2018) described that during abiotic stress some highly abundant amino acids such as glutamine, proline and gamma-aminobutyric acid (GABA) are synthesized to act as osmolytes or as nitrogen storage molecules, while the less abundant branched-chain amino acids, *i.e.* leucine, valine and isoleucine are accumulated and catabolized as a consequence of the increased protein turnover during the stress. Interestingly, levels of alpha-aminoadipic acid (Aad) decreased in all saline stressed plants. It has been reported that Aad is a metabolite derived from lysine catabolism via the saccharopine pathway, accumulated when plants are submitted to biotic and abiotic stresses (ARRUDA; NESHICH, 2012; HARTMANN; ZEIER, 2018; MICHALETTI et al., 2018). However, our findings suggest that lysine catabolism was not significant to alleviate the effects of saline stress in tomato plants.

Besides compounds belonging to the primary metabolism, among the VIP compounds, a wide amount of discriminant secondary metabolites were also detected, mostly flavonoids and phenolics, modulated by both bacterial inoculation and salinity. To avoid the oxidative damage caused by salt stress, plants have evolved different adaptative mechanisms including the synthesis and regulation of metabolites through the shikimate, phenylpropanoid, flavonoid or isoprenoid pathway (e.g., phenolic acids, flavonoids, anthocyanins, lignans and saponins) which play an important role in salt tolerance (HICHEM; MOUNIR; NACEUR, 2009). In a salt-stressed rhizosphere, modulations in the levels of phenolics and flavonoids have an important role in the preservation of osmotic balance and as metal chelators (KHAN et al., 2017). In addition they could also be relevant in plant nutrient acquisition processes and as chemoattractants for plant-microbe interactions (WESTON; MATHESIUS, 2013). Moreover, salt-tolerant beneficial microorganisms are able to act as elicitors of root exudates, stimulating the production of several compounds related to the secondary metabolism. For instance, esculin (a coumarin) was highly decreased in the NaCl treatment, whereas PGPB inoculation elicited an accumulation of this metabolite (**Fig. 5.4E**). It has been reported that solubility and transport of iron (Fe) is notably reduced in saline soils (NOUCK et al., 2016). In dicots plants like tomato, Fe acquisition comprises three principal steps: Fe solubilization and mobilization; reduction of Fe(III) to Fe(II); and subsequent Fe(II) import into the cell (BRUMBAROVA; BAUER; IVANOV, 2015). In reducing the salt stress, Fe plays a fundamental role by activating antioxidative enzymes that act as major scavengers of ROS (TRIPATHI et al., 2018). Recent studies have unravelled a crucial role of secreted coumarins in the acquisition of iron by chelation and/or reduction of Fe(III) (SCHMID et al., 2014; TSAI; SCHMIDT, 2017). Additionally, coumarins may be necessary to stimulate the production of systemic elicitors by rhizobacteria which trigger induced systemic tolerance to stresses (ZAMIOUDIS; HANSON; PIETERSE, 2014). Accordingly, although Fe concentrations in tomato plants were not evaluated here, our results suggest that inoculation with PGPB can improve Fe acquisition acting as elicitors of compounds related to salt tolerance. Indeed, previous studies have highlighted that PGPB might affect positively the Fe nutrition in dicot plants, both by modulating Fe availability in the rhizosphere and by altering the expression of the plant molecular machinery devoted to the micronutrient acquisition at root level (PII et al., 2015; PII et al., 2016; MARASTONI et al., 2019). Furthermore, to reinforce this hypothesis, it could be observed that avenic acid, classified as phytosiderophore, was found to be up-accumulated only in the treatment inoculated with *Pseudomonas* 16S. This strain was previously described to be a promising bacteria for siderophore production (ZULUAGA et al., 2020). Many significant roles have been described for siderophores, including Fe chelation, virulence in pathogens, oxidative stress tolerance and regulation of other biomolecules (KHAN; SINGH; SRIVASTAVA, 2018). In this regard, further investigation is required to clarify if one of the possible mechanisms governing the response of *Pseudomonas* 16S to salinity in dicot plants is correlated to Fe acquisition.

Similarly, a strong up-accumulation of petunidin 3-*O*-glucoside (an anthocyanin) and avenacin A1 (a saponin) were also observed only in both PGPB inoculated treatments. Eryılmaz (2006) reported that increased levels of anthocyanins were related to the amelioration of the saline stress in tomato seedlings, whereas Mishra et al. (2011) described that inoculation with *Pseudomonas* strains improved the level of anthocyanin and total phenolics which resulted in a higher tolerance of wheat plants to cold stress. On the other hand, saponins are known to be synthetized due to a general defense mechanism in plants against stresses, and their concentration is higher in roots (PUENTE-GARZA et al., 2017). Moreover, in a recent study Xie et al. (2019) reported that plant inoculation with beneficial bacteria promoted an increased content of saponins and flavonoids improving plant tolerance to drought. Conversely, coumestrol (an isoflavone) was strongly down-accumulated in the inoculated treatments. It has been reported that coumestrol is often elicited in legumes produced as one of the plant's responses to exogenous invaders but also during the recognition process in the legume-rhizobium symbiosis (DAKORA, 1993; LEE et al., 2012). Additionally, during drought stress, Tripathi et al. (2016) reported an increase of coumestrol in soybean roots which promoted the growth of mycorrhizal fungi, improved water use efficiency, and thereby enhanced plant tolerance to drought stress. However, our findings led us to speculate that the synthesis of coumestrol in non-leguminous plants is regulated by a different signaling pathway; thus, in this study, it could be downregulated to facilitate the interaction between the PGPB and the saline-stress tomato plants.

