Identifying the genetic determinants of barley colonisation by the growth-promoting bacteria *Pseudomonas fluorescens*

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A thesis submitted to the University of East Anglia for the degree of Doctor of Philosophy

John Innes Centre

September 2021

Abstract

The global population will increase dramatically during this century which, together with the climate crisis, will pose an enormous challenge for food security. We will need to balance boosting food production with reducing agrochemical use in order to preserve natural ecosystems. To do this, exploiting the benefits that the rhizosphere microbiome has on plant health, growth and resistance to biotic and abiotic stresses has been proposed as a feasible strategy. In my project, I examined the interaction between barley, the fourth most important cereal crop, and the widespread beneficial bacteria *Pseudomonas fluorescens.* Plants can actively shape their rhizosphere microbiomes mainly through the secretion of root exudates whose composition depends greatly on host genotype. The ability of different microbes to utilise these exudates determines their success in the rhizosphere, which results in a shift in the rhizobiome composition. In my PhD, I first investigated the effect that barley genotype exerts on the overall rhizosphere microbiome and on the soil *Pseudomonas* population. I next examined the genetic features of the *Pseudomonas* populations linked to two particular barley cultivars, Chevallier and Tipple, and identified a set of cultivar-specific genes among the rhizosphere *Pseudomonas* isolates. These genes fell mainly into two categories: substrate transport and central metabolism, supporting the role of root exudates as the primary drivers of rhizosphere microbiome assembly. Next, I analysed the exudates composition of Chevallier and Tipple, and observed that the secretion of sugars and organic acids were the major differences between them. Finally, I screened a Chevallier x Tipple population for the secretion of fructose as a potential determinant of *Pseudomonas* recruitment, highlighting two large QTL in the barley genome. Overall, the ultimate objective of this project is to provide a basis to select more efficient biocontrol strains, and breed plants with an improved ability to recruit beneficial *Pseudomonas*.

Acknowledgments

I definitely have to start by thanking my supervisor, Jake Malone. During these last four years, he has always offered advice, guidance and support in every step of this long way and for that, I am incredibly grateful. Being part of the Malone lab has been a fantastic experience, not only professionally, but also personally. They all contributed massively to motivate me throughout these 4 years and made great coming to work every day. Thank you all, Richard, Rosaria, Cat, Javi, Ainelén, Danny, Sankar and Anna. Also, to the past members of the lab who helped, one way or another, to push this project forward.

Thanks to my whole supervisory team, Chris Ridout, Sarah de Vos and Paul Nicholson. Your input on this project has been incalculable. I should mention too, how much I have learnt being part of the NHB team during all this time, from being out in the field to participating in all the outreach activities.

Special thanks to everybody who has collaborated on this project. To my proof readers, Cat Thompson and Natalia Miguel Vior, to Anita Bollmann-Giolai for dealing with the amplicon sequencing data, to Govind Chandra for being the bioinformatics guru, to Phil Poole and Vinoy Ramachandra for the *Rhizobium* biosensors, to Rachel Goddard for her help with the QTL analysis and to Jack Davies and Owen Thornton for being such outstanding students.

I want to extend my gratitude to everyone in MolMicro. This department welcomed me with open arms and made me feel part of this big family since day 1. Thank you, MolMicro, for all the coffees shared, the causal corridor catch-ups, the laughs, the Christmas lunches and in general, for being there. We have gone together through a lot this last year and a half, and I truly believe we have helped each other more than ever. Very well deserved thanks must also go to the scientific support platforms, without them our daily work would be much more complicated and definitely, less efficient.

When I first started my PhD back in 2017, I did not imagine all the things that were about to happen to me. Certainly, I did not imagine going through a pandemic either, but overall, I could not be happier to have made the decision to move to the UK and pursue my PhD at the John Innes Centre. I have met fantastic people, had great experiences and grown a lot as a person. Thanks to those who have shared all these moments with me, who have brought light into my days, you all know who you are.

Ahora, me gustaría que parte de mis agradecimientos se dirigieran a todos aquellos que hicieron posible que yo haya llegado hasta aquí. En primer lugar, a mis padres, Encarna y Miguel. Ellos me han brindado las herramientas necesarias, muchas veces sacrificándolo todo en el camino, para que yo pudiera perseguir mis metas. Gracias mamá por ser tan luchadora, gracias papá por tu inquietud innata. Sin vosotros, yo no sería nadie. Os quiero muchísimo. Gracias a mis abuelos, Paqui, Vicente y Encarna, que siempre se sintieron tan orgullosos de mí, me encantaría poderos mostrar hasta donde he llegado. Os echo de menos. Gracias a toda mi familia, por siempre hacerme sentir en casa. Gracias a mis amigas y amigos de España, que me habéis acompañado en la distancia durante todo este tiempo y me habéis recibido con los brazos abiertos cuando he vuelto. Gracias a mi familia de Norwich, a los que están y los que se fueron, sin vosotros los días grises habrían pesado más. Qué suerte que la vida nos cruzara.

Es necesario mencionar a aquellas personas que despertaron en mí el gusanillo este de la ciencia. Millones de gracias a mis primeros mentores, Luis Rey Navarro, Jose Manuel Palacios y Tomás Ruiz Argüeso, quien desafortunadamente ya no está con nosotros. Ellos hicieron posible mis primeros pasos en la investigación y por ello, siempre les estaré agradecida. Gracias a todas aquellas personas que, en esa época que parece ya una vida pasada, me animasteis a seguir adelante con esta locura.

Por último, pero desde luego no menos importante, gracias a ti, Miguel. Eres mi mejor amigo, mi compañero y mi mayor apoyo. Un día, hace más de 4 años ya, dejaste todo atrás y empezaste de cero por mí, para estar a mi lado mientras yo perseguía mis sueños. En cierto modo, un pedacito de esto te pertenece, por apoyarme día tras día y por quererme tanto. Te quiero. Gracias también por haberme regalado una segunda familia. Gracias Ana y Tomás por tratarme como a una hija. ¡Ah! Y gracias al perrito Tres por hacer que nuestros días nunca sean aburridos.

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Abbreviations

ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1-carboxylate
AFLPs	Amplified fragment length polymorphisms
AHL	Homoserine lactones
APC	Amino acid-polyamine-organocation
ASVs	Amplicon sequence variants
CCR	Carbon catabolite repression
CDS	Coding DNA sequence
CER	controlled environment room
CFC	cetrimide/fucidin/cephalosporin
CFUs	Colony forming units
CIM	Composite interval mapping
CIMMYT	International Maize and Wheat Improvement Centre
CPs	SynCom of Chevallier-derived *Pseudomonas*
CRB	Congo Red binding
CRh	Chevallier rhizosphere microbiome extract
CxT	Chevallier x Tipple mapping population
DAPG	2,4-diacetylphloroglucinol
DNA	Difco™ Nutrient Agar
dpi	days post-inoculation
DW	Dry weight
ED	Entner-Doudoroff pathway
EPS	Exopolysaccharides
ESTs	Expressed sequence tags
FAO	Food and Agriculture Organisation of the United Nations
FE	Fluorescence emission
FHB	Fusarium Head Blight
FP	Fahraeus agar
GABA	γ-aminobutyric acid
GC-MS	Gas chromatography - Mass spectrometry
gDNA	Genomic DNA
HCN	Hydrogen cyanine

HMW-C	High-molecular weight compounds
HYV	High-yielding crop varieties
IAA	Indole-3-acetic acid
InSeq	Insertion sequencing
iPBS	Inter-primer binding site
IRAP	Inter-Retrotransposon Amplified Polymorphism
IRRI	International Rice Research
ISR	Induction of systemic resistance
ISSR	Inter-simple sequence repeats
ITS	Internal transcribe spacer
IVET	In vitro expression technology
KB	King's medium B
KDPG	2-keto-3-deoxy-6-phosphogluconate
LB	Lysogenic broth
LMW-C	Low-molecular weight compounds
LPS	Lipopolysaccharides
LTTRs	LysR-Type family transcriptional regulators
MAMPs	Microbe-associated molecular patterns
ML	Maximum Likelihood method
MO	Swarming motility
MYM	Maltose-yeast extract-malt extract medium
NGS	Next-generation sequencing
OD	Optical density
OTUs	Operational taxonomic units
PA	Protease activity
PA	Protease activity
PBS	phosphate-buffered saline
PGPF	Plant growth–promoting fungi
PGPR	Plant growth-promoting rhizobacteria
Phl+	*Pseudomonas* producing DAPG
PTI	Plant pattern-triggered immunity
Pvd	Pyoverdine
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restricted fragment length polymorphism

RILs	Recombinant inbred lines
Rlv3841	*Rhizobium leguminosarum* bv. viciae 3841
Rlv3841_lux	RLv3841 based lux biosensors
RNA-seq	RNA sequencing
ROS	reactive oxygen species
RT	Room temperature
SAR	Systemic acquired resistance
SEM	Standard error of the mean
SNPs	Single-nucleotide polymorphisms
SS	*Streptomyces venezuelae* suppression
SSR	Simple sequence repeats
SynCom	Synthetic community
TPs	SynCom of Tipple-derived *Pseudomonas*
TRh	Tipple rhizosphere microbiome extract
TY	Tryptone-yeast
UMA	UMS medium supplemented with 1.5% agar
UMS	Universal minimal salts
WT	Wild type

CHAPTER 1:

General introduction

1.1 Background

World population is expected to reach almost 10 billion by 2050, which equates to an increase of approximately 40% (http://www.fao.org/faostat). To sustain such numbers, it is predicted that an increase of 70 % in global food production will be required (FAO, 2009). This situation will pose an enormous challenge for traditional agriculture: increasing the production of food, fibres and fuel without expanding the current farmland and respecting the environment. In 2017 the Food and Agriculture Organisation of the United Nations (FAO) described the 10 challenges that the World will face in the upcoming years as a result of the population dynamics as follows:

1. Sustainably improving agricultural productivity to meet increasing demand
2. Ensuring a sustainable natural resource base
3. Addressing climate change and intensification of natural hazards
4. Eradicating extreme poverty
5. Ending hunger and all forms of malnutrition
6. Making food systems more efficient, inclusive and resilient
7. Improving income earning opportunities in rural areas
8. Building resilience to protracted crises, disasters and conflicts
9. Preventing transboundary and emerging agriculture and food system threats
10. Addressing the need for coherent and effective national and international governance

Focusing only on the agricultural related challenges, the efforts will have to be directed towards making agriculture more respectful to the environment considering huge increase in population, climate change and social inequalities. Or in other words, what has been described as sustainable intensification. The term sustainable intensification was first coined by Pretty in 1997 when he claimed that sustainable agriculture aims to manage pests, fertilisers, soil, and water using a variety of approaches and technologies. It seeks to promote higher enterprise diversity within farms as well as increased links and flows between them. By-products or wastes from one component are used as inputs in another. Given that natural processes increasingly replace external inputs, the environmental impact is eventually diminished (Pretty, 1997, Struik and Kuyper, 2017).

One of the major achievements of modern agriculture has been the rapid acceleration in global food production over the last 50 years. In the mid-60s, production of cereal crops such

as wheat and rice experienced spectacular increases during the phenomenon known today as the Green Revolution (Borlaug, 2000, Borlaug and Dowswell, 2003, Rajaram, 2011).

The Green Revolution not only symbolised how agricultural science could be applied to implement modern farming techniques in developing countries, but also the success of a more equitable distribution of food resources throughout society (Evenson and Gollin, 2003, Hedden, 2003, Rajaram, 2011). This new revolution provided the World with new ways to improve food production by increasing the yields of the main cereal crops. It all started with wheat breeding and the creation of the International Maize and Wheat Improvement Centre (CIMMYT) in Mexico, under the lead of Dr Norman Borlaug. Later, this was followed by rice with the International Rice Research Institute (IRRI) in the Philippines, both founded by the Rockefeller Foundation in the 1950s. The greatest achievement of these two institutions was the development of modern high-yielding crop varieties (HYVs) (Evenson and Gollin, 2003, Hedden, 2003, Rajaram, 2011). HYVs showed an impressive rise in yield that was mostly achieved through breeding incorporation of dwarfing genes, resulting in shorter and stiff-strawed varieties with better harvest indices. These plants dedicated more energy on producing grain than creating straw or leaf material, besides showing better responses to fertilisers than traditional varieties (Evenson and Gollin, 2003, Hedden, 2003, Pingali, 2012).

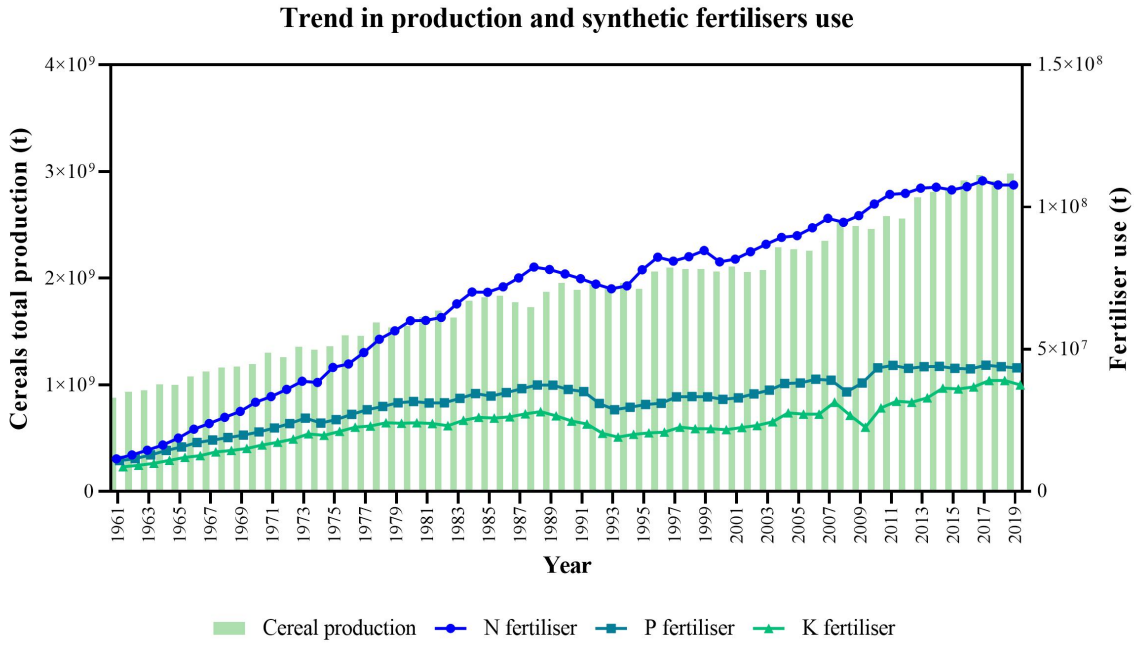

Figure 1.1. Trend in cereal production and fertiliser use. Data obtained from FAOSTAT (http://www.fao.org/faostat).

Borlaug himself recognised that the enormous agricultural output achieved during the Green Revolution could not have been possible without the use of synthetic fertilisers, mainly nitrogen and phosphorous. According to some authors up to 50% of crop yield is attributable to commercial nutrient inputs (Stewart et al., 2005, Pingali, 2012, Stewart and Roberts, 2012). Plotting both trends together over time, cereal production and total fertiliser use (figure 1.1), it is remarkable how they have increased in parallel since the Green Revolution took place.

The desire and necessity to maximise grain yield has led farmers to (i) apply higher amounts of fertilisers than the minimum required for maximum crop production and (ii) to overexploit their farmland. As a consequence, the uncontrolled release of agrochemicals, as well as the intensive overworking of arable land, are causing negative effects on farm agroecosystems. These negative effects include contamination of water reserves and soil, eutrophication of aquatic systems, deforestation, deterioration of soil fertility, land degradation, over-extraction of groundwater, loss of biodiversity and emissions of greenhouse gases (Adesemoye and Kloepper, 2009, Adesemoye et al., 2009, FAO, 2009, FAO, 2017).

It is clear that a bidirectional relationship has been established between agriculture and climate change. On the one hand, agricultural yields could suffer tremendously from the impacts of climate change as a consequence of extreme weather conditions, water scarcity, increased weeds and pests, higher carbon dioxide concentration and more diseases. On the other hand, agriculture *per se* contributes to around 10 % of the anthropogenic greenhouse gas emissions as well as to approximately 70% of land-use emissions (FAO, 2009, Franks, 2014). Therefore, it is a requirement that agriculture adapts to climate change while contributing to mitigate its effects at the same time.

Within this scenario, sustainable agriculture, environment preservation and feeding a population of 10 billion will be some of the biggest issues to be faced by humanity in the 21st century. It is vital that we learn how to increase yields from land which is already cultivated and understand how we can improve plant growth in a new agricultural era with lower chemical inputs. According to Beddington et al. (2012) there are some promising areas of research that could contribute to the implementation of sustainable intensification such as breeding of new varieties of crops and livestock better adapted to adverse climates, diet change in ruminants to reduce methane emissions and increase productivity gains, improving soil management, integration of agro-ecological approaches or promotion of engineering technologies that increase water efficiency (Beddington et al., 2012). Alongside these areas, some authors have highlighted the importance of focusing our scientific efforts on the natural

abilities of plants to produce more (Tkacz and Poole, 2015). One possible tool to achieve such a goal in the long term is the exploitation of plant-associated microorganisms, especially those in the belowground organs, in order to increase resilience to biotic and abiotic stresses, nutrient uptake or general plant health.

All in all, the idea that plant nutrition and general fitness are highly influenced by the microbial composition of the rhizosphere is increasingly accepted. Therefore, a better understanding of the dynamics and interactions of the microbial community living in the rhizosphere, i.e., the rhizosphere microbiome, has been in the scientific spotlight over the past few years as a potential tool to enhance crop production (Sanchez-Canizares et al., 2017, Singh et al., 2020). Using the ability of rhizospheric microbes to provide plants with essential nutrients as an important target of new plant-microbe studies, as well as for new breeding programs, we might contribute to the generation of plant varieties with an enhanced capability to interact with beneficial microbes (Hartmann et al., 2007, Berendsen et al., 2012, Tkacz and Poole, 2015).

1.2 The plant microbiome

Plants grow in close contact with distinct microbial communities, termed the plant microbiome (Turner et al., 2013a, Muller et al., 2016). The plant microbiome comprises several types of organisms such as bacteria, fungi, protozoa, archaea and viruses that can benefit the plant host in different ways by improving growth, protecting against pathogens or conferring adaptative advantages (Orozco-Mosqueda et al., 2018).

Since the microbiome and the plant host establish an incredibly close relationship, different authors suggest that they should be considered as a whole, referring to it as the pan-genome or the holobiont of the plant (Turner et al., 2013a, Sanchez-Canizares et al., 2017). The holobiont is the combination of the host plus all the microbial communities inhabiting the host tissues. Host-microbe interactions have been proven to affect host development, immunity, metabolism, behaviour and even speciation (Shropshire and Bordenstein, 2016). Given the influence on the overall host fitness, some authors have coined the term of "the hologenome theory of evolution" in which they described the holobiont "as a single dynamic entity in which a vast amount of the genetic information and variability is contributed by the microorganisms. Evolution of the holobiont can occur by changes in the host genome and/or in any of the associated microbial genomes" (Zilber-Rosenberg and Rosenberg, 2008). Even though co-evolution has been observed for specific interactions, such as in host-pathogen or

between symbionts, to what extent the aforementioned co-evolution process occurs for all the microbial consortia or only for certain partners remains unclear (Hassani et al., 2018).

Microbial communities can inhabit different niches within the plant such as the phyllosphere, the endosphere or the rhizosphere. The phyllosphere is constituted by all the aboveground organs, mainly dominated by leaves but including stems, fruits and flowers, and represents a variable habitat exposed to environmental conditions that can change very rapidly, among others, radiation, moisture or temperature (Orozco-Mosqueda et al., 2018). The endosphere is occupied by those microorganisms that colonise the internal plant tissues, also called endophytes. Lastly, belowground organs are surrounded by the bulk soil and the rhizosphere, the layer of soil that is influenced by plant roots (Turner et al., 2013a, Muller et al., 2016). Although each plant compartment bears a different microbial composition, sometimes different communities can show certain overlap, for example in the case of rhizosphere communities and the root endosphere (Turner et al., 2013a).

Among all the plant compartments described above, the understanding of the rhizosphere microbiome has gained more popularity in recent years as a potential tool to enhance plant yield and health and therefore, the rest of this section will focus on this compartment. The main reason for this is that the rhizosphere acts as the interface between the microorganisms inhabiting the bulk soil and the plant, which makes it the most diverse set of microorganisms directly interacting with the plant (Compant et al., 2010, Bulgarelli et al., 2013).

1.2.1 Plant rhizosphere

The term “rhizosphere” was coined in 1904 by Lorenz Hiltner as the soil influenced by roots. According to him, the extension of the rhizosphere depends on the soil structure, but mostly on the plant species (Hiltner, 1904). Nowadays, the rhizosphere is defined as a thin layer of soil firmly attached to plant roots and influenced by plant-derived compounds (Berendsen et al., 2012).

The microbiome of the rhizosphere constitutes the most diverse set of microorganisms directly interacting with the plant. Consequently, the impact of this community on plant fitness and adaptation could be enormous (Coats and Rumpho, 2014). The relative abundance of microorganisms in the rhizosphere is much higher but less diverse than in the surrounding bulk-soil because of a phenomenon known as the rhizosphere effect. Plants can secrete an average of 21% of their photosynthates into the rhizosphere, which triggers a specialised shift in microbial composition and increases microbial density 10 to 1000 times

within the root influenced area (Berendsen et al., 2012, Lundberg et al., 2012, Bulgarelli et al., 2013, Haichar et al., 2014).

The composition of the rhizosphere microbiome is determined by multiple hierarchical factors that act as a filter of specialisation: climate (temperature and rainfall), soil (physicochemical properties such as pH or texture and nutrient availability), plant characteristics (genotype, exudates, signals and self-induced soil modifications) and lastly, symbiont effect (interactions at microorganism level) (Uroz et al., 2019). Some authors have proposed a two-step selection process to explain the phenomenon by which the microbial composition from bulk soil to the root endosphere experiences a dramatic rearrangement. The first step of specialisation occurs from bulk soil to rhizosphere, and is driven by rhizodeposition and root exudates. These processes fuel a substrate-driven community shift which would benefit fast growing bacterial taxa (Bulgarelli et al., 2013, Philippot et al., 2013). The second step is responsible for the selection of microorganisms from the rhizosphere to the root endosphere and here other host mechanisms, such as the innate immune system, might play an important role (Bulgarelli et al., 2013).

Over the past decade, the development of more affordable next-generation sequencing (NGS) techniques has made possible to gain new and deeper insights into the composition of microbial communities of different plant hosts, especially for bacterial communities. Despite the large number of bacterial phyla described in nature, these studies have shown that the bacterial microbiota of plants is dominated by only a few phyla: Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Lundberg et al., 2012, Bulgarelli et al., 2013, Muller et al., 2016).

The rhizosphere microbial consortia may be neutral, deleterious or beneficial for the plant host. When these microbes stimulate plant fitness, they are called plant growth–promoting fungi (PGPF) or plant growth-promoting rhizobacteria (PGPR) (Compant et al., 2010, Pieterse et al., 2014) and they are of special interest as they can be applied in agricultural production or in phytoremediation processes. Due to the greater presence of the so-called PGPR and the economic and scientific interest attracted by the bacterial members of the plant microbiome, the next section will focus on this group of organisms.

1.2.2 Plant growth-promoting rhizobacteria (PGPR)

PGPR are able to colonise plant roots in significant numbers, $10^5 – 10^7$ CFU/g of fresh root. We can distinguish between two types of PGPR: rhizosphere colonisers and root colonisers or endophytes. Both groups can affect plant growth directly by biological nitrogen fixation, phytohormone production and nutrient solubilisation; or indirectly, mainly by the suppression of pathogenic microorganisms (figure 1.2). The best studied examples of PGPR belong to several genera of bacteria such as *Azospirillum*, *Gluconacetobacter*, *Pseudomonas*, *Rhizobium* and *Bacillus*, and the most represented phyla are Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria (Bulgarelli et al., 2013).

The benefits of PGPR have been widely studied and we can find well known examples such as nitrogen-fixing bacteria (Oldroyd, 2013, Turner et al., 2013a, Tkacz and Poole, 2015) or some members of the genus *Pseudomonas* (Combes-Meynet et al., 2011, Mendes et al., 2011, Bulgarelli et al., 2013). However, in most cases, their contributions to the host phenotype are not yet totally understood.

Currently, the exact mechanism by which communities of commensal rhizobacteria are assembled is unknown. As mentioned above, many factors have been shown to be involved: host genotype, developmental stage, root morphology or root exudates. Special importance has been given to rhizodeposition or root exudation as this process is responsible for the nutrient enrichment occurring in the rhizosphere, therefore the ability to utilise plant-derived compounds more effectively than others is crucial for root and rhizosphere colonisation (Bulgarelli et al., 2013, Sasse et al., 2018).

PGPR colonisation success depends on various aspects such as motility, chemotaxis, substrate specificity, competitiveness, cooperativeness, attachment or overcoming the plant immune system. Bacteria constituting the rhizosphere consortium must be able to actively catabolise sugars and organic acids present in the exudates, sense those molecules and move towards them, compete for the substrates as well as for the physical niche with other microbes and lastly, they need to successfully attach to the root surface or enter the root tissue, in the case of the endophytes (Bulgarelli et al., 2013, Backer et al., 2018, Sasse et al., 2018).

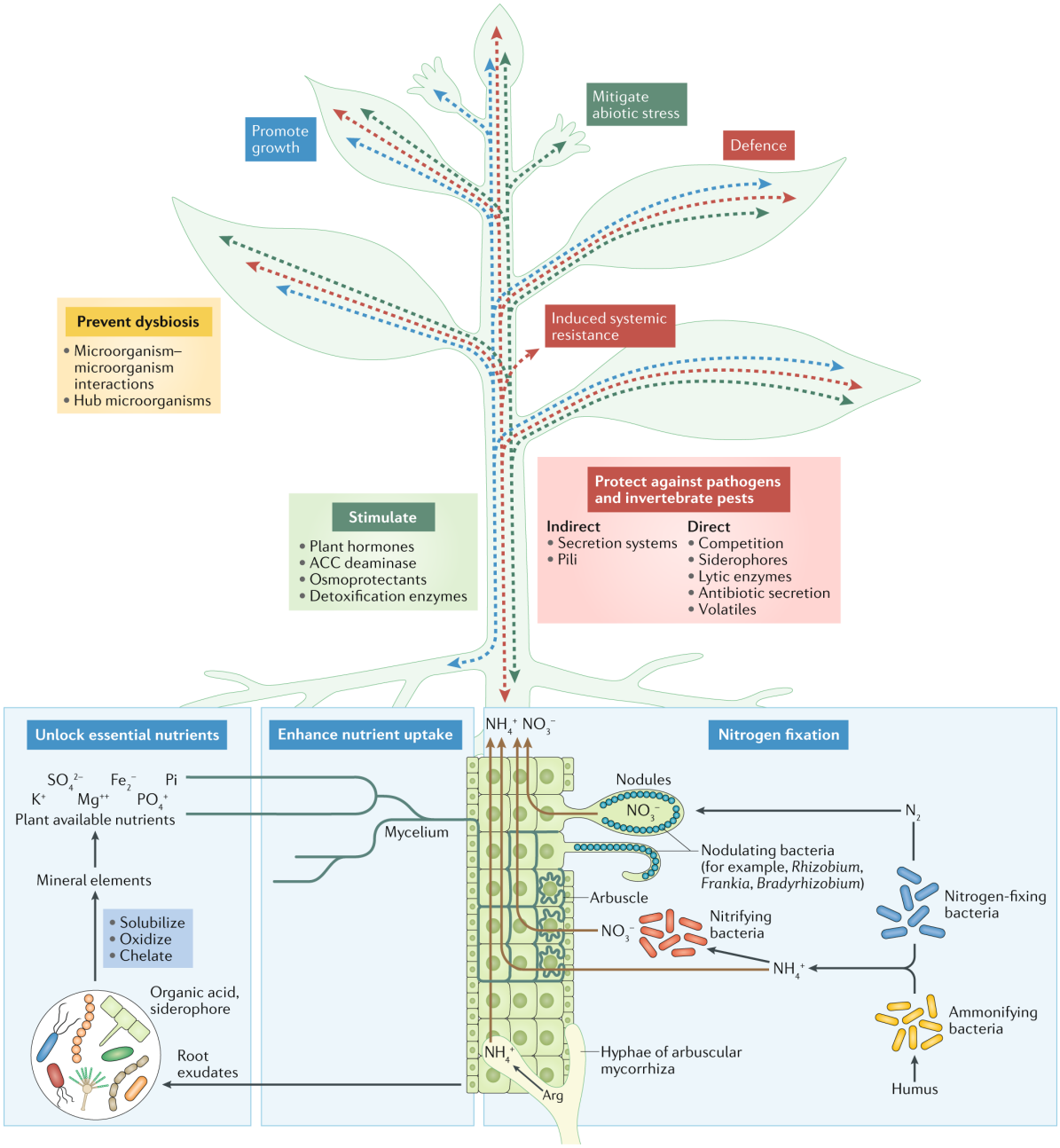

Figure 1.2. Beneficial effects of PGPR. These benefits include growth promotion (blue), stress control (green) and defence against pathogens and pests (red). Taken from Trivedi et al. (2020).

1.2.2.1 Nutrient acquisition by PGPR

One of the best-known mechanisms by which rhizobacteria promote plant growth is biological nitrogen fixation. Biological nitrogen fixation is the process of reducing atmospheric dinitrogen (N_2) into ammonia via the nitrogenase enzymatic complex. Bacteria can fix N_2 in association with plants, as is the case for *Rhizobium, Frankia* and *Actinobacteria*; or as free-living organisms, such as *Pseudomonas, Azoarcus, Beijerinckia, Cyanobacteria, Klebsiella, Pantoea, Azotobacter, Azospirillum,* etc. (Bulgarelli et al., 2013, Alori et al., 2017). Symbiotic N_2 fixation takes place in legume and non-legume plants, however the contribution to agroecosystems by legume-rhizobium associations is remarkably high, accounting for up to 48 million tonnes of fixed N_2 per year (Alori et al., 2017). When rhizobia interact with legumes, bacterial infection of the roots leads to the formation of root nodules. This specialised new organ provides an optimal environment for the bacterial N_2 fixation, mainly by controlling free oxygen flow given the extreme sensitivity to O_2 of nitrogenase. This protective micro-environment is crucial for catalysing the reduction of atmospheric nitrogen into ammonium (Berges and Mulholland, 2008, Oldroyd, 2013). During this interaction, rhizobia provide legumes with bioavailable forms of nitrogen, while the plant delivers fixed carbon to the bacteroids inhabiting the nodules (Backer et al., 2018).

Plant-available phosphorous is relatively scarce in the soil, therefore the role of phosphate-solubilising organisms is key to facilitate its uptake by plants (Owen et al., 2015). Phosphate-solubilisers synthetize phosphatases and phytases that dephosphorylate phytates, the most abundant form of organic P in the soil, making it accessible for the plant. Also, they secrete organic acids that lower pH, such as succinic, citric or gluconic acids; chelate the cations bound to phosphate and release it into the soil solution (Bulgarelli et al., 2013, Owen et al., 2015, Backer et al., 2018).

Absorption of other micronutrients can be also improved by rhizobacteria. This is the case of Fe and Zn solubilising bacteria, which through production of organic acids, such as 2-ketogluconic acid and gluconic acid, or siderophore biosynthesis can help to increase the availability of these micronutrients in the soil, ultimately leading to an enhancement in the plant biomass (Mumtaz et al., 2017, Stamenković et al., 2018).

1.2.2.2 Phytohormone production by PGPR

Phytohormones are key chemicals that regulate plant growth and development as well as the response to limiting environmental factors. The most well-studied hormones are auxins, cytokinins, gibberellins, abscisic acid and ethylene. (Bulgarelli et al., 2013, Backer et al., 2018).

Many rhizobacteria possess the ability to produce auxins, with indole-3-acetic acid (IAA) producers a particularly well studied case. IAA is able to directly promote plant growth by enhancing cell division, elongation and differentiation, which results in an increase in xylem formation, seed germination rate and root morphogenesis (Singh et al., 2016, Egamberdieva et al., 2017). In bacteria, tryptophan has been identified as the main precursor for IAA biosynthesis, an amino acid that can be found in root exudates (Spaepen et al., 2007, Lugtenberg and Kamilova, 2009). The ultimate effect of exogeneous bacterial IAA will depend very much on the endogenous IAA levels in the plant. Therefore, bacterial IAA may have neutral, beneficial, or even, negative effects on plant growth promotion, in addition to acting as a bacterial signalling molecule (Spaepen et al., 2007, Singh et al., 2016).

PGPR can also modulate the levels of certain phytohormones such as ethylene. Ethylene is a volatile hormone, very active in low concentrations and commonly known as a stress hormone. Ethylene biosynthesis is triggered upon various biotic and abiotic stresses and controls different aspects of plant development, such as senescence, abscission or pathogen-defence signalling (Bulgarelli et al., 2013, Backer et al., 2018, Stamenković et al., 2018). Some microorganisms are able to manipulate the ethylene levels present in the rhizosphere by converting its precursor 1-aminocyclopropane-1-carboxylate (ACC) into α-ketobutyrate and ammonia. The enzyme responsible of this process is the ACC deaminase encoded by the gene *acdS* (Danish and Zafar-Ul-Hye, 2019). As a consequence of the reduction in extra-radicular ACC concentration, ACC exudation increases and eventually leads to a decrease in ethylene biosynthesis due to the lack of its precursor. This phenomenon appears to control ethylene levels below the level that would trigger growth arrest in plants, an effect that is even greater under stress conditions, such as drought (Pérez-Montaño et al., 2014, Danish and Zafar-Ul-Hye, 2019).