Finally, it is important to highlight that although metabolomics brings the opportunity to elucidate some possible mechanisms taking place in the plant-microbe-stress interactions, some other factors such as the intensity and duration of the stress, as well as the method used to collect root exudates, are also crucial and might determine the ecological relevance of the metabolic profile obtained.

5.5 CONCLUSIONS

Our results reinforce the findings of previous studies indicating that inoculation with PGPB is a feasible technology for increasing crop productivity, enhancing stress tolerance and helping to foment environmental sustainability. Both approaches used in our study allowed determining a distinctive functional activity of the rhizosphere-associated microbiome and root exudation signature upon inoculation of tomato roots with PGPB and salt stress imposition. Specifically, by performing Biolog EcoPlates method, we identified that microorganisms dwelling the rhizosphere of tomato in the saline treatments exhibited a higher metabolic activity, where carbohydrates, carboxylic acids, and amino acids were the main kind of substrates which contributed to their functional diversity. On the other hand, the UHPLC-QTOF-MS metabolomic approach allowed to identify a distinctive metabolic profile of root exudates, dependent of the PGPB strain used (*Pseudomonas* 16S or *Enterobacter* 15S) under saline conditions in tomato, indicating that each bacteria possess distinct machinery to confer tomato plant adaptation strategies to stress. Crucial compounds from primary and secondary metabolism related to salinity stress tolerance and PGPB colonization were herein detected. Proline, was significantly accumulated in the NaCl treated plants. Notwithstanding, the most represented among differential compounds were flavonoids and phenolics (including anthocyanins, isoflavones, flavonols, hydroxycinnamics and coumarins). Interestingly, some findings led us to speculate that tomato plants inoculated with *Pseudomonas* 16S exhibited a higher tolerance to salinity correlated to mechanisms comprising Fe acquisition. However, further physiological and molecular studies are recommended to understand the mechanisms whereby such bacteria improved plant growth and seemingly facilitated the maintenance of the homeostatic balance in salt-stressed tomato.

5.6 ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Supplementary Table 5.1. The AWCD of utilized carbon substrates with incubation time in Biolog EcoPlates in the different treatments (mean ± SE, n = 3). 15S+NaCl: tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application, 16S+NaCl: tomato plants inoculated with *Pseudomonas* 16S and 100 mM NaCl application, NaCl: uninoculated plants with 100 mM NaCl application, Control: uninoculated plants without NaCl application. Significant differences ($p < 0.05$) according to Skott-Knott's test are indicated by lower case letters across treatments.

Average Well Color Development (AWCD)				
Incubation time (h)	**Treatments (mean ± SE, n = 3)**			
	15S+NaCl	**16S+NaCl**	**NaCl**	**Control**
0	0.000 ± 0.00 a	0.000 ± 0.00 a	0.000 ± 0.00 a	0.000 ± 0.00 a
24	0.054 ± 0.01 a	0.046 ± 0.03 a	0.064 ± 0.01 a	0.028 ± 0.00 a
48	0.336 ± 0.03 a	0.291 ± 0.08 a	0.329 ± 0.02 a	0.200 ± 0.05 b
72	0.715 ± 0.01 a	0.620 ± 0.01 a	0.696 ± 0.01 a	0.515 ± 0.02 b
96	1.012 ± 0.02 a	0.953 ± 0.06 a	0.973 ± 0.03 a	0.867 ± 0.18 b
120	1.250 ± 0.02 a	1.162 ± 0.04 a	1.162 ± 0.00 a	1.137 ± 0.10 a
144	1.442 ± 0.03 a	1.350 ± 0.05 a	1.325 ± 0.02 a	1.335 ± 0.15 a
168	1.594 ± 0.03 a	1.485 ± 0.07 a	1.459 ± 0.08 a	1.531 ± 0.11 a

Supplementary Table 5.2. Loadings of the first (PC1) and second (PC2) principal components based on the 31 carbon sources in Biolog EcoPlates (factor loadings ≥|0.20| are shown in bold). The original data to perform PCA are available in **Supplementary Table 5.5**.

Substrates	**Abbreviations**	**PC1 (45.3%)**	**PC2 (30.8%)**
Phenylethylamine	Phe ami	**0.2545**	-0.0722
Putrescine	Put	**0.2065**	**-0.2231**
L-arginine	L-arg	**-0.2401**	0.0564
L-asparagine	L-asp	0.0463	0.1523
L-phenylalanine	L-phe	-0.0657	**-0.3099**
L-serine	L-ser	**-0.2598**	0.0671
L-threonine	L-thr	**-0.2094**	-0.1732
Glycyl-L-glutamic acid	Gly-L-glu ac	-0.1288	-0.1556
D-cellobiose	D-cel	**0.2623**	0.0178
α-D-lactose	α-D-lac	**0.2624**	-0.0541
β-methyl-D-glucoside	β-Met-D-Glu	**0.2576**	-0.0237
D-xylose	D-xyl	0.0064	0.1487
i-erythritol	i-ery	**0.2083**	0.1757
D-mannitol	D-man	-0.0583	-0.1315
N-acetyl-D-glucosamine	N-ace-D-glu	-0.1742	**-0.2985**
Glucose-1-phosphate	Glu-1-pho	-0.1448	**-0.2631**
D,L-α-glycerol phosphate	D,L-α-gly pho	**0.2032**	0.1515
D-Galactonic acid γ-lactone	D-gal ac γ-lac	-0.0864	**0.2092**
Pyruvic acid methyl ester	Pyr ac met est	**-0.2348**	0.1497
D-glucosaminic acid	D-glu ac	0.1534	0.1883
D-galacturonic acid	D-gal ac	**-0.2584**	0.0245
γ-Hydroxybutyric acid	γ-hyd ac	-0.1316	0.1475
Itaconic acid	Ita ac	-0.0703	**0.2012**
α-ketobutyric acid	α-ket ac	-0.0676	**-0.2970**
D-malic acid	D-mal ac	-0.1666	0.1955
2-hydroxy benzoic acid	2-hyd ben ac	0.1173	**0.2893**
4-hydroxy benzoic acid	4-hyd ben ac	-0.1533	-0.1740
Tween 40	Twe 40	**0.2131**	-0.0160
Tween 80	Twe 80	**0.2475**	0.0541
α-cyclodextrin	α-cyc	-0.0780	**-0.2917**
Glycogen	Glyg	0.1225	-0.0341

Supplementary Table 5.3. Metabolic profile assessed by the Biolog EcoPlates at 72 h incubation time for the tomato rhizosphere microbial communities. 15S+NaCl: tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application, 16S+NaCl: tomato plants inoculated with *Pseudomonas* 16S and 100 mM NaCl application, NaCl: uninoculated plants with 100 mM NaCl application, Control: uninoculated plants without NaCl application. Capital letters compare each substrate across treatments and lower-case letters compare all the substrates in each treatment. Equal letters do not differ with Skott-Knott's test ($p \leq 0.05$).

Average Well Color Development (AWCD)					
Carbon group	**Substrate**	**Treatments**			
		15S +NaCl	**16S+NaCl**	**NaCl**	**Control**
Amines	Phenylethylamine	0.415 Ae	0.269 Ad	0.424 Ae	0.305 Af
	Putrescine	0.347 Be	0.588 Ac	0.893 Ad	0.051 Bh
Amino acids	L-arginine	0.855Ad	0.825 Ab	0.514 Ae	0.791 Ad
	L-asparagine	2.263 Aa	1.692 Ba	1.551 Ba	1.728 Ba
	L-phenylalanine	0.536 Ad	0.686 Ac	0.333 Bf	0.367 Bf
	L-serine	1.646 Ab	0.373 Cc	0.893 Bd	0.380 Cf
	L-threonine	0.657 Ad	0.388 Bc	0.353 Bf	0.604 Ae
	Glycyl-L-glutamic acid	0.295 Ae	0.076 Ad	0.207 Af	0.192 Ag
Carbohydrates	D-cellobiose	0.604 Bd	0.839 Ab	1.002 Ad	0.397 Cf
	α-D-lactose	0.393 Be	0.656 Ac	0.735 Ae	0.054 Ch
	β-methyl-D-glucoside	0.425 Be	0.763 Ab	0.688 Ae	0.061 Ch
	D-xylose	0.047 Ae	0.047 Ad	0.039 Ag	0.034 Ah
	i-erythritol	0.320 Ae	0.406 Ac	0.369 Af	0.247 Ag
	D-mannitol	2.624 Aa	1.569 Ba	1.307 Bb	1.394 Bc
	N-acetyl-D-glucosamine	1.781 Ab	1.023 Bb	1.285 Bb	0.826 Bd
	Glucose-1-phosphate	0.966 Ad	0.523 Bc	1.107 Ac	0.068 Ch
	D,L-α- glycerol phosphate	0.386 Ae	0.447 Ac	0.261 Bf	0.454 Ae
	D-galactonic acid γ-lactone	0.472 Be	0.952 Ab	0.619 Be	0.345 Bf
Carboxylic acids	Pyruvic acid methyl ester	1.255 Ac	0.879 Bb	0.473 Be	1.574 Ab
	D-glucosaminic acid	0.571 Ad	0.508 Ac	0.632 Ae	0.686 Ad
	D-galacturonic acid	0.430 Ce	0.861 Bb	1.559 Aa	1.795 Aa
	γ-hydroxybutyric acid	0.370 Ae	0.264 Ad	0.274 Af	0.225 Ag
	Itaconic acid	0.166 Be	0.147 Bd	0.560 Ae	0.709 Ad
	α-ketobutyric acid	0.064 Ae	0.116 Ad	0.213 Af	0.220 Ag
	D-malic acid	1.631 Ab	0.435 Dc	1.367 Bb	0.666 Cd
Phenolic compounds	2-hydroxy benzoic acid	0.036 Ae	0.028 Ad	0.029 Ag	0.027 Ah
	4-hydroxy benzoic acid	0.226 Ce	0.810 Ab	0.537 Be	0.484 Be
Polymers	Tween 40	1.247 Bc	1.561 Aa	1.187 Bc	0.482 Ce
	Tween 80	0.604 Bd	1.165 Ab	1.511 Aa	0.515 Be
	α- cyclodextrin	0.288 Ae	0.065 Bd	0.118 Bg	0.057 Bh
	Glycogen	0.250 Be	0.246 Bd	0.545 Ae	0.238 Bg