1.2.2.3 Plant protection against pathogens by PGPR

Multiple PGPR have biocontrol capabilities against a broad range of plant pathogens which ultimately has an indirect effect on plant growth. The mechanisms by which PGPR can control plant pathogens are diverse and include (1) competition for nutrients and

environmental niches, for example through the secretion of siderophores in iron-limited conditions, ; (2) production of antimicrobial molecules, such antibiotics or extracellular lytic enzymes including hydrogen cyanine (HCN), phenazines or surfactants; (3) induction of systemic resistance (ISR), a phenomenon triggered in the plant upon local contact with the PGPR that induces a defensive response in the entire plant as a consequence of the recognition of certain bacterial components (lipopolysaccharides -LPS-, flagella or certain volatiles); or (4) interference with quorum sensing mechanisms, for example by degrading homoserine lactones (AHLs), key for bacteria to express virulence factors at a high cell density (Lugtenberg and Kamilova, 2009, Muller et al., 2016, Romera et al., 2019).

One example of the antagonist effect exerted by the PGPR community is shown by Carrión and colleagues. They studied in detail the inhibition mechanism of *Rhizoctonia solani*, a wide host range fungal pathogen, by sugar beet root endophytes in disease-suppressive soils. They observed that the fungal inhibition phenotype could be attributed to two bacterial genera, *Chitinophaga* and *Flavobacterium*. By utilising a combination of metagenomics, functional characterisation and mutagenesis, the authors were able to pinpoint a secondary metabolite encoding gene cluster as the genetic mechanism responsible of *Flavobacterium* antagonism towards the fungal pathogen (Carrión et al., 2019).

1.3 *Pseudomonas fluorescens*

The genus *Pseudomonas* includes more than 200 species of Gamma-proteobacteria. They are widely spread throughout the environment and are characterised by metabolic versatility, diversity, motility due to one or several polar flagella, aerobic respiration and a high genomic G + C content (59 – 68%) (Haas and Defago, 2005).

P. fluorescens is a widespread soil bacterium and a major constituent of the plant microbiome. It is able to colonise a vast range of different plants in a non-specific manner, benefiting from root exudates as a source of nutrients. *P. fluorescens* are capable of occupying several environmental niches and metabolising many different carbon sources (Mauchline and Malone, 2017). Many fluorescent pseudomonads can live as commensals or beneficial bacteria with plants and in turn, they can have an important impact on plant health by enhancing access to nutrients, suppressing some pathogens or inducing systemic resistance in the plant host (Seaton and Silby, 2014). *P. fluorescens* belongs to what is known as the *Pseudomonas fluorescens* complex, which represents one of the most diverse bacterial groups with more than fifty named species. Originally, this group owed its named to the production of the fluorescent pigment pyoverdine (Pvd), also known as pseudobactin. Pvd

is extracellularly diffusible and has high affinity for Fe^{3+} ions, acting as a siderophore for the producer strain. Therefore, Pvd-producing *Pseudomonas* spp. may inhibit the growth of bacteria and fungi with less efficient siderophores when iron is a limiting factor (Haas and Defago, 2005). Nowadays, thanks to NGS, the *P. fluorescens* complex is defined based on the phylogenetic relationships between its group members, which has made possible the establishment of eight phylogenomic groups: *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata*, *P. chlororaphis* and *P. protegens* (figure 1.3) (Garrido-Sanz et al., 2016).

The diverse lifestyles of the *P. fluorescens* complex are also reflected in their genetic repertoire. They are usually characterised by relatively large genomes, 5.51 to 7 Mbp, a huge percentage of strain-specific genes and an incredibly large pan-genome observed in the sequenced members of the group (Seaton and Silby, 2014). In fact, Garrido-Sanz et al. (2016) evaluated a total of 93 sequenced genomes within the *P. fluorescens* complex and found that only 1334 CDSs could be considered part of the core genome of the complex. This represented only about 23 % of the individual genomes gene content, which averaged 5,839 CDSs. On the other hand, the pan-genome of the complex was estimated as 30,848 CDSs, substantially bigger than others described for *P. aeruginosa* or *Escherichia coli*. All in all, the large size of the pan-genome and the reduced amount of core genomes are clearly a consequence of *P. fluorescens*' complex environment, colonisation versatility and adaptation ability (Garrido-Sanz et al., 2016, Garrido-Sanz et al., 2017).

One example of this versatility is found in the fact that *Pseudomonas* spp. are one of the most widespread colonisers of plant tissues. For example, Thiergart et al. studied the distribution of the microbial communities in *Arabidopsis thaliana* and co-occurring grasses across multiple sample sites in Europe. They found that a very small subset of conserved taxa was present in the plant roots around the whole continent, among which one *Pseudomonas* OTU was found as the most dominant and prevalent, providing evidence of the relevant role of *Pseudomonas* as a member of the plant-associated bacterial communities (Thiergart et al., 2020). *P. fluorescens* as a PGPR

One the most interesting features of the *P. fluorescens* complex is their ability to act as a PGPR thanks to their particular capacity to produce a huge variety of bioactive secondary metabolites, such as antibiotics, bacteriocins or siderophores (Haas and Defago, 2005, Seaton and Silby, 2014). The production of the phenolic compound 2,4-diacetylphloroglucinol (DAPG) is a good example of this trait. Not all the members of the fluorescent group are able to produce this metabolite and those carrying the gene cluster

responsible of the production of DAPG are generally called Phl$^+$ pseudomonads. DAPG is a key factor in the control of several fungal diseases, for example it causes membrane damage to *Pythium* spp. and inhibition of its zoospores (Haas and Defago, 2005, Loper et al., 2012).

One of the best studied examples is the prevalence of *Pseudomonas* populations in disease-suppressive soils. A study on *R. solani* showed that members of the *Pseudomonadaceae* family conferred protection against fungal infection (Mendes et al., 2011). Likewise, some other *Pseudomonas* spp. have been described as involved in the control of take-all, a fungal disease of cereals caused by *Gaeumannomyces graminis*. After successive cultivation of a particular cultivar of wheat, the abundance of Phl$^+$ pseudomonads densities increases, which results in a reduction of the disease severity known as take-all decline. The mechanism of action, although not uniquely, is linked to the production of DAPG. Either when a different cultivar is planted, or a different crop is included in the rotation, a negative effect on the population densities of Phl$^+$ pseudomonads is observed, impairing the suppression of take-all in subsequent years (Haas and Defago, 2005, Turner et al., 2013a, Mauchline et al., 2015).

In addition to natural product biosynthesis, other mechanisms related to *Pseudomonas'* ability as biocontrol agents are their efficient competitiveness, the capacity of some strains to interrupt the quorum sensing systems of pathogenic bacteria, and the production of phytohormones that directly enhance plant growth (Haas and Defago, 2005, Pieterse et al., 2014, Seaton and Silby, 2014).

In this study, *P. fluorescens* SBW25 will be used as a model organism, given its good colonisation ability of different plant species and the availability of several genetic manipulation resources. *P. fluorescens* SBW25 was originally isolated from sugar beet in 1989 in Oxfordshire (Bailey et al., 1995) and since then it has been utilised as a model to investigate plant-microbe interactions. For example, Silby et al. (2009b) carried out one of the first gene expression profiling experiments *in vivo* to study plant colonisation traits. By using IVET (*In vitro* expression technology) strategy, they characterised a diverse array of genes relevant for sugar beet colonisation that have provided the basis for several further studies within the plant-microbe interaction field (Mauchline et al., 2015, Little et al., 2019, Prudence et al., 2021).

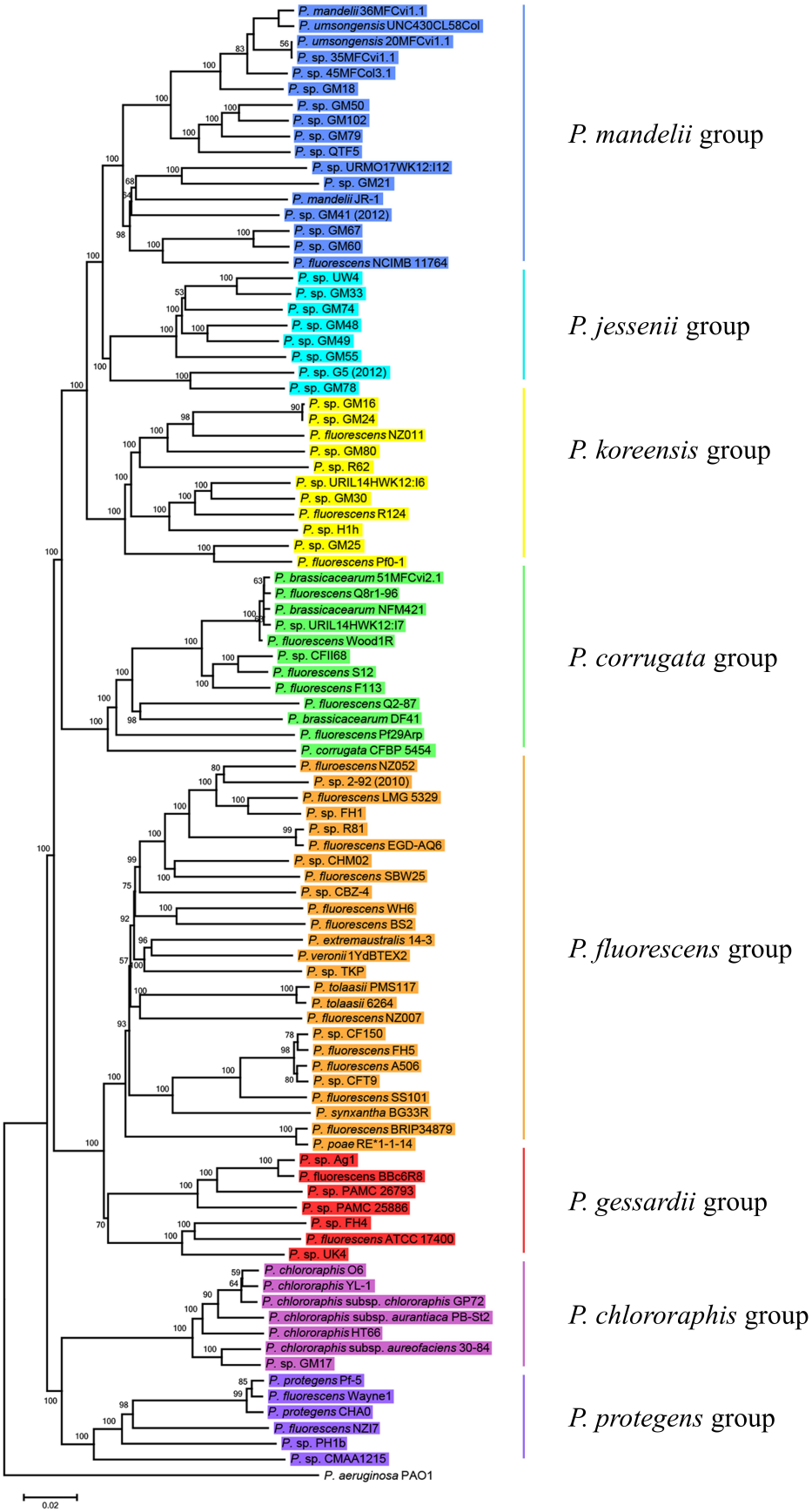

Figure 1.3. *P. fluorescens complex* phylogeny divided into the eight phylogenomic groups. Modified from Garrido-Sanz et al. (2016).

1.4 Barley (Hordeum vulgare L.)

Cultivated barley (*Hordeum vulgare* spp. *vulgare*) belongs to the *Poaceae* family and is the fourth most important annual cereal crop ($1.59 \cdot 10^8$ t) after maize ($1.15 \cdot 10^9$ t), wheat ($7.66 \cdot 10^8$ t) and rice ($7.55 \cdot 10^8$ t) (http://www.fao.org/faostat, 2020). Nowadays, it is mostly employed as feed for livestock, but it also used in the food and brewery industries (Gozukirmizi and Karlik, 2017). Moreover, barley is a temperate crop, well adapted to extreme environments where other cereals will not grow, acting as a main food crop or livestock feed and forage all over the world (Sato, 2020). Barley was domesticated very early, more than 10,000 years ago, and was considered a staple food for ancient civilisations in the Fertile Crescent, the area now occupied by Israel, Jordan, south Turkey, Iraqi Kurdistan and southwestern Iran. (Sato, 2020). Its importance in former times is also highlighted by the fact that Sumerian and Babylonian cultures used barley grains as currency (Gozukirmizi and Karlik, 2017).

The wild ancestor of barley, *H. vulgare* ssp. *spontaneum*, is also commonly found in the habitats defined as the Fertile Crescent, which supports the idea that at least one of the domestication centres of barley was indeed the Near East Fertile Crescent, probably along with some regions in Central Asia (Badr et al., 2000, Wang et al., 2015). This variety of habitats led to a huge genetic variation among different barley cultivars, opening the doors to the development of breeding programs to obtain new cultivars with higher productivity, enhanced quality grain and resistance to stresses, both biotic (diseases and pests) and abiotic (heat, drought or salinity) (Gozukirmizi and Karlik, 2017). As a consequence of this variability, more than 485,000 accessions of the genus *Hordeum* are kept in different institutions all over the world, with more than 299,000 corresponding to new and old cultivars, as well as landraces, of cultivated barley (Sato, 2020).

Cultivated barley is a self-pollinating diploid species with $2n = 2x = 14$. In 2012, its genome was sequenced which, together with its huge genetic diversity, has contributed to the use of barley as a model organism over the years in plant genetics studies of the Triticeae tribe. This tribe includes other important cereals with more complex genomes such as wheat or rye. The barley genome contains 5.1 Gb of DNA, with 26,159 ‘high-confidence’ genes, whose homology is supported from other plant genomes, and distributed in seven chromosomes (Gecheff, 1989, Mayer et al., 2012).

In the genus *Hordeum*, there are three spikelets (flowers) attached to the rachis of the spike. Based on its structure, barley can be divided into two main groups: two-row or six-row

varieties. In six-rowed barley, all three spikelets are fertile whereas in two-rowed types, only the central spikelet is fertile. As a consequence of this arrangement, the size and symmetry of the grain is different for the two types, in six-rowed barley only the median grains are symmetrical with a variable level of asymmetry on the lateral ones; on the contrary, grains in two-rowed cultivars are all uniformly symmetrical (Briggs, 1978). This in turn impacts their downstream use. In the brewery industry homogenous germination rates are needed, so the tendency is to use two-rowed cultivars for malting purposes, leaving the six-rowed ones for food or livestock feed (Ullrich, 2010). Depending on the sowing season, they can be further divided into spring or winter cultivars, which will impact the developmental period. Hence, a typical spring barley will require between 100 – 140 days to reach maturity, whereas a winter variety may take up to nine months to complete its cycle (Briggs, 1978).

The development of genomic tools as well as NGS technologies have facilitated the identification of the genetic basis of several agronomic traits in many crops, including barley. For example, multiple quantitative trait loci or QTL have been discovered for disease resistance against several pathogens, abiotic stress tolerance, malting quality, grain weight or plant development (Backes et al., 1995, Bezant et al., 1996, Angessa et al., 2017, Honsdorf et al., 2017, Goddard et al., 2019).

Over the last years, thanks to the great development experienced in the microbiome study field, many authors have started to elucidate the mechanisms underpinning the relationship between the plant host and its rhizosphere microbial community (Lundberg et al., 2012, Mendes et al., 2013, Song et al., 2021). In barley, the effect of plant genotype and domestication stage were investigated by Bulgarelli et al. (2015). In this work, they investigated the structure and functions of the bacterial microbiome on three barley genotypes: the wild ancestor *H. vulgare* ssp. *spontaneum*, a landrace and an elite cultivar. They concluded that the barley rhizosphere microbiome was dominated by three main families, Comamonadaceae, Flavobacteriaceae, and Rhizobiaceae in the tested conditions. Additionally, they could attribute a 5.7% of the overall variance in the microbiome composition to the host genotype alone, which partially would have its origin in the domestication stage of each of the three accessions tested. Lastly, they identified specific functions of the root-associated taxa that were enriched in the rhizosphere and the roots, such as siderophore production, regulation of virulence or sugar transport.

Within this scenario, the hypothesis that domestication and modern agricultural techniques could have also affected microbiota profiles seems highly plausible (Bouffaud et al., 2012, Bulgarelli et al., 2013, Bulgarelli et al., 2015). In fact, lower percentages of mycorrhizal

colonisation were observed in modern bread wheat cultivars in comparison to native grasses or diploid ancestors, which may demonstrate that high-yielding cultivars are less responsive to mycorrhizal colonisation (Hetrick et al., 1995). Furthermore, common agricultural practices, such as tillage, have been shown to impact the structure of barley rhizosphere microbial communities (Bziuk et al., 2021). In addition, a study on common bean (*Phaseolus vulgaris*) domestication history showed that wild and modern bean accessions differed in certain phenotypic traits, such as root length or nodule number. Root length was then proven to impact the relative abundance of specific bacterial taxa. Thus, as a consequence of the breeding process, Bacteroidetes decreased gradually, whereas Actinobacteria and Proteobacteria, increased. Taken all together, the authors were able to link domestication status, root morphology and rhizosphere microbiome structure (Pérez-Jaramillo et al., 2017).