Supplementary Table 5.4. Discriminant metabolites in root exudates of tomato plants identified by VIP (variable importance in projection) selection method as function of the inoculation with *Enterobacter* 15S or *Pseudomonas* 16S and 100 mM NaCl application. Metabolites were thereafter subjected to fold-change analysis (each treatment vs Control). FC: fold-change; Accum: Accumulation.

Class	**Cluster**	**Compound**	**VIP score**	**15S+NaCl**		**16S+NaCl**		**NaCl**	
				FC	**Accum**	**FC**	**Accum**	**FC**	**Accum**
Flavonoids	Anthocyanins	cinnamtannin A2	1.47 ± 0.77	0.15	up	0.75	up	0.24	up
		petunidin 3-O-glucoside	1.25 ± 0.96	17.83	up	17.50	up	0.00	down
		pelargonidin 3-O-	1.21 ± 0.30	-0.10	down	0.01	up	-0.35	down
		cyanidin 3-O-(6"-malonyl-3"-glucosyl-glucoside)	1.50 ± 0.43	2.01	up	0.95	up	1.25	up
		delphinidin 3-O-feruloyl-glucoside	1.49 ± 1.25	-20.43	down	0.25	up	1.38	up
		vitisin A	1.12 ± 0.48	-0.57	down	0.49	up	0.80	up
		peonidin 3-O-galactoside	1.10 ± 1.06	0.40	up	-19.46	down	0.63	up
	Flavanones	8-prenylnaringenin	1.21 ± 0.61	-0.59	down	-0.27	down	-1.79	down
		isosakuranetin	1.59 ± 0.90	2.86	up	0.44	up	0.10	up
		6-prenylnaringenin	1.44 ± 0.86	-21.89	down	-0.45	down	-21.89	down
		sakuranetin	1.31 ± 0.93	0.37	up	0.10	up	-0.54	down
	Flavones	apigenin	1.29 ± 0.56	-0.16	down	-0.12	down	-0.63	down
		nepetin	1.26 ± 0.31	-0.05	down	0.38	up	0.36	up
		apigenin 7-O-(6"-malonyl-apiosyl-glucoside)	1.12 ± 1.36	0.05	up	0.74	up	-0.48	down
		sinensetin	1.13 ± 0.81	-0.44	down	0.43	up	0.40	up
	Flavonols	myricetin	1.13 ± 1.33	-0.20	down	0.93	up	-1.23	down
		galangin	1.13 ± 0.50	-0.16	down	-0.17	down	-0.49	down
		quercetin	1.27 ± 0.78	-19.60	down	0.00	down	-19.60	down
		quercetin 3-O-xylosyl-glucuronide	1.26 ± 1.74	1.26	up	3.37	up	-17.15	down
		quercetin 3-O-glucosyl-xyloside	1.18 ± 0.88	17.81	up	17.87	up	0.00	down
		isorhamnetin 7-O-	1.10 ± 0.39	-0.02	down	0.60	up	0.62	up
	Isoflavones	coumestrol	1.29 ± 0.99	-17.77	down	-17.77	down	-1.05	down
		formononetin	1.15 ± 1.34	1.61	up	0.45	up	1.00	up
	Rutin	quercetin 3-O-rutinoside	1.19 ± 1.11	2.32	up	2.48	up	1.26	up
	Others	butein	1.17 ± 0.55	0.51	up	0.26	up	-0.18	down
		(+)-catechin	1.14 ± 0.39	3.03	up	3.12	up	3.04	up
		patuletin 3-O-(2"-feruloylglucosyl)(1-6)-[apiosyl(1-2)]-glucoside	1.42 ± 0.85	0.16	up	0.52	up	0.32	up
		kaempferol 3-O-glucosyl-rhamnosyl-galactoside	1.37 ± 0.40	-1.78	down	-1.71	down	-0.67	down
		chrysoeriol 7-O-(6"-malonyl-apiosyl-glucoside)	1.34 ± 1.36	-0.63	down	0.57	up	0.98	up
		dihydromyricetin 3-O-rhamnoside	1.11 ± 0.97	-0.59	down	0.19	up	-0.28	down
		5,3',4'-trihydroxy-3-methoxy-6,7-methylene-dioxyflavone 4'-glucuronid	1.11 ± 0.48	0.00	down	18.15	up	17.76	up
		chrysoeriol 7-O-glucoside	1.10 ± 0.39	-0.02	down	0.60	up	0.62	up
Phenolics	Acids, carbocyclic	dihydro-p-coumaric acid	1.26 ± 0.77	-2.53	down	-2.99	down	-3.62	down
		methoxyphenylacetic acid	1.26 ± 0.79	-2.53	down	-2.99	down	-3.55	down
	Caffeic acids	caffeoyl tartaric acid	1.21 ± 0.67	1.72	up	-0.48	down	1.57	up

		caffeic acid	1.17	± 0.56	-0.77	down	-1.43	down	-0.83	down
	Catechols	catechol	1.49	± 0.52	0.31	up	1.34	up	1.86	up
		curcumin	1.25	± 0.69	-0.26	down	-1.07	down	-1.34	down
	Cinnamates	5-O-galloylquinic acid	1.30	± 0.48	0.14	up	0.04	up	-18.43	down
	Coumaric acids	m-coumaric acid	1.11	± 0.49	0.57	up	-0.03	down	-0.44	down
	Coumarins	esculin	1.26	± 1.52	1.38	up	0.42	up	-17.89	down
	Hydroxy-benzoates	3-hydroxybenzoic acid	1.24	± 0.79	-0.99	down	-1.87	down	-1.01	down
		2-hydroxybenzoic acid	1.23	± 0.81	-0.99	down	-2.24	down	-1.01	down
	Ortho-aminobenzoates	avenanthramide 2p	1.33	± 0.42	0.17	up	1.12	up	1.00	up
	Phenols	4-vinylphenol	1.24	± 0.78	2.37	up	2.02	up	2.95	up
	Terpenes	epirosmanol	1.11	± 0.87	-0.72	down	-0.41	down	-0.98	down
	Others	1,2'-disinapoyl-2-feruloylgentiobiose	1.54	± 0.24	-0.03	down	0.24	up	-0.20	down
		1-sinapoyl-2,2'-diferuloylgentiobiose	1.52	± 0.96	21.36	up	0.00	up	0.00	down
		2-S-glutathionyl caftaric	1.43	± 0.76	-8.48	down	1.11	up	1.37	up
		2,3-dihydroxy-1-guaiacylpropanone	1.42	± 0.67	1.59	up	0.90	up	1.15	up
		1,2,2'-	1.29	± 1.09	0.52	up	1.03	up	-0.08	down
		feruloyl tartaric acid	1.26	± 0.48	0.46	up	-0.11	down	-0.67	down
		3,4-dihydroxyphenyl-2-oxypropanoic acid	1.17	± 0.56	-0.76	down	-1.43	down	-0.83	down
		p-coumaric acid 4-O-	1.17	± 1.11	-17.64	down	-17.64	down	-17.64	down
		24-methylenecholestanol ferulate	1.15	± 1.35	1.26	up	0.81	up	1.24	up
Amino acids	Amino acids	gamma-aminobutiric acid	1.33	± 0.75	0.33	up	-0.04	down	0.03	up
	Amino acids, basic	L-glutamine	1.10	± 0.95	-1.18	down	-0.42	down	-0.52	down
	Amino acids, branched-chain	L-isoleucine	1.14	± 0.65	-0.13	down	0.07	up	-1.86	down
		L-leucine	1.14	± 0.65	-0.13	down	0.07	up	-1.86	down
	Amino acids, cyclic	L-proline	1.37	± 0.77	2.92	up	1.98	up	2.76	up
		L-histidine	1.10	± 1.93	1.20	up	1.63	up	1.25	up
	Amino acids, sulfur	L-homocysteine	1.32	± 0.17	-0.37	down	0.31	up	-1.14	down
		L-cysteine	1.29	± 0.93	3.38	up	3.64	up	4.16	up
		L-methionine	1.10	± 0.66	1.83	up	0.94	up	1.40	up
Organic acids	Citrates	citrate	1.50	± 0.23	-1.55	down	-1.60	down	-1.97	down
	Dicarboxylic acids	succinate	1.42	± 0.36	0.17	up	0.15	up	0.10	up
		alpha-aminoadipic acid	1.10	± 1.08	-1.06	down	-2.07	down	-1.49	down
Glycosides	Glucosides	galloyl glucose	1.37	± 1.03	4.00	up	-12.04	down	2.40	up
		cyanidin 3-O-(6"-dioxalyl-glucoside)	1.25	± 1.08	18.31	up	0.00	down	19.15	up
		gallic acid 4-O-glucoside	1.23	± 1.04	3.48	up	1.41	up	2.55	up
		arbutin	1.17	± 0.66	-0.91	down	-0.67	down	-0.72	down
	Glucosinolates	4-methylthiobutyl glucosinolate	1.37	± 0.60	0.94	up	0.22	up	0.54	up
		7-methylsulfinylheptyl glucosinolate	1.23	± 0.65	0.16	up	0.66	up	1.12	up
	Saponins	avenacin B2	1.41	± 0.83	2.44	up	0.28	up	2.42	up
		avenacin A2	1.30	± 0.61	0.41	up	-0.11	down	0.55	up
		avenacin A1	1.61	± 0.63	15.84	up	16.00	up	0.00	down
		soyasaponin III	1.28	± 0.20	0.25	up	0.24	up	0.30	up
Lignans	Lignans	dimethylmatairesinol	1.57	± 0.51	2.56	up	2.00	up	0.23	up
		lariciresinol	1.42	± 0.72	0.50	up	-0.65	down	-0.20	down
		demethoxycurcumin	1.16	± 0.74	-0.63	down	-0.61	down	-0.36	down
		cyclolariciresinol	1.42	± 0.72	0.50	up	-0.65	down	-0.20	down
Others	Furans	tetronic acid	1.17	± 0.86	0.00	down	18.89	up	19.72	up
	Lactones	5-deoxystrigol	1.10	± 0.65	-1.04	down	-0.74	down	-2.19	down
	Stilbenes	pinosylvin	1.17	± 0.88	-0.05	down	0.09	up	-0.58	down
	Sugar Acids	ascorbic acid	1.36	± 1.20	-0.46	down	3.43	up	0.64	up