In collaboration with my iCASE partner (New Heritage Barley Ltd, Norwich, UK) barley cultivars with distinct phenotypes, a different origin and a different state of domestication have been chosen in order to ensure the most diverse genetic pools (figure 1.4). By examining the composition of the rhizosphere microbiome and the colonisation differences in barley cultivars with distinct genotypes, the current knowledge about the genetic determinants controlling this process may be expanded.

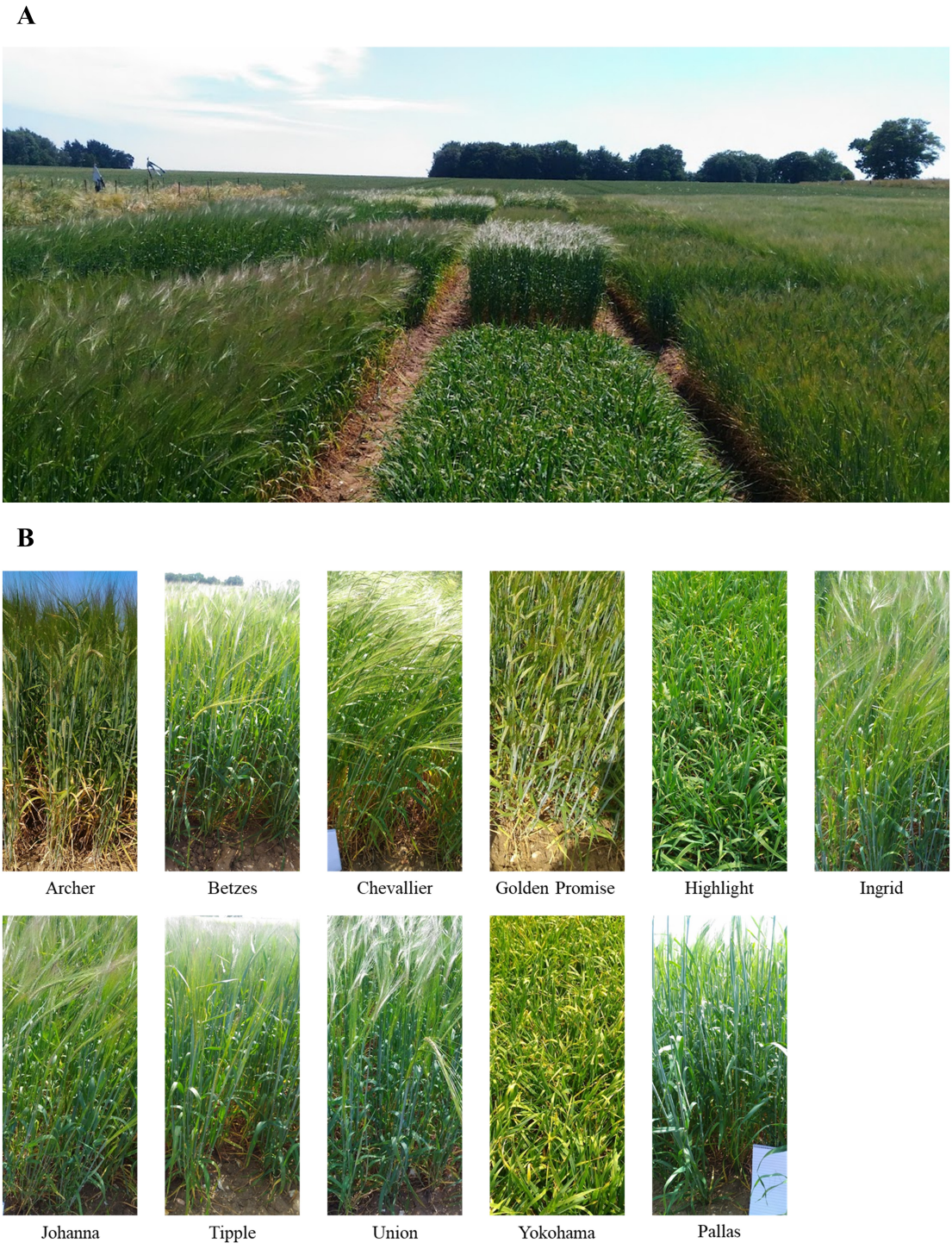

Figure 1.4. Barley diversity panel growing in the field at Church Farm, Bawburgh, Norwich. **A.** General view of the plots. **B.** Individual cultivars part of the diversity panel used in this study. Note that SusPtrit is not included here. Pictures taken by myself in June 2018. All the cultivars were photographed the same day. In the pictures the differences in height, developmental stage and size of the spike are noticeable.

1.5 Aims and objectives

The current climate crisis urges us to develop new ways of producing food to feed a growing global population in a more sustainable way. This process has been described above as sustainable intensification. In this project, the main aim is to identify genetic determinants that control the colonisation of barley roots by *P. fluorescens*. Ultimately, this information could be used to breed new barley cultivars with an enhanced capability to recruit beneficial microbial associations, which would contribute to increasing plant yield with fewer agrochemical inputs.

To investigate this process, four objectives were defined:

a) To examine the barley genotype effect on rhizosphere microbial community assembly. To do so, two different approaches were used: (1) a culture-dependant method in which the rhizosphere *Pseudomonas* populations from a barley diversity panel were examined and (2) a culture-independent characterisation by amplicon sequencing of the overall microbial community, bacteria and fungi, inhabiting the rhizosphere.

b) To identify putative genetic traits among the *Pseudomonas* populations selected in the rhizosphere environment as a consequence of adaptation to the plant host and to investigate the barley genotype effect on this process. With this aim, whole genome sequencing of the *Pseudomonas* rhizosphere collection and RNA-seq of *P. fluorescens* SBW25 under the influence of the distinct cultivars were employed.

c) To explore the root exudates composition of two distinct barley cultivars and their putative role in influencing rhizosphere microbial shaping. This was investigated *in vivo* by bacterial biosensors and *in vitro* by metabolomic analysis.

d) To map the potential root colonisation QTL by using the F7 progeny lines from a Chevallier x Tipple (CxT) crossing population and the understanding gained from previous objectives.

CHAPTER 2:

Material and methods

2.1 Plant material

A diversity panel of 12 different barley cultivars was used (Table 2.1). A F7 Chevallier x Tipple (CxT) mapping population of 188 lines was used to perform the QTL screening (Table 2.1). Seeds were surface disinfected with 70% ethanol for 1 min, 5% sodium hypochlorite for 2 min and several washes with sterile distilled water. If their germination rate was uneven, they were left overnight at 4 °C. All seeds were subsequently germinated in water-agar 1.5% for 48 h or until germination in darkness at room temperature (RT) before being taking forward for further use.

Table 2.1. Plant material used in this study

Cultivar	**Sow season**	**Origin**
Chevallier	Spring	United Kingdom
Tipple	Spring	United Kingdom
Golden Promise	Spring	United Kingdom
Highlight	Spring	Germany
Union	Spring	Germany
Yokohama	Spring	Japan
Johanna	Spring	Germany
Archer	Winter	United Kingdom
Betzes	Spring	Poland
SusPtrit	Spring	Experimental line (Atienza et al. 2004)
Ingrid	Spring	Sweden
Pallas	Spring	Sweden
Mapping population	**Source**	
F7 CxT	Dr Rachel Goddard and D**r** Paul Nicholson, JIC (Goddard et al., 2019)	

2.2 Bacterial strains and culture conditions

For routine experiments, *Pseudomonas* spp. were grown overnight at 28 °C with shaking in lysogenic broth (LB, tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) (Miller, 1972). For selective *Pseudomonas* isolation, plates made with *Pseudomonas* Agar Base supplemented with CFC (cetrimide/fucidin/cephalosporin) (Oxoid, UK) were prepared according to the manufacturer's instructions. When stated, *Pseudomonas* cultures were grown in M9 minimal media supplemented with the appropriate carbon source at a final concentration of 0.4% w/v (Miller, 1972) or King's medium B (KB, protease peptone 20 g/L, glycerol 10%, K_2HPO4 1.6 g/L). *Rhizobium leguminosarum* bv *viciae* strain 3841 lux (Rlv3841_*lux*) biosensors

(Pini et al., 2017) were grown overnight at 28 °C with shaking in tryptone-yeast (TY) (Beringer, 1974) or universal minimal salts (UMS) (Pini et al., 2017). UMS was supplemented with 30mM pyruvate and 10mM ammonium chloride unless specified otherwise. Antibiotics were added when necessary at the following concentrations: streptomycin (500 μg/ml) and tetracycline (2 μg/ml in UMS, 5 μg/ml in TY).

Escherichia coli was grown on LB at 37 °C, supplemented with tetracycline 12.5 μg/ml when necessary. *Streptomyces venezuelae* ATCC 10712 was grown for up to 5 days or until sporulation on maltose-yeast extract-malt extract medium (MYM, maltose 4 g/L, yeast extract 4 g/L, malt extract 10 g/L) prepared with 50% tap water and 50% reverse osmosis water and supplemented with R2 trace element solution at 1:500 (Bibb et al., 2012). When agar was necessary, it was added at a concentration of 1.5%, with the exception of MYM where 2% agar was used. All the strains used in this work are listed in table 2.2.

Table 2.2. Bacterial strains used in this thesis

Bacterial strains	Description	Reference
Escherichia coli		
E. coli DH5α	Strain used for cloning and plasmid storage	New England Biolabs
Pseudomonas fluorescens		
P. fluorescens SBW25	Environmental *P. fluorescens* isolate	(Rainey and Bailey, 1996)
P. fluorescens SBW25 Δ*rccR*	*rccR* (*PFLU_6073*) deletion strain	(Campilongo et al., 2017)
P. fluorescens SBW25 Δ*hexR*	*hexR* (*PFLU_4840*) deletion strain	(Campilongo et al., 2017)
P. fluorescens SBW25-*lacZ*	WT strain expressing *lacZ*	(Campilongo et al., 2017)
P. fluorescens SBW25 Δ*rccR-lacZ*	Mutant *rccR* background expressing *lacZ*	This work
P. fluorescens SBW25 Δ*gabP*	*gabP* (*PFLU_0315*) deletion strain	This work
P. fluorescens SBW25 Δ*vanR*	*vanR* (*PFLU_3295*) deletion strain	This work
P. fluorescens SBW25 Δ*2583*	*PFLU_2583* deletion strain	This work
P. fluorescens SBW25 Δ*5080*	*PFLU_5080* deletion strain	This work
P. fluorescens SBW25 Δ*6072*	*PFLU_6072* deletion strain	This work
P. fluorescens SBW25 Δ*dctA*	*dctA* (*PFLU_3500*) deletion strain	This work
P. fluorescens SBW25 Δ*1533*	*PFLU_1533* deletion strain	This work
P. fluorescens SBW25 Δ*2414*	*PFLU_2414* deletion strain	This work
P. fluorescens SBW25 Δ*3091*	*PFLU_3091* deletion strain	This work
P. fluorescens SBW25 Δ*4463*	*PFLU_4463* deletion strain	This work
Streptomyces		
S. venezuelae ATCC 10712	Laboratory strain	(Bibb et al., 2012)

***R. leguminosarum* bv viciae 3841 *lux* operon fusions (Rlv3841_*lux*)**		
LMB483	Phenylalanine biosensor	
LMB590	Xylose biosensor	
LMB592	*myo*-Inositol biosensor	
LMB593	Sucrose biosensor	
LMB608	Tartrate biosensor	
LMB610	Formate biosensor	
LMB613	Salicylic acid biosensor	(Pini et al., 2017)
LMB614	C4-dicarboxylates biosensor	
LMB617	Mannitol biosensor	
LMB619	Erythritol biosensor	
LMB638	Malonate biosensor	
LMB639	GABA biosensor	
LMB667	Fructose biosensor	
OPS0650	Proline biosensor	(Rubia et al., 2020)

2.3 Isolation of root-associated *Pseudomonas*

Barley seedlings were transplanted into 9 cm pots containing JIC Cereal Mix (40% Medium Grade Peat, 40% Sterilised Soil, 20% Horticultural Grit, 1.3kg/m³ PG Mix 14-16-18 +Te Base Fertiliser, 1kg/m³ Osmocote Mini 16-8-11 2mg + Te 0.02% B, Wetting Agent, 3kg/m³ Maglime, 300g/m³ Exemptor). Plants were grown for three weeks at 25°C in a controlled environment room (CER) with a 16 h light cycle and watered twice a week with sterile tap water. The rhizospheres of three independent plants from each cultivar were sampled individually. Gloves were used throughout the whole process which were disinfected with 70% ethanol between samples within the same cultivars and changed between different cultivars. Flame-sterile scissors were used to remove aerial parts from roots and excess of soil was eliminated by energetic shaking of the root system prior to transferring to sterile 50 ml tubes. Bulk soil samples of around 30 ml were taken from the centre of unplanted pots as a control. Each tube was filled up to 50 ml with sterile phosphate-buffered saline (PBS, per litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO4, 0.24 g KH_2PO4, pH 7.4) and vortexed at 4°C for 10 min. Dilution series were performed with sterile PBS and plated onto *Pseudomonas* selective medium (*Pseudomonas* agar base with CFC). Plates were incubated at 28°C until colonies arose and single colonies were randomly selected and transferred to fresh *Pseudomonas* agar base. Next, the isolates were streaked to single colonies on KB and preserved for further analysis. 20 isolates were randomly selected per individual plant, which composed a total of 60 isolates per cultivar studied.

2.4 Phenotypic characterisation of *Pseudomonas* spp.

Up to 60 strains isolated from each barley cultivar were characterised for polysaccharide and siderophore production, protease activity, motility, Hydrogen Cyanide (HCN) production and *S. venezuelae* suppression. Given the high number of isolates to be studied in this work, a high throughput screening protocol was developed. For that reason, 500 cm^2 square plates were used to test polysaccharide and siderophore production as well as for protease activity assays. 140 mm petri dishes were used to study *S. venezuelae* suppression and motility and 96-multiwell plates were employed to test HCN production. To transfer the overnight cultures of the rhizosphere isolates onto each plate, a microplate replicator was employed in each case. Siderophore production was studied by measuring the fluorescence emission of the isolates under UV light after 24-48 h of growing on King's medium B agar (KB) (King et al., 1954). To test polysaccharide differences, Congo Red 0.005% was added to KB agar and differences in colony pigmentation were assessed after 24-48 h. To address the variances in protease production, milk powder 1% was added to KB agar and absence or presence of a degradation halo was observed after 24-48 h. Motility was studied by observing the different spreading patterns on 0.5% agar KB plates after 8 and 24 h. Cyanogenic bacteria were detected following a method adapted from that described by Castric and Castric (1983) where 96-multiwell plates containing LB liquid cultures were used instead of standard petri dishes. Regarding *S. venezuelae* suppression, 200 µl of a 1:25 *Streptomyces* spore suspension was spread onto Difco™ Nutrient Agar (DNA) (Thermo Fisher, USA) and allowed to dry. Overnight *Pseudomonas* cultures were then placed on top using the replicator and growth development for both organisms was recorded after 3, 5, 7 and 10 days post-inoculation (dpi).

For each assay, ordinal values between 0 and 3 were assigned to each sample within each phenotypic assay, except for protease activity and motility assays where, for simplicity, only Yes or No values were assigned. A representation of the scale used for the assessment in each case is found in figure 2.1.

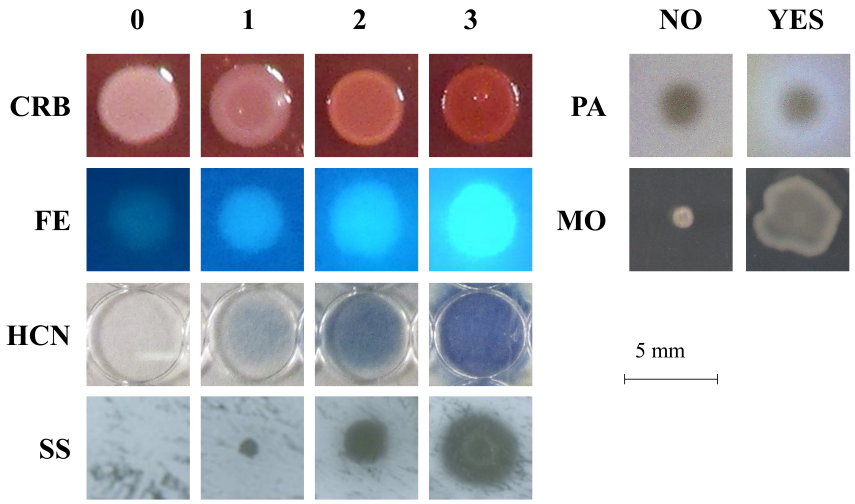

Figure 2.1. Scale used for scoring bacterial phenotypes: Congo Red binding (CRB), fluorescence emission (FE), Hydrogen Cyanide production (HCN), *Streptomyces* suppression (SS), protease activity (PA) and motility (MO).

2.5 *gyrB* genotyping of *Pseudomonas* spp.