Unsaturated FA	5-heneicosenylresorcinol	1.24	± 1.58	0.13	up	0.88	up	0.20	up
Others	avenic acid	1.59	± 1.14	-0.20	down	1.26	up	-0.44	down
	deoxymugineic acid	1.39	± 0.60	-0.38	down	-0.35	down	-1.67	down
	strigol-derivatives (strigol,orobanchol)	1.29	± 0.55	-0.55	down	-1.13	down	-1.38	down
	ribose/xylose	1.28	± 0.51	-1.33	down	-1.40	down	-0.85	down
	benzoxazinone	1.11	± 0.86	0.00	down	-1.60	down	-0.05	down

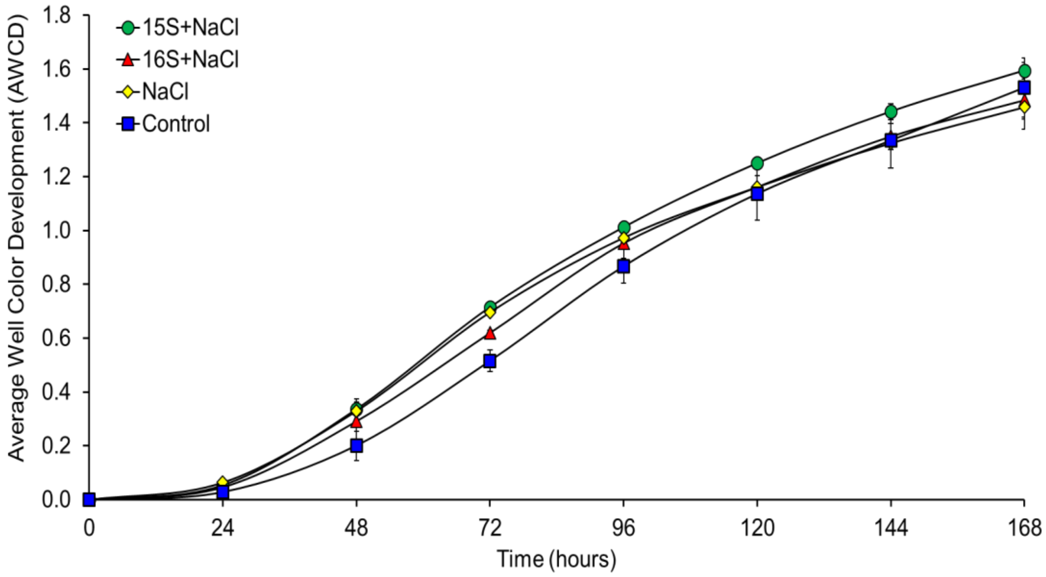
Supplementary Table 5.5. The original data to perform PCA (**Fig 5.1 and Supplementary Table 5.2**). Biolog EcoPlates data at 72 h were normalized dividing each OD_{590} corrected value by the AWCD according to (GE et al., 2018).