Chevallier and Tipple rhizosphere isolates were selected for comparison by genotyping. The *gyrB* housekeeping gene was used to identify bacterial isolates, enabling resolution to the strain level. Briefly, colony PCR was performed using the primers described by Yamamoto and Harayama (1995) and PCR products were sent for Sanger sequencing using both forward and reverse primers (Eurofins Genomics, Germany). Sequence alignments were performed in MEGA X 10.1 (Kumar et al., 2018) and Geneious (Geneious Prime 2021.1.1, https://www.geneious.com) by MUSCLE (Edgar, 2004). Sequences were all trimmed at the maximum length of the shortest query, 816 bp, and subsequent phylogenetic analyses were carried out. MEGA X 10.1 (Kumar et al., 2018) and iTOL (Letunic and Bork, 2021) were used for the construction of the resulting tree, utilising the Maximum-Likelihood method and Tamura-Nei model with a bootstrap value of 1000.

2.6 Illumina® whole genome sequencing

A total of 42 *Pseudomonas* strains isolated from the rhizospheres of Tipple and Chevallier were sent for whole genome sequencing. 22 Chevallier-derived isolates and 20 from Tipple were selected according to three criteria i) their phylogenetic distribution to ensure maximum diversity, ii) their growth efficiency in different carbon sources and iii) random selection to minimise bias. Genomic DNA (gDNA) was extracted from overnight LB liquid cultures using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) following the manufacturer's instructions. The quality of the gDNA was evaluated by the 260:280 and

260:230 absorbance ratios using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA) and the quantity was measured with a Qubit® 2.0 Fluorometer (Thermo Scientific, USA). The concentration of the samples was normalised to 30 ng/µl and 20 µl of each sample were sent for genome sequencing to the Earlham Institute (Norwich Research Park, UK). In order to aim for 30x coverage, a LITE library was constructed and Illumina NovaSeq6000 SP with 150 paired-ends technology was used.

2.7 Reciprocal BLAST analysis

Dr Govind Chandra (Molecular Microbiology, John Innes Centre) performed the reciprocal analysis as follows. Genomes were assembled and a database of genes of interest screening was created. 410 *P. fluorescens* SBW25 genes of interest were selected based on a combination of previously published plant-induced genes -including transporters, biofilm formation regulators, chemotaxis proteins or siderophores (Silby et al., 2009a)-, and relevant substrate transporters and transcriptional regulators, traits shown to be key for adaptation to the rhizosphere environment (Compant et al., 2010, Campilongo et al., 2017). The reference genome, *P. fluorescens* SBW25, was individually BLAST-searched against all the other 42 genomes searching for the interesting genes and reciprocal BLAST hits were recorded. Finally, genomes were manually examined for the presence or absence of individual genes. Decisions were made based on a combination of sequence identity (> 66%), alignment coverage (> 66%) and whether or not the hits were reciprocal best hits. Candidate genes for further analyses were selected based on their significance level (Chi-Squared test, $p < 0.05$).

2.8 16S/ITS amplicon sequencing

Tipple and Chevallier barley plants were grown in JIC Cereal Mix (see 2.3) for three weeks at 25°C in a CER with a 16 h light cycle and watered twice a week with sterile tap water. Six plants were selected for rhizosphere and root endosphere gDNA extraction to ensure that at least five high-quality DNA extractions were achieved in each case. Bulk soil controls were maintained under the same conditions as the rest of the samples. Gloves were used throughout the whole process, which were disinfected with 70% ethanol between samples within the same cultivars and changed in between different cultivars. Flame-sterile scissors were used to remove aerial parts from roots and excess of soil was eliminated by energetic shaking of the root system prior transferring to sterile 50 ml tubes.

About 5 g of the rhizosphere-root sample were used in each case. Roots were covered with 10-20 ml of 0.1M KH_2PO_4 pH 8.0 buffer and placed in a shaker at 200 rpm for 30 minutes

at RT. This first suspension was kept and roots were transferred into a fresh 50 ml tube. Another 10-20 ml of buffer were added and this washing process of the roots was repeated a total of three times, every time keeping the supernatant separately (wash 1, wash 2 and wash 3). For the last step, 10-20 ml were added, and tubes were vortexed twice for 30 s, roots were removed and placed in a new tube and this last wash (wash 4) was collected. Wash 1 and wash 2 were pooled together and centrifuged for 10 min, at 29000 x g, RT. Supernatant was discharged, wash 3 and 4 were combined into this mix and a final centrifugation was carried out. The resulting pellet is considered here as the total rhizosphere. Washed roots are assumed to contained only closely associated epiphytes and endophytes.

gDNA extraction was performed using a FastDNA™ SPIN Kit for soil (MP Biomedicals, UK) according to the manufacturer's instructions. The quality of the gDNA was evaluated by the 260:280 and 260:230 absorbance ratios using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA) and the quantity was measured with a Qubit® 2.0 Fluorometer (Thermo Scientific, USA). The regions targeted were V4 for the bacterial 16S rRNA gene and ITS1-1F for fungal ITS. The samples were sent for PCR-free library preparation and sequencing to Novogene Co. Ltd. (Hong Kong, CN) using Illumina NovaSeq6000 with 250 paired-ends and 50000 reads.

The analysis of the amplicon sequencing data was done by Dr Anita Bollmann-Giolai (Department of Evolutionary Biology and Environmental Studies, University of Zurich) as described previously on the demultiplexed files supplied by Novogene with some modifications (Bollmann-Giolai et al., 2020). Briefly, on the demultiplexed files supplied with primers and sequence adapters already removed, fastp version 0.20.0 (Chen et al., 2018) was run on 16S as well as ITS data with disabled length filter and trim_poly_g to trim polyG read tails off. Those can occur due to the 2-colour system of NovaSeq data. Following that, the data quality was controlled by R-3.6.3 and DADA2 version 1.14.1 according to the workflow version 2 previously described by Callahan et al. (2016). The truncation length for forward reads was set to 200 bp and the truncation length for the reverse reads to 180 bp for 16S and 210 bp for ITS, respectively. For 16S and ITS libraries the following parameters were used: maxN=0, maxEE=c(2, 2) and truncQ=2 and a minimum length of 50 bp. and in both cases, forward and reverse reads were merged with default settings. Silva database (silva_nr_v132) was used to classify bacterial reads (Quast et al., 2012) and UNITE (sh_general_release_dynamic_s_01.12.2017), for fungal (Nilsson et al., 2019) and reads without a match in the databases used were removed. Alpha-diversity analysis was based on

Shannon index and Observed measure and was calculated on pre-normalised data (package "phyloseq," R-3.6.3, version 1.30.0). Regarding beta-diversity analysis, ASVs with a mean lower than 10-5 were ignored for subsequent analysis, and the filtered data of ASVs was used to calculate Bray-Curtis beta-diversity (R-3.6.3 "vegan" package, version 2.5.6). Statistical analyses were also performed on filtered data by using the package "vegan", ANOSIM and PERMANOVA: adonis function (Dixon, 2003). For data visualization ggplot2 (version 3.3.0) was used. Lastly, to test differentially present taxa between the two cultivars, a DESeq2 analysis was performed (Love et al., 2014) in R-3.6.3 using untransformed data (package 'DESEq2', version 1.26.0).

2.9 Bacterial growth assays

Barley rhizosphere isolates from Tipple and Chevallier cvs. were grown in minimal M9 media with acetate or glucose as a sole carbon source to test for differences in growth kinetics at the population level. Bacterial growth was monitored in a microplate spectrophotometer SPECTROstar Nano (BMG LABTECH Ltd., UK) or PowerWave (BioTek Instruments, USA). This assay was carried out in 96-multiwell plates containing 199 µl of M9 medium supplemented with the appropriate carbon source and 1 µl of overnight LB cultures was inoculated into each well using a multichannel pipette providing an initial OD_{600} of 0.01. Plates were incubated statically at 28 **°C** for 48 h. Each experiment was independently repeated at least twice. *P. fluorescens* SBW25 WT and mutants were grown overnight in triplicates in LB-medium with shaking at 28 °C. They were then inoculated in 200 µl of the media tested at a final OD_{600} of 0.01 and bacterial growth was measured in a microplate spectrophotometer as described above.

2.10 Bacterial genetic manipulation

Gene deletions were made by allelic exchange following a two-step homologous recombination process (Hmelo et al., 2015). To summarise, 500-700 bp homologous flanking regions of the target regions were either PCR-amplified and Gibson-assembled into the BamHI site of the suicide vector pTS1 (Scott et al., 2017) or synthetically designed by Twist Biosciences (Ca, USA). The pTS1 vector is an adaption of pME3087 (Voisard et al., 1994) containing a *sacB* gene which allows counter-selection on sucrose plates.

P. fluorescens SBW25 electrocompetent cells were prepared by growing the cells overnight and washing with 300 mM sucrose three times at RT. These cells were then electroporated at 2500 V with 100-300 ng of the gene deletion constructs. Cells were recovered in 3 ml of

LB and incubated for 2 h at 28 °C with shaking to enable expression of antibiotic resistance genes. Single crossovers were selected on 12.5 µg/ml tetracycline and re-streaked to obtain individual colonies. These were then grown overnight without selection in 50 ml of LB at 28 °C with shaking, then dilution series were plated on LB 10% sucrose to counter-select bacteria without a second homologous recombination event, leading to the loss of the *sacB*-containing plasmid. Individual *sacB*- colonies were subsequently picked and patched on LB plus or minus tetracycline to confirm double recombinants. Tetracycline sensitive colonies were PCR-checked with primers external to the deletion constructs to confirm gene deletion.

The *P. fluorescens* SBW25Δ*rccR-lacZ* deletion mutant was made in a SBW25-*lacZ* background, already available in our lab collection in order to the perform competitive colonisation assays. The deletion of the *rccR* in the *lacZ* background was done using the original pME3087 construct for *rccR* deletion published by our group in 2017 (Campilongo et al., 2017). Briefly, PCR products ligated into pME3087 between EcoRI-BamHI were transformed by electroporation into the target strain SBW25, and single crossovers were selected on 12.5 µg/ml tetracycline and re-streaked. Cultures from single crossovers were grown overnight in LB medium, then diluted 1:100 into fresh medium. After 2 hours, 5 µg/ml tetracycline was added to inhibit the growth of cells that had lost the tetracycline cassette. After a further hour of growth, samples were pelleted and re-suspended in fresh LB containing tetracycline and 2 µg/ml piperacillin and phosphomycin to kill growing bacteria. Cultures were grown for a further 4–6 hours, washed once with LB and dilution series were plated onto LB agar. Overnight individual colonies were patched onto LB plates plus and minus tetracycline, and Tet-sensitive colonies tested for gene deletion by colony PCR with external primers.

All the vectors and primers used in this work can be found in tables 2.3 and 2.4.

Table 2.3. Plasmids used in this study.

Plasmid	Description	Antibiotic Resistance	Reference
pTS1	Suicide plasmid, pME3087 derivative, *sacB*	Tet	(Scott et al., 2017)
pME3087_Δ*6073*	pME3087 suicide plasmid containing 500 bp flanking regions of *PFLU_6073*	Tet	(Campilongo et al., 2017)
pTS1_Δ*PFLU0315*	pTS1 based synthetic construct for deletion of *PFLU_0315*	Tet	This work
pTS1_Δ*PFLU3295*	pTS1 based synthetic construct for deletion of *PFLU_3295*	Tet	This work
pTS1_Δ*PFLU5080*	pTS1 based synthetic construct for deletion of *PFLU_5080*	Tet	This work
pTS1_Δ*PFLU6072*	pTS1 based synthetic construct for deletion of *PFLU_6072*	Tet	This work
pTS1_Δ*PFLU3500*	pTS1 based synthetic construct for deletion of *PFLU_3500*	Tet	This work
pTS1_Δ*PFLU1533*	pTS1 based synthetic construct for deletion of *PFLU_1533*	Tet	This work
pTS1_Δ*PFLU2414*	pTS1 based synthetic construct for deletion of *PFLU_2414*	Tet	This work

Table 2.4. Oligonucleotides used in this work.

ID	Name	Sequence (3'-5')	Description
1998	gyrB-UP1	CAYGCNGGNGGNAARTTYGA	To amplify *gyrB* gene as described by (Yamamoto and Harayama, 1995)
1999	gyrB-UP2R	CCRTCNACRTCNGCRTCNGTCAT	To amplify *gyrB* gene as described by (Yamamoto and Harayama, 1995)
136	PFlu6073UPOUT	CGGTGCACCAACTGCGTCAA	To confirm *PFLU_6073* deletion with outside primers
137	PFlu6073DNOUT	AACGCGTGCACTTCACCCAG	To confirm *PFLU_6073* deletion with outside primers
2000	KOPFLU3500_F	TCTTGGCCCGATTGCGCA	To confirm *PFLU_3500* deletion with outside primers
2001	KOPFLU3500_R	CTTCGCGCATGTCCATCA	To confirm *PFLU_3500* deletion with outside primers
2002	KOPFLU0315_F	TCATTGCGATCTTCGCTG	To confirm *PFLU_0315* deletion with outside primers
2003	KOPFLU0315_R	ATGGCCTCGCCGAACAAG	To confirm *PFLU_0315* deletion with outside primers
2004	KOPFLU3295_F	AAGACTGTCTGCACCGCG	To confirm *PFLU_3295* deletion with outside primers
2005	KOPFLU3295_R	TGCGTGACAGCCGTATCG	To confirm *PFLU_3295* deletion with outside primers
2006	KOPFLU5080_F	GGAGCAAAGAACCCAACC	To confirm *PFLU_5080* deletion with outside primers
2007	KOPFLU5080_R	TGAGCCGCTTTGTTCTCG	To confirm *PFLU_5080* deletion with outside primers
2008	KOPFLU6072_F	CCATGCGGCGGATGACTT	To confirm *PFLU_6072* deletion with outside primers
2009	KOPFLU6072_R	GTGCATGAAGACACCGAG	To confirm *PFLU_6072* deletion with outside primers

2010	KOPFLU1533_F	GTTTGAAACCACCGCGCG	To confirm *PFLU_1533* deletion with outside primers
2011	KOPFLU1533_R	GGGTTGGCCATCATGTCG	To confirm *PFLU_1533* deletion with outside primers
2012	KOPFLU2414_F	TTCAAGGTGCGTTTGCGC	To confirm *PFLU_2414* deletion with outside primers
2013	KOPFLU2414_R	ATCATGCTCATGGCGGTG	To confirm *PFLU_2414* deletion with outside primers
2054	up700_2583_F	ATTCGAGCTCGGTACCCGGG CCGCGTTTCAGGGTCAGTG	SBW25 deletion of *PFLU_2583*, for Gibson assembly into pTS1
2055	up700_2583_R	GCTACTTGAAACGGATCGGG GTCTTCATG	SBW25 deletion of *PFLU_2583*, for Gibson assembly into pTS1
2056	down700_2583_F	CCCGATCCGTTTCAAGTAGC CCTGACATAAC	SBW25 deletion of *PFLU_2583*, for Gibson assembly into pTS1
2057	down700_2583_R	CCTGCAGGTCGACTCTAGAG GACGATGCCTTTGCCCTG	SBW25 deletion of *PFLU_2583,* for Gibson assembly into pTS1
2058	KO2583_mutantcheckF	CTTGCAAAACAGCCAGGCCC	To confirm *PFLU_2583* deletion with outside primers
2059	KO2583_mutantcheckR	ATGGCAAACACTTCGTTCAT TTT	To confirm *PFLU_2583* deletion with outside primers
2064	up3091_F	ATCGATCCGAATTCGAGCTC GGTACCCGGGAAAACGCTC CACCTGTTGG	SBW25 deletion of *PFLU_3091*, for Gibson assembly into pTS1
2065	up3091_R	GGAGCCTTAAAGCTTCATCG GCACCGGTTAGCG	SBW25 deletion of *PFLU_3091*, for Gibson assembly into pTS1
2066	down3091_F	TAACCGGTGCCGATGAAGCT TTAAGGCTCCAGC	SBW25 deletion of *PFLU_3091*, for Gibson assembly into pTS1
2067	down3091_R	AGCTTGCATGCCTGCAGGTC GACTCTAGAGCAAGGGTTTG CGGTGTAG	SBW25 deletion of *PFLU_3091,* for Gibson assembly into pTS1
2072	up4463_F	ATCGATCCGAATTCGAGCTC GGTACCCGGGGACCCGCTG ATCGCCCAG	SBW25 deletion of *PFLU_4463*, for Gibson assembly into pTS1
2073	up4463_R	TGTAGATCACGCCGTGGGAG TCATTGCAGTCCTTG	SBW25 deletion of *PFLU_4463,* for Gibson assembly into pTS1
2074	down4463_F	ACTGCAATGACTCCCACGGC GTGATCTACACTTTAAAC	SBW25 deletion of *PFLU_4463*, for Gibson assembly into pTS1
2075	down4463_R	AGCTTGCATGCCTGCAGGTC GACTCTAGAGGACCCGCAG CGTGACCTG	SBW25 deletion of *PFLU_4463,* for Gibson assembly into pTS1
2076	KO4463_test_F	CAGTCGCCGCCTGCTCGA	To confirm *PFLU_4463* deletion with outside primers
2077	KO4463_test_R	ATCTGGGAGCGTAAGGCCG	To confirm *PFLU_4463* deletion with outside primers
2082	KO3091_test_F	GGCAATCATGAACGGCCAG T	To confirm *PFLU_3091* deletion with outside primers
2083	KO3091_test_R	AAGCCTTCGCCATACAGTGC	To confirm *PFLU_3091* deletion with outside primers

2.11 Root colonisation assays

Germinated seedlings were placed into sterile 50 ml plastic tubes containing washed, medium grain vermiculite and rooting solution (1 mM $CaCl_2 \cdot 2H_2O$, 100 µM KCl, 800 µM $MgSO_4$, 10 µM FeEDTA, 35 µM H_3BO_3, 9 µM $MnCl_2 \cdot 4H_2O$, 0.8 µM $ZnCl_2$, 0.5 µM $Na_2MoO_4 \cdot 2H_2O$, 0.3 µM $CuSO_4 \cdot 5H_2O$, 6 mM KNO_3, 18.4 mM KH_2PO_4, and 20 mM Na_2HPO_4). They were transferred to the CER at 25 °C with 16 h light cycle and grown for 7 days, after which time that they were inoculated with a 1:1 mix of $1x10^3$ CFU WT and mutant SBW25 strains. Barley plants were grown up to 5 days, after which shoots were removed and 20 ml of PBS were added to each tube and vortexed for 10 min at 4 °C to resuspend bacteria. Eight to ten plants per condition were collected and 100 µl of the PBS suspension were used to make dilution series which were then plated onto LB supplemented with 100 µg/ml carbenicillin, 0.1 mM IPTG and 50 µg/ml X-Gal. Plates were incubated at 28 °C until blue and white colonies were clearly distinguishable. Colony counting was undertaken and final ratios were calculated for all the mutants tested. Experiments were repeated at least twice independently.