Rsi values				
Substrate	**Treatments**			
	15S+NaCl	**16S+NaCl**	**NaCl**	**Control**
Pyruvic acid methyl ester	1.755	1.419	0.680	3.054
Tween 40	1.744	2.520	1.705	0.935
Tween 80	0.844	1.880	2.170	0.999
Cyclodextrin	0.403	0.106	0.169	0.110
Glycogen	0.349	0.398	0.783	0.462
D-cellobiose	0.845	1.355	1.439	0.771
D-lactose	0.549	1.059	1.056	0.104
β-methyl-D-glucoside	0.594	1.232	0.988	0.119
D-Xylose	0.066	0.075	0.056	0.065
i-erythritol	0.448	0.655	0.530	0.478
D-mannitol	3.669	2.532	1.877	2.705
N-acetyl-D-glucosamine	2.490	1.651	1.846	1.604
D- glucosaminic acid	0.799	0.820	0.908	1.332
Glucose-1-phosphate	1.351	0.843	1.590	0.131
D,L-α-glycerol phosphate	0.540	0.721	0.374	0.882
D-galactonic acid γ-lactone	0.660	1.536	0.888	0.669
D-galacturonic acid	0.601	1.390	2.238	3.483
2-hydroxy benzoic acid	0.050	0.045	0.042	0.052
4-hydroxy benzoic acid	0.317	1.308	0.772	0.939
Hydroxybutyric acid	0.518	0.426	0.394	0.437
Itaconic acid	0.232	0.237	0.805	1.377
Ketobutyric acid	0.090	0.187	0.305	0.426
D-malic acid	2.281	0.702	1.963	1.293
L-arginine	1.196	1.331	0.738	1.536
L-asparagine	3.164	2.731	2.228	3.353
L- phenylalanine	0.749	1.108	0.478	0.711
L-serine	2.301	0.601	1.282	0.738
L-threonine	0.918	0.626	0.507	1.171
Glycyl-L-glutamic acid	0.412	0.123	0.298	0.372
Phenylethylamine	0.580	0.434	0.609	0.591
Putrescine	0.485	0.949	1.283	0.099

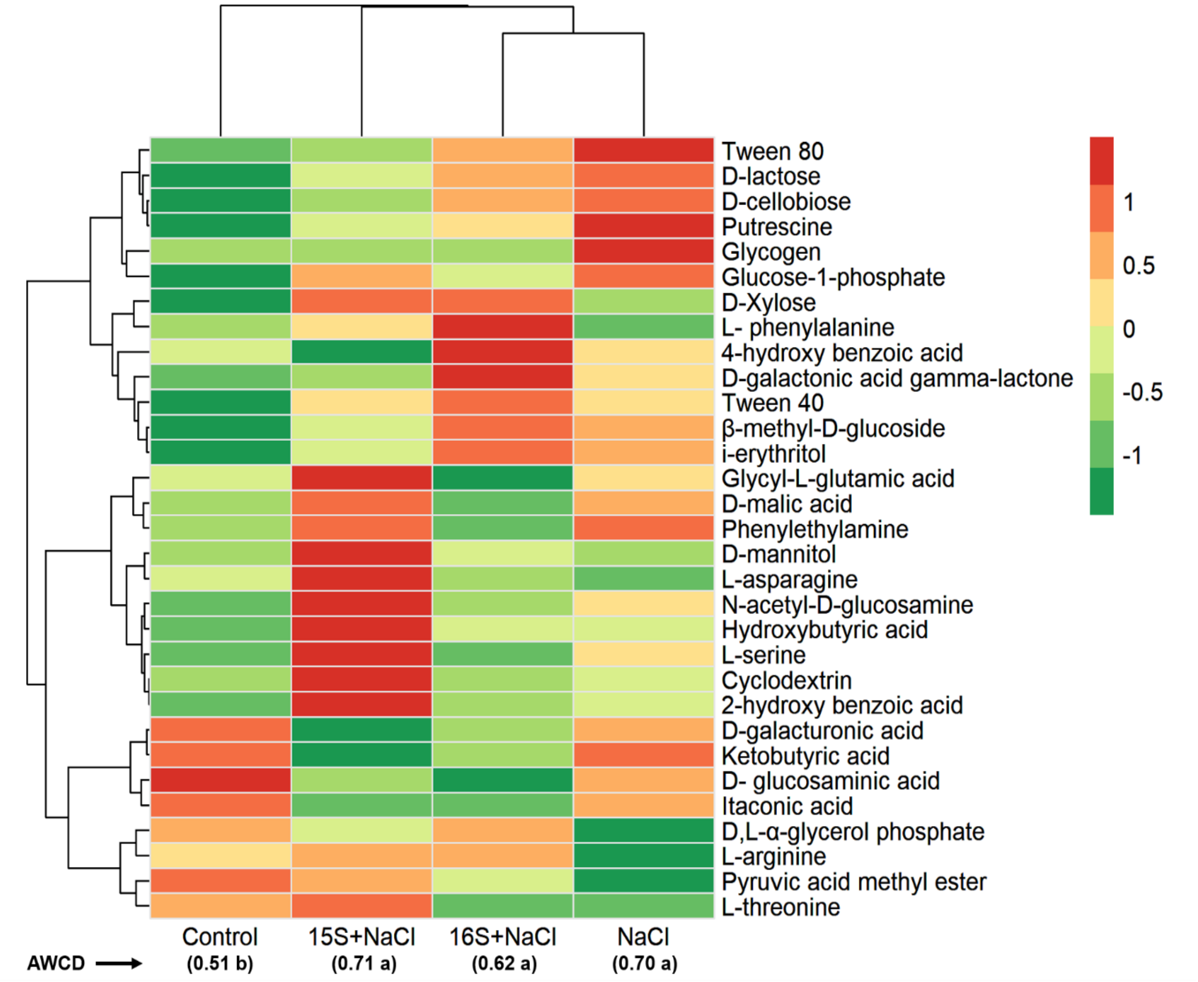
Supplementary Table 5.6. The original data of **Fig 5.2**. Effect of bacterial inoculation and NaCl application on carbon source utilization rates by the tomato rhizosphere microbial community at 72 h incubation based on specific carbon groups in the Biolog EcoPlates (amines, amino acids, carbohydrates, carboxylic acids, phenolics and polymers). Capital letters compare all group of substrates for each treatment and lower-case letters compare each group of substrates across treatments. Equal letters do not differ with Skott-Knott's test ($p \leq 0.05$). 15S+NaCl: tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application, 16S+NaCl: tomato plants inoculated with *Pseudomonas* 16S and 100 mM NaCl application, NaCl: uninoculated plants with 100 mM NaCl application, Control: uninoculated plants without NaCl application.