2.12 Root exudates screening with Rlv3841_*lux* biosensors

To perform this analysis, the method described by Pini et al. (2017) was followed and adapted for optimal results in barley plants. Square 100 mm plates were filled in angle with 75 ml Fahraeus agar (FP) (Somasegaran and Hoben, 1994), a small square section was pierced on top of the plate allowing the growth of the barley seedling and the medium was covered with sterile filter paper upon which one seedling was placed. Biosensors were grown on UMA (UMS medium supplemented with 1.5% agar) slopes for 3 d at 28 °C, washed three times in UMS without any additions, and inoculated directly on the seedling root with 200-300µl of the Rlv3841_lux biosensor (Pini et al., 2017) suspension adjusted to an OD_{600} of 0.1. A second filter paper was applied on top of the inoculated seedling and the plates covered with aluminium foil were placed in the CER at 25 °C with 16 h light cycle until photographed. A NightOWL II LB 983 CCD camera-box (Berthold Technologies GmbH & Co., Germany) was used to take pictures of plates after 2, 5 and 7 dpi to determine the appropriate time point for further analyses. ImageJ software (Rueden et al., 2017) was employed to analyse the resulting pictures.

2.13 QTL screening for fructose secretion in barley

Fructose Rlv3841 biosensor was used to screen a subset of 110 individual lines from the CxT F_7 RIL population previously genotyped (Goddard et al., 2019) for fructose secretion into the rhizosphere. Lines were analysed in batches of 20 lines in triplicates, with the exception of the first 10 lines as they were used as a pilot experiment. To make comparisons possible across all lines and eliminate any sample variation effect, a normalisation step was performed. All values were normalised according to the following formula:

$$x_{normalised} = \frac{(x_i - \bar{x}_{10\%})}{Range}$$

Where $Range = \bar{x}_{90\%} - \bar{x}_{10\%}$, $\bar{x}_{10\%}$ is the 10th percentile average of the six batches, $\bar{x}_{90\%}$ the 90th percentile average of the six batches and x_i is the raw value.

Genstat 19th edition (VSN International Ltd., UK) was used for QTL analysis as described by Goddard et al. (2019). Briefly, single-trait single environment analysis was performed on the mean values of luminescence for all the lines studied and a LOD score of 3.0 was established to detect significant QTLs. Simple interval mapping (SIM) was used for initial QTL detection, followed by composite interval mapping (CIM) to finalise the QTL location using the detected candidate QTL as co-factors. A final QTL model was then fitted to produce the estimated QTL effects.

2.14 Cross-inoculation experiment

In order to assess how the specific rhizosphere consortium affects plant growth, a cross-inoculation experiment was performed with i) a rhizosphere extract of the two barley cultivars tested and ii) a synthetic community (SynCom) of the rhizosphere *Pseudomonas* spp. isolated above (see section 2.3) (Figure 2.2).

The rhizospheres of 3 weeks old barley plants grown in soil were extracted by pulling the plants from the pots and firmly shaking the roots. Shoots were removed and up to 5 g of roots were placed in 50 ml Falcon tubes, which were filled up with PBS buffer and vortexed for 10 min at 4 °C to resuspend bacteria. Roots were discarded and the remaining PBS was transferred to a larger container. The rhizosphere inoculum of each barley cultivar consisted of a pull-down of 4 plants. Heavier particles were removed, and the rhizosphere suspension was centrifuged at 2700 x g and 4 °C for 30 minutes, washed with PBS and the process was repeated three times. The final volume was adjusted so that sufficient inoculum could be

produced. Each seedling was inoculated with 5ml of the rhizosphere suspension. If long-term storage was required, glycerol was added at 40% final concentration and samples were flash frozen and stored at -80 °C.

The Chevallier and Tipple SynComs were created by mixing the 60 strains in a one to one ratio as described elsewhere (Bodenhausen et al., 2014) but with some modifications. Briefly, overnight cultures were adjusted using PBS instead of $MgCl_2$ to an OD_{600} of 0.2, mixed together in a 1:1 ratio, then the final mixture was re-adjusted to $OD_{600} = 0.2$. Seedlings were inoculated with 5 ml of the SynCom inocula.

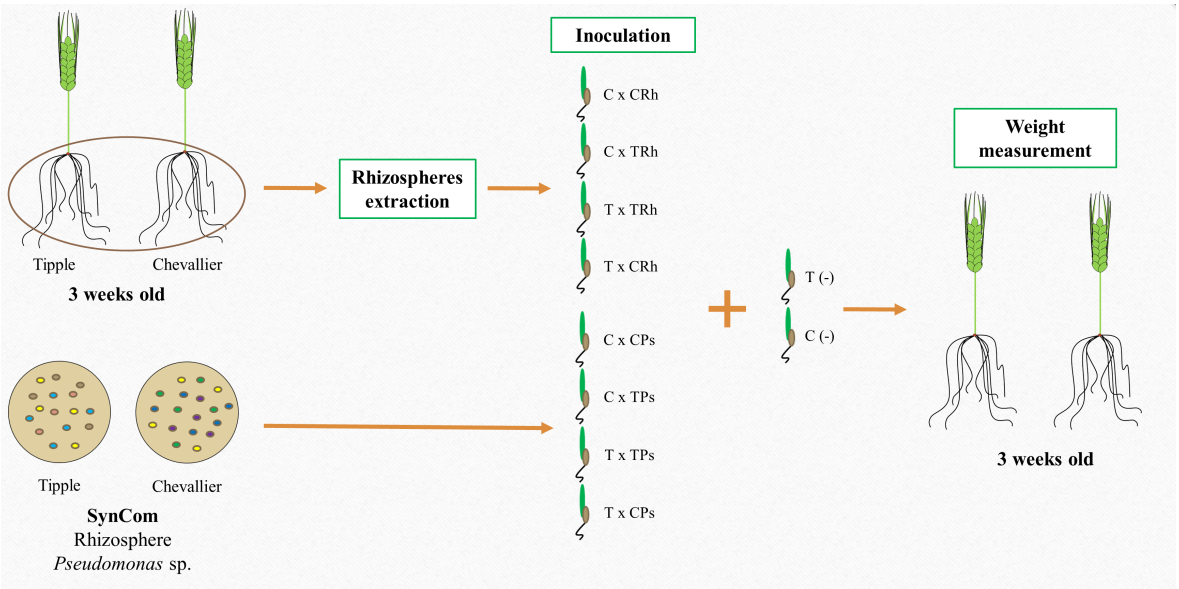

Figure 2.2. Schematic representation of the cross-inoculation experiment.

The whole experiment was performed under controlled conditions at 25 °C with 16 h light cycle in hydroponic systems using vermiculite and the same rooting solution as described above. Plants were placed in trays separated by treatment and watering took place from the bottom every two or three days, 200 ml during the first week and 500ml for the rest of the experiment. After three weeks, vermiculite was removed from the roots, plants were placed in an oven at 60 °C for up to six days and dry weight was recorded.

2.15 Root exudates extraction

A hydroponic system was created in order to grow barley plants for up to three weeks (figure 2.3). Two 50 ml plastic tubes were connected with muslin fabric and the bottom part was filled with rooting solution. The whole system was autoclaved to ensure sterility. Barley seedlings were transferred into the tubes and grown in a CER at 25 °C with 16 h light cycle for 3 weeks. Filter-sterilised fresh rooting solution was added once a week. The root exudates extraction was performed as described elsewhere (Zhalnina et al., 2018). Plants were removed from the tubes and roots were carefully washed with deionised water. Four plants were transferred into 200 ml of mili-Q water and incubated for 2 h in the same growing conditions. The liquid fractions were then freeze-dried and kept for further use.

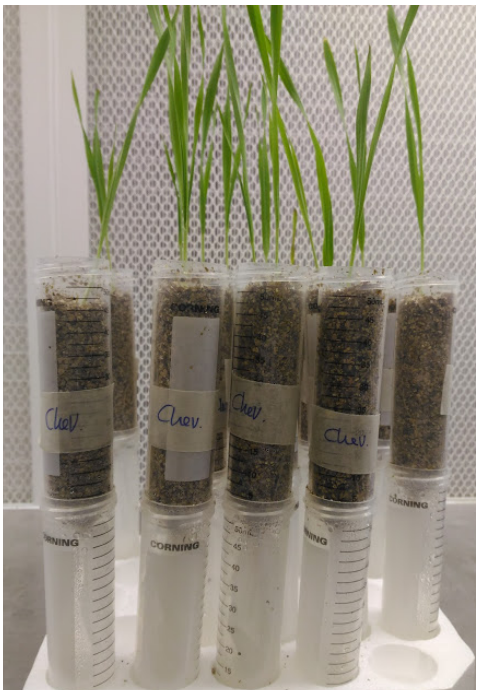

Figure 2.3. Hydroponic system for exudates collection.

2.16 Root exudates GC-MS analyses

Freeze-dried root exudates fractions were diluted with 80% methanol to a concentration of 10 µg/µl. Organic solvent was removed by placing samples on a GeneVac (SP Scientific) prior to being processed. Samples were analysed by using Agilent GC-MS Single Quad (7890/5977) plus Gerstel MPS (Agilent Technologies, USA). The MultiPurpose Sampler or MPS enables the automated derivatisation of the samples and their subsequent processing. The identification of the molecules was based on comparison with the metabolic libraries Agilent Fiehn 2013 GC-MS Metabolomics RTL (Agilent Technologies, USA) and NIST

(http://www.nist.gov) and the Agilent MassHunter Workstation package, particularly Qualitative, Unknowns and Mass Profiler Professional software were used for identification, analysis and data visualisation (Agilent Technologies, USA).

2.17 Rhizosphere RNA extraction and RNA-seq analysis

P. fluorescens SBW25 RNA was extracted from the rhizospheres of Chevallier and Tipple growing in axenic conditions as described above for the colonisation assays. 7 days old seedlings were inoculated with 1 ml of a PBS-washed *P. fluorescens* SBW25 culture adjusted to OD_{600} = 1.0 and RNA was extracted at 1 and 5 dpi. A control was also included represented as the adjusted culture before plant inoculation. For the control, 5 ml were taken and spun down for 5-10 min at 9000 x g and 4 °C. Pellets were flash-frozen and kept for later RNA extraction.

For the rhizosphere samples, up to 12 plants were combined to provide a sufficient RNA amount and three rhizosphere samples were extracted per time point. Aerial parts of the plants were removed, and 8 ml of PBS and 12 ml of RNA later were added. Root systems were vortexed for 10 min at 4 °C to resuspend bacteria and combined to create a single rhizosphere sample. Immediately, the mix was filtered through four layers of previously autoclaved muslin cloth placed in a sterile glass funnel and collected into sterile centrifuge bottles. The filtrate was centrifuged at 170 x g at 4 °C, the supernatant was transferred to a new bottle and the centrifugation step was repeated to remove any excess of vermiculite particles. The supernatant was transferred to a new bottle and centrifuged for 10 min 17000 x g and 4 °C. The obtained pellets were then flash-frozen for further use. To lyse the cells, 400 µl of 10mM Tris-Cl pH 8.0 and 700 µl of RLT buffer with β-mercaptoethanol (RNeasy® Mini kit, Qiagen, GE) were used to resuspend the pellet which was then transferred to Matrix B tubes (MP Biomedicals, UK). Two pulses of 30 s were performed in the FastPrep machine (MP Biomedicals, UK) with 90 s rest on ice in between. Samples were centrifuged for 15 min at 16000 x g and 4 °C, and the supernatant was used as instructed by the manufacturer (RNeasy® Mini kit, Qiagen, GE), including the on-column digestion with RNAse-Free DNase Set (Qiagen, GE). RNA quality and concentration were checked by a nanodrop spectrophotometer (Thermo Scientific, USA) and a Qubit® 2.0 Fluorometer (Thermo Scientific, USA). If concentrations were too high, a dilution step was carried out prior to a second DNase treatment by TURBO DNA-free™ Kit (Thermo Scientific, USA). One final concentration check was carried out as described before and RNA integrity was tested on a 1 % agarose gel.

Samples were sent to Novogene Co. Ltd. (Hong Kong, CN) for rRNA depletion, strand-specific library construction and sequencing on an Illumina Novaseq 6000. Data analysis were carried out by Dr Govind Chandra (Molecular Microbiology, John Innes Centre) as follows. A package named Subread (Liao et al., 2013) was used to align the reads in the fastq files to the reference genome, *P. fluorescens* SBW25. Then, BAM files were sorted and indexed using SAMtools (version 1.8) (Li et al., 2009). Mapping of the reads were counted from the BAM files using the featureCounts program, part of the Subread package, which resulted in a table of counts with one row for each gene and one column for each sample. The table of counts was used as the input to the R package edgeR (Chen et al., 2016) to test differential expression of each gene and a differential log fold changes table was produced.

CHAPTER 3:

Identifying differences between the rhizosphere *Pseudomonas* populations of the barley diversity panel

3.1 Introduction

3.1.1 Plant species as a factor determining microbiota assembly

The plant microbiome field has exploded over the last few years. Initial studies in the era of next-generation sequencing focused mainly on describing which bacterial taxa were prevalent in association with the plant and how this could affect certain traits, such as plant growth or disease resistance (Mendes et al., 2011, Philippot et al., 2013). Later on, the number of plant species whose microbiota had been characterised increased sufficiently that a general observation could be made: every plant species possesses a unique microbial signature that extends their capacity to adapt to the surrounding environment and enhances their ability to withstand biotic and abiotic stresses (Bulgarelli et al., 2013, Tkacz and Poole, 2015, Pfeilmeier et al., 2021). It is important to highlight that plant compartment also plays a fundamental role in the differentiation of the microbiota. Four main compartments have been previously defined (Bulgarelli et al., 2013, Muller et al., 2016): phyllosphere, leaf endosphere, rhizosphere and root endosphere. Each of these micro-habitats within the plant provides a specific niche to which microbes must adapt. This will also impact the final composition of the microbiota within the same plant species (Hacquard, 2016).

Many authors have illustrated the microbiome diversity of several plant species, and although the host species is not the only factor shaping plant-associated microbial communities, it has been shown to have a major influence (Bulgarelli et al., 2013, Tkacz et al., 2015). For example, Lundberg et al. (2012) showed how the *Arabidopsis thaliana* root associated microbiome was greatly influenced by the soil type but certain taxonomic groups, such as the family Streptomycetaceae and certain Proteobacteria families, were selected in all cases, especially in the endophytic root compartment (Lundberg et al., 2012). A later study by Tkacz et al. (2015) compared three model plants, *A. thaliana*, *Medicago truncatula and Brachypodium distachyon*, and three crops, *Brassica rapa*, *Pisum sativum* and *Triticum aestivum*, growing under different soil conditions. They demonstrated that the six plant species studied recruited very distinct bacterial and fungal rhizosphere communities, and also that soil composition is critical for stability and maintenance of microbial diversity. They also observed that *A. thaliana* exerted the weakest influence on its microbiota in comparison to the other five plant species studied (Tkacz et al., 2015).

3.1.2 Plant genotype effect on microbiome establishment

Besides the effect that the plant species has on microbiota assembly, it has been demonstrated that the plant genotype also plays an important role in this process. Different cultivars of the same crop species can differ in their resistance profile against certain phytopathogens, their root architecture, or their domestication level, among other agronomic traits (Bodenhausen et al., 2014, Schlaeppi and Bulgarelli, 2015, Muller et al., 2016).

In barley, Bulgarelli et al. (2015) investigated the differences not only between genotypes but also across different stages of crop domestication. They found that the host genotype influenced both root and rhizosphere microbiota, which manifests in variations of many OTUs (Operational Taxonomic Units) from diverse phyla. The total variation, at least at genus level, explained by the host genotype is only about 6%, although to what extent this has a major impact on plant health is yet to be determined. Another study, based on five commercial barley cultivars, showed that genotype was a significant, albeit not the only determinant in shaping the seed associated microbiome (Yang et al., 2017).

At this stage, the mechanisms underpinning the differential recruitment process in distinct host genotypes remain largely unknown. In *A. thaliana*, cuticle formation and ethylene signalling have been identified as potential plant determinants in shaping the phyllosphere microbiome (Bodenhausen et al., 2014). Some studies have also started to shed light on this phenomenon in the context of crop domestication, for instance, Kavamura et al. (2020) were able to show how the selection for dwarf wheat genotypes has indirectly modified the root structure which ultimately has influenced the recruitment capability of such cultivars.

Over the last few years, the hypothesis that microbial selection in different plant genotypes occurs at the level of bacterial strain has been increasingly accepted. Mauchline et al. (2015) studied the *Pseudomonas* diversity in take-all infected wheat fields over the course of two years and observed that wheat cultivar profoundly affects the *Pseudomonas* community composition as a whole, as well as the distribution of specific genetic traits important for wheat rhizosphere colonisation. In a very recent publication, it was shown how the colonisation of *A. thaliana* by specific beneficial bacteria, such as *P. fluorescens* is shaped at the strain level by plant genes. In this work, Song et al. (2021) described how a previously characterised receptor kinase, FERONIA (Guo et al., 2018), negatively modulates *P. fluorescens* colonisation by controlling the basal levels of reactive oxygen species (ROS).

Altogether, these findings provide the foundation towards engineering the plant microbiota, especially in the rhizosphere compartment since it acts as the specialising interface between the bulk soil communities and the plant. By understanding the mechanisms responsible for the recruitment of specific microbes, we can better exploit the microbial benefits for plant health and growth promotion under various biotic and abiotic stresses. This can be achieved in two main ways (1) creating personalised synthetic communities to serve specific purposes, e.g., biocontrol of certain phytopathogens; (2) identifying the plant determinants of selection and developing specific breeding programs to select for cultivars with an improved capability for the recruitment of beneficial microorganisms (Tkacz and Poole, 2015, Muller et al., 2016). .

In this context, this chapter focusses on two main goals. Firstly, I aim to characterise how barley genotype exerts a measurable effect on the microbial population under controlled conditions. And secondly, to show the effects of the plant genotype on the community composition of one particular bacterial genus, *Pseudomonas.* In order to that, I performed a culture-dependant approach to phenotypically screen root-associated *Pseudomonas* from 12 barley cultivars. This low-cost method provided a general picture of the phenotypic traits recruited by all the cultivars studied and allowed the selection of two interesting cultivars for subsequent analysis, Tipple and Chevallier. I then performed 16S and ITS amplicon analyses on the rhizosphere and root compartments of the two chosen cultivars to obtain the overall bacterial and fungal composition of the microbiome. Finally, I genetically characterised the *Pseudomonas* rhizosphere isolates from these two cultivars and performed a phylogenetic analysis based on the housekeeping gene *gyrB* to study the differences in the *Pseudomonas* community composition at a strain level driven by the plant genotype.

3.2 Results

3.2.1 Isolation of *Pseudomonas* strains from the barley rhizosphere

In order to investigate how barley genotype affects rhizosphere *Pseudomonas* spp. composition, the rhizospheres of 12 distinct barley cultivars were isolated and root associated pseudomonads were selected on *Pseudomonas* CFC Agar and taken forwards for further investigation (figure 3.1).

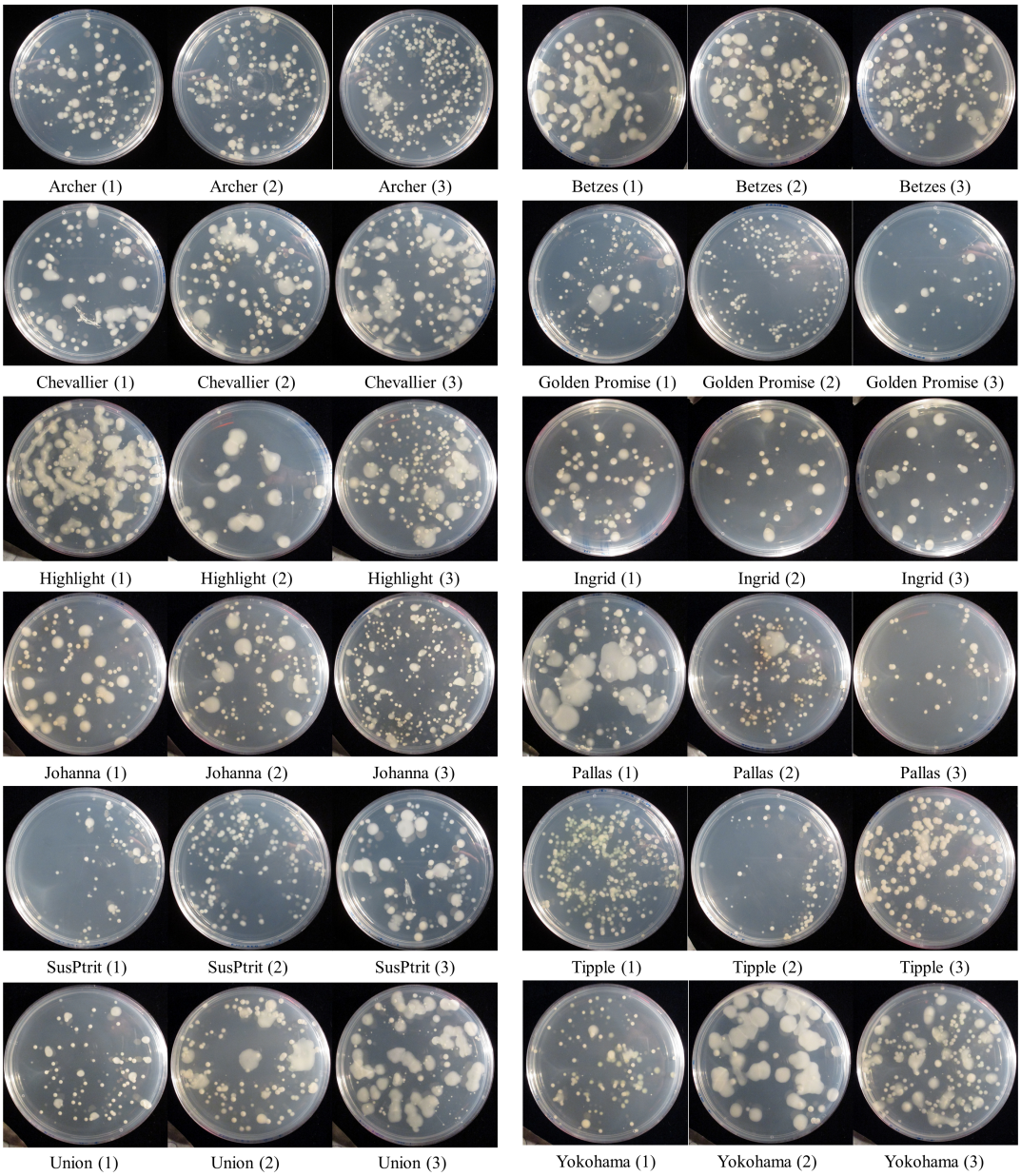

Figure 3.1. *Pseudomonas* spp. isolates obtained from the rhizospheres of a barley diversity panel of 12 different cultivars on *Pseudomonas* CFC Agar. Representative plates of three different plants are shown here as an example.

A total of 60 isolates was randomly selected per cultivar, with isolates taken from three different plants to try and minimise any individual plant effect. The isolates obtained from the three different plants were combined and treated as a representative population for the corresponding cultivar.

Substantial morphological differences were observed between *Pseudomonas* colonies isolated from different members of the barley diversity panel. For example, *Pseudomonas* spp. isolated from Tipple rhizospheres in general produced colonies with smaller and well-defined margins than those generally observed across the wider experiment. Isolates from Highlight rhizospheres produced large colonies that were markedly raised above the surface of the agar.

In general, bacterial morphology was quite consistent across the individual plants sampled for each cultivar. This is particularly evident in the case of isolates from Chevallier, Ingrid or Golden Promise. However, some expected morphological variations of the colonies could be observed from plant to plant within the same cultivar. For example, the isolates obtained from Pallas rhizosphere were, in general, small and with well-defined margins in plant 2 and 3, whereas a larger number of bigger colonies with viscous aspect was isolated in plant 1. Nevertheless, this is not entirely surprising since *Pseudomonas* have been shown to undergo a phenomenon known as phenotypic variation or phase variation. This phenomenon is defined as a reversible process during which a mixed bacterial population with different phenotypes is generated that is mediated by DNA mutations, reorganisations or modifications. Phase variation is very common among bacterial populations that inhabit complex environmental niches, and it facilitates rapid adaptation to sudden changes in the ecosystem (Sánchez-Contreras et al., 2002, Van Den Broek et al., 2005).

3.2.2 *Pseudomonas* strains isolated from barley rhizosphere were phenotypically different

To gain insight into the differences in the *Pseudomonas* community composition between the different barley cultivars, several phenotypic assays were carried out. In total, 733 isolates taken from 12 cultivars, plus a bulk-soil control set, were tested for polysaccharide and siderophore production, protease activity, motility, Hydrogen Cyanide (HCN) production and *S. venezuelae* suppression (Figure 3.2). These traits were used to characterise their morphological differences in order to investigate how the different cultivars shape their rhizosphere *Pseudomonas* communities and, to some extent, they might be used to assess

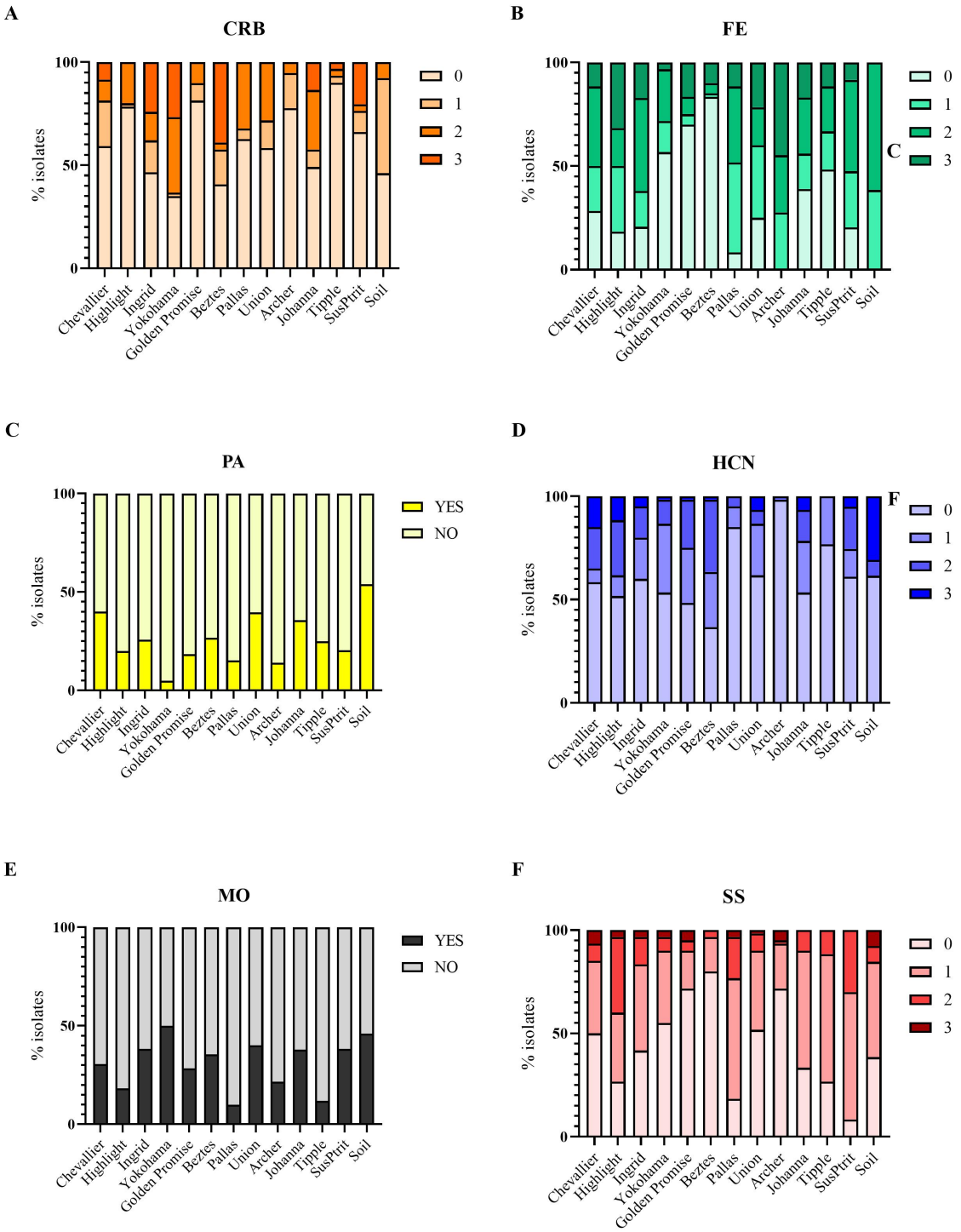

Figure 3.2 Phenotypic analyses of the *Pseudomonas* rhizosphere strains. **A.** Congo Red binding (CRB). **B.** Fluorescence emission (FE). **C.** Protease activity (PA). **D.** Hydrogen cyanide production (HCN). **E.** Motility (MO). **F.** *Streptomyces* suppression (SS). Data is shown as the total percentage *of isolates presenting* the score given over 60 isolates per cultivar, except for the control soil where only 13 isolates were obtained. Significant differences according to Chi-square test can be found in the supplementary table (Table S1).

the general diversity of soil pseudomonads (Mauchline et al., 2015). Each phenotype was scored on an ordinal scale between 0 (no phenotype observed) and 3 (strong phenotype) or as YES/NO as specified above (see Material and Methods section, figure 2.1).

Congo Red binding (CRB) was used here as a proxy for the level of biofilm forming ability of the isolates, as described previously (Mauchline et al., 2015). All the *Pseudomonas* spp. isolates were rated and are presented in figure 3.2A. Given the fact that in some cases differences between categories 2 and 3 were difficult to evaluate, they were considered together to establish three different groups of isolates: high binding (more than 25 % of the isolates bind Congo Red), medium (between 25 % and 15%) and low (less than 15%). The first group (high binding) included the isolates from cultivars Ingrid, Yokohama, Betzes, Pallas, Union and Johanna; the medium binding group consisted of those from Chevallier, Highlight and SusPtrit and the low binding group comprised Golden Promise, Archer, Tipple and the soil isolates.

Siderophore secretion was evaluated by examining colonies under UV light. The level of fluorescence (FE) emitted by the macrocolony is a direct proxy for siderophore secretion, as these molecules are UV fluorescent (Figure 3.2B). Since both 3 and 2 categories (described in material and methods, section 2.4) exhibited a strong FE phenotype sometimes difficult to distinguish, they were considered together to make comparisons simpler, and therefore two groups can be distinguished: high FE with more of 40% of fluorescent strains and low, with less than 40%. Cultivars showing high recruitment of siderophore-producers were Chevallier, Highlight, Ingrid, Pallas, Archer, Union, Johanna and SusPtrit. Yokohama, Golden Promise, Betzes, Tipple and the soil isolates had less than 40 % of fluorescent strains.

Protease activity (PA) of the isolates was analysed by the presence (YES) or the absence (NO) of a degradation halo on milk-KB plates (Figure 3.2C). This allowed me to identify those cultivars with a higher occurrence of protease positive isolates (more than 25%), such as Chevallier, Ingrid, Betzes, Union, Johanna, Tipple and the soil isolates; and those with less than 25%: Highlight, Yokohama, Golden Promise, Pallas, Archer and SusPtrit.

Hydrogen cyanine (HCN) producers were identified using the Feigl Anger colorimetric detection reagent (Castric and Castric, 1983) (Figure 3.2D). These isolates were scored from 0, no HCN being produced, to 3, HCN largely produced, as described in the materials and methods section (see figure 2.). After scoring, cultivars were classified as efficient HCN recruiters (more than 30%), decent (between 15 and 30%) and poor (less than 15%). Cultivars with a high incidence of HCN producers were Chevallier, Highlight, Beztes and

soil isolates. Those presenting intermediate levels were Ingrid, Golden Promise, Johanna and SusPtrit. Lastly, the lowest percentages of HCN^+ isolates were observed in Yokohama, Pallas, Union, Archer and Tipple.

Swarming motility (MO) was examined by observing the migration patterns on soft agar of the soil pseudomonads (Figure 3.2E), as described in the materials and methods section (see figure 2.1 for an example). Chevallier, Ingrid, Yokohama, Betzes, Union, Johanna, SusPtrit and bulk soil showed greater numbers of motile bacteria, more than 30% of the total isolates. On the other hand, Highlight, Golden Promise, Pallas, Archer and Tipple showed a lower motility profile, less than 30%.