C utilization rate (×10^{-3} h^{-1})				
Substrate group	**Treatments (mean ± SD)**			
	15S+NaCl	**16S+NaCl**	**NaCl**	**Control**
Amines	5.3 ± 2.1 Cb	5.9 ± 1.7 Bb	9.1 ± 2.3 Aa	2.5 ± 1.2 Db
Amino acids	14.5 ± 1.8 Aa	9.4 ± 0.2 Ab	8.9 ± 0.6 Ab	9.4 ± 0.4 Bb
Carbohydrates	11.1 ± 1.9 Ba	10.0 ± 1.1 Aa	10.3 ± 0.7 Aa	5.4 ± 0.4 Cb
Carboxylic acids	8.9 ± 1.4 Bb	6.4 ± 0.5 Bc	10.1 ± 0.8 Ab	11.7 ± 0.4 Aa
Phenolics	1.8 ± 1.6 Dc	5.8 ± 0.4 Ba	3.9 ± 0.9 Bb	3.5 ± 1.1 Db
Polymers	8.3 ± 0.4 Bb	10.5 ± 1.5 Aa	11.7 ± 1.8 Aa	4.5 ± 0.8 Cc

Supplementary Fig 5.1. The AWCD of utilized carbon substrates with incubation time in Biolog EcoPlates in the different treatments (mean ± SE, n = 3). 15S+NaCl: tomato plants inoculated with *Enterobacter* 15S and 100 mM NaCl application, 16S+NaCl: tomato plants inoculated with *Pseudomonas* 16S and 100 mM NaCl application, NaCl: uninoculated plants with 100 mM NaCl application, Control: uninoculated plants without NaCl application (the original data available in **Supplementary Table 5.1**).



Supplementary Fig 5.2. Metabolic profile showing the utilization of the 31 carbon substrates for the microbial communities assessed by the Biolog EcoPlates (at 72 h incubation) in tomato plants inoculated with *Enterobacter* and 100 mM NaCl application (15S+NaCl), *Pseudomonas* and 100 mM NaCl application (16S+NaCl) or uninoculated plants with 100 mM NaCl application (NaCl), and without NaCl application (Control) after growing in a rhizobox system for 40 days. The highest consumption is represented by a dark red color and the lowest consumption by a dark green color. AWCD for each treatment is given in parentheses (lower-case letters following the AWCD values indicate a significant difference across treatments ($p < 0.05$) according to Skott-Knott's test). The original data available in **Supplementary Table 5.3**.



6 CONCLUSÃO GERAL

Nossos resultados fornecem informações sobre as interações entre tomate e bactérias benéficas associativas, que podem ser exploradas para desenvolver estratégias úteis em prol de uma agricultura sustentável. A inoculação com ambas bactérias promoveu a regulação de uma alta quantidade de compostos do metabolismo vegetal, envolvidos nos processos de colonização bacteriana, na promoção do crescimento das plantas, bem como à tolerância ao estresse salino. Além disso, a microbiota associada à rizosfera do tomate exibiu um metabolismo diferencial em resposta à inoculação e à salinidade. O efeito de *Enterobacter* 15S foi mais pronunciado nas condições de interação em que não houve imposição do estresse, enquanto *Pseudomonas* 16S teve uma significância mais clara relacionada com a tolerância do tomate à salinidade, possivelmente relacionada com a adquisição de ferro.