To test the competitive ability of the rhizosphere isolates against other common soil dwelling bacteria, *S. venezuelae* suppression (SS) assays were conducted (Figure 3.2F). Isolates were evaluated at 10 dpi and scored as described in the materials and methods section 2.4. Only the Highlight-derived *Pseudomonas* populations was considered highly suppressive, with 40% of the isolates showing a strong inhibition phenotype. Chevallier, Ingrid, Pallas, SusPtrit and soil isolates contained intermediate number of suppressors, with between 15 and 40% of total isolates inhibiting *S. venezuelae*. And lastly, with less than 15% of the total isolates suppressing *S. venezuelae*, were Yokohama, Golden Promise, Betzes, Union, Archer, Johanna and Tipple.

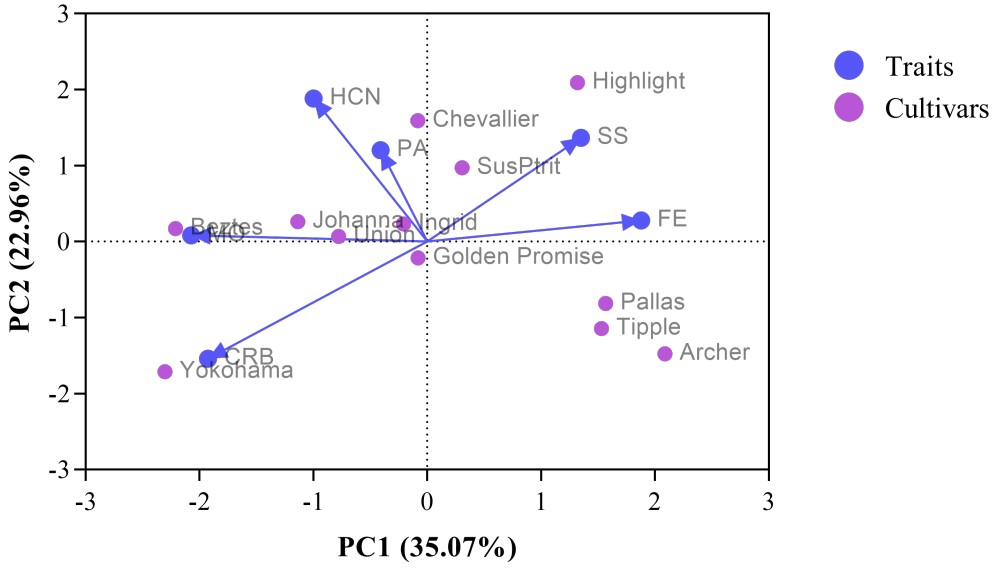

Figure 3.3. Principal components analysis including all the phenotypic traits examined in each barley cultivar. The two most significant components are shown, accounting together for the 58.03% of the total variance of the data. Congo Red binding (CRB). Fluorescence emission (FE). Protease activity (PA). Hydrogen cyanide production (HCN). Motility (MO). Streptomyces suppression (SS).

To study how the bacterial traits influenced the overall population composition, a principal components plot was created (Figure 3.3). These two components explain almost 60% of the total variance of the phenotypic dataset. High values of PC1 are associated with high values of FE and low values of CRB, MO. On the other hand, PC2 is mostly positively associated with HCN, SS and PA. Therefore, we would expect to find that cultivars with high percentages of FE do not efficiently recruit large numbers of CRB and MO positive isolates. Similarly, we could predict that traits such as HCN, SS and PA would co-occur within the *Pseudomonas* populations in the barley rhizosphere. By dissecting the cultivars based on their bacterial phenotypes, I was also able to observe clustering of cultivars and a total of four distinct groups were identified: (1) Pallas, Tipple and Archer; (2) Chevallier, SusPtrit and Highlight; (3) Johanna, Union, Ingrid and Golden Promise; and (4) formed by the two outliers Yokohama and Betzes.

Finally, in order to obtain a general profile of overall *Pseudomonas* populations isolated from the rhizospheres of the 12 diversity panel members, the phenotypic traits associated with the *Pseudomonas* isolates from each cultivar were represented by radial graphs (Figure 3.4). Each cultivar is represented here by the percentage of the isolates over the total population studied showing a strong phenotype for the trait studied, i.e., scored as 2 or 3 in the case of CRB, FE, HCN and SS; or as a YES in PA and MO. This creates a unique microbiological profile that is linked to each of the barley cultivars examined in this work. These microbial IDs provide a quick visualisation of the general *Pseudomonas* composition as well as a good approximation to determine what interesting bacterial traits might be present in the rhizosphere and how this relates to the plant genotype. It is interesting to highlight how extreme the differences between cultivars can be, such is the case for Chevallier and Tipple. Chevallier isolates showed higher percentages for PA, FE and MO, whereas Tipple isolates presented a much lower incidence for almost all the traits studied. These differences are not surprising given the fact that these cultivars have a markedly distinct history and genetic background, Chevallier is an English landrace cultivar first planted in 1820, whereas Tipple is a modern cultivar released in 2004 by Syngenta Seeds, Ltd (Goddard et al., 2019).

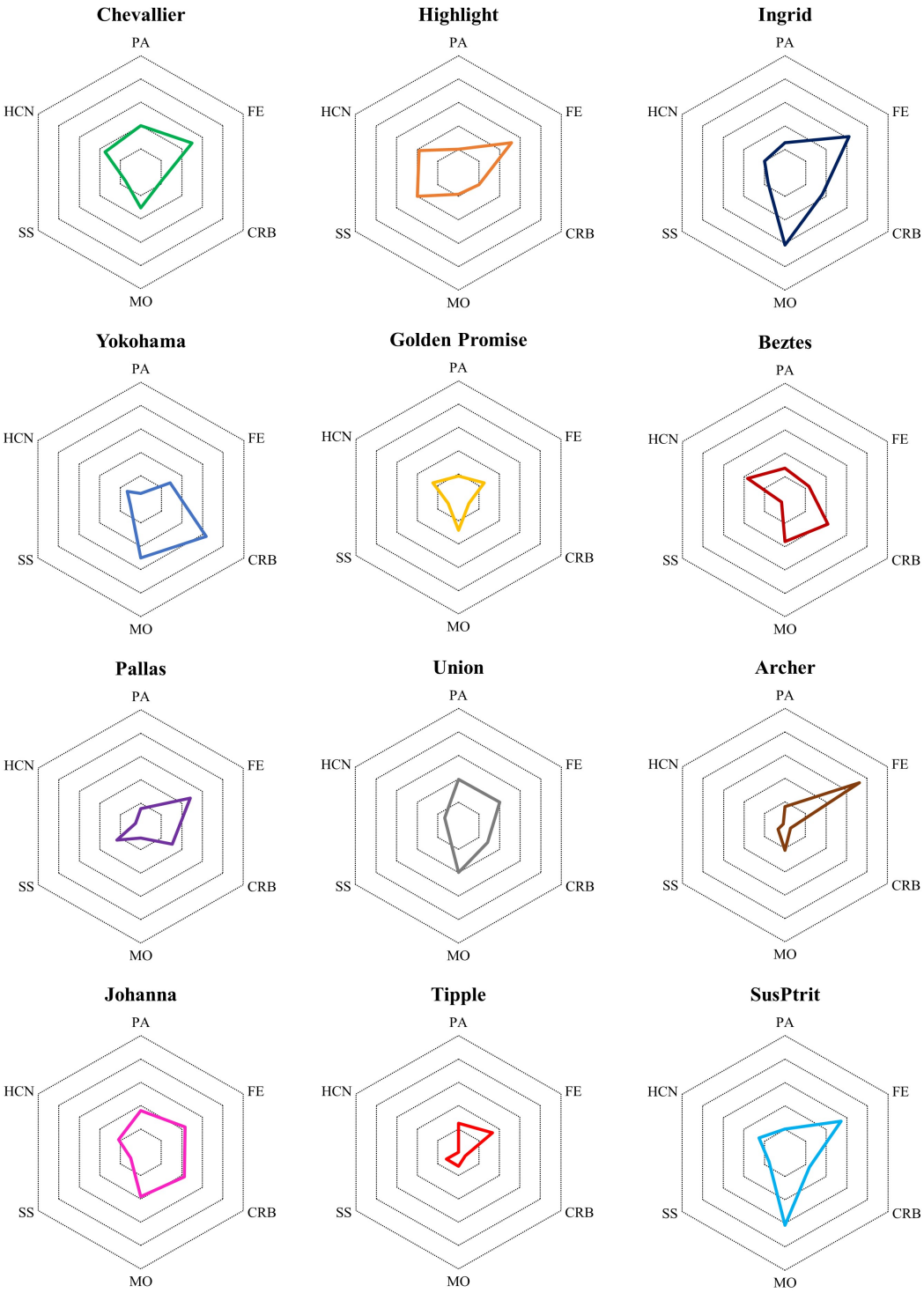

Figure 3.4. Microbial IDs of the diversity panel members. The radial graphs of each barely cultivar represent the percentage of isolates over the total rhizosphere *Pseudomonas* population presenting a strong phenotype, scored as 2 or 3, or as "YES", for the trait studied (HCN, SS, MO, CRB, FE or PA).

3.2.3 16S RNA gene and ITS amplicon sequencing

The phenotypic screening identified pronounced differences in the *Pseudomonas* populations seen for different members of the barley diversity panel. Given the marked differences shown between Chevallier and Tipple, their distinct origin, and the availability of genetic resources for downstream studies, these two barley cultivars were selected for further investigation of their overall fungal and bacterial microbiome composition in a culture-independent way. To do this, I performed amplicon sequencing based on the 16S rRNA gene to identify bacterial members of the microbial community and the internal transcribe spacer or ITS to capture fungal diversity of the two cultivars selected. Two plant compartments per cultivar, root endosphere and rhizosphere, along with a bulk soil, were studied and a total of five plants per cultivar were used. The subsequent sequencing data analysis was carried out with the support of Dr Anita Bollmann-Giolai (Department of Evolutionary Biology and Environmental Studies, University of Zurich).

First of all, the abundance of amplicon sequence variants (ASVs) was analysed. The preference of these over OTUs is due to their better resolution, down even to single-nucleotide differences (Callahan et al., 2017). Figure 3.5 shows a summary of the relative abundance of ASVs and a PCoA with Bray-Curtis distance matrix for both, bacterial and fungal barley associated communities. In figure 3.5A, the 30 most represented bacterial genera are shown as the relative abundance of ASVs, of which a 63.3 % were Proteobacteria, 26.7 % Actinobacteria, 6.7 % Acidobacteria and 3.3 % Bacteroidetes. The most immediate conclusion is the visual differences that can be observed between the bar charts. These differences were statistically confirmed, and found that both cultivars (adonis test, *p*-value 0.001) and the plant compartment (ANOSIM test, *p*-value 0.001) differed in their bacterial composition. In the bulk soil, the most abundant genera were *Rhodanobacter* (8.35 %), *Acidothermus* (6.82 %) and *Conexibacter* (5.11 %). Regarding Chevallier rhizosphere, *Massilia* (7.67 %), *Acidothermus* (5.65 %), Burkholderiaceae family members (5.53 %), *Rhodanobacter* (5.12%) and *Pseudomonas* (4.87 %), and were the most abundant genera; in the root compartment, the most representative genera were *Massilia* (17.03 %), *Pseudomonas* (7.15 %), members of the Burkholderiaceae family (7.04 %) and *Klebsiella* (6.49 %). In Tipple rhizosphere, *Pseudomonas* (7.55 %), *Massilia* (6.62 %), *Rhodanobacter* (5.13 %) and *Acidothermus* (4.76 %) presented the greatest relative abundances; with regard to Tipple roots endosphere, *Pseudomonas* (13.43 %), *Klebsiella* (8.82 %) and *Massilia* (8.36 %) were enriched in comparison to the other compartments.

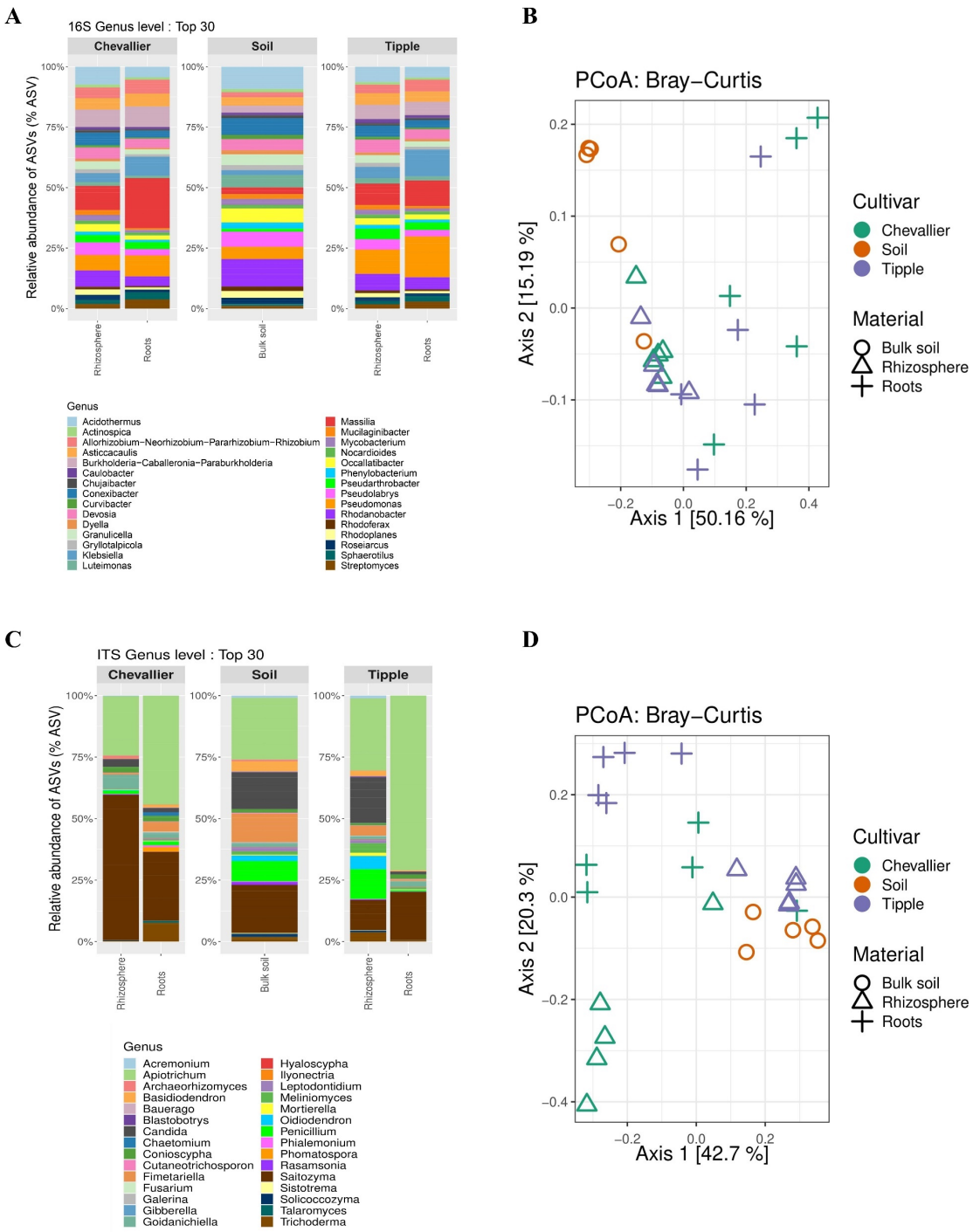

Figure 3.5. PCoA comparison of ASV abundances and relative abundance bar chart for bacterial and fungal communities of Chevallier, Tipple and bulk soil. **A.** Top 30 bacterial community composition. **B.** PCoA showing beta-diversity by Bray-Curtis distance for bacterial community composition. **C.** Top 30 fungal community composition. **D.** PCoA showing beta-diversity by Bray-Curtis distance for fungal community composition. Two plants compartment, root endosphere and rhizosphere, and the bulk soil were analysed. Five replicates were used per condition. Significant differences observed between the rhizospheres of the two cultivars and soil for both bacterial (adonis test, *p*-value 0.001) and fungal (ANOSIM test, *p*-value 0.005) communities, but also between the different compartments (bacterial ANOSIM test, *p*-value 0.001; fungal adonis test, *p*-value 0.002).

Then, the samples were analysed by PCoA based on Bray-Curtis distance as a measure of the variation of bacterial taxonomic profiles between samples (figure 3.5B). The first component accounted for about 50 % of the variance and although the samples clustered better by their origin, i.e., rhizosphere, root or bulk soil, there was also certain level of clustering according to host cultivar. In fact, when these differences were examined, the bacterial composition between cultivars was significantly different (adonis test, *p*-value 0.001) as well as between roots, rhizosphere and bulk soil (ANOSIM test, *p*-value 0.001). The differences in bacterial composition between plant compartments of the same and different cultivars were also examined. When they were compared within each cultivar, root endosphere and rhizosphere communities were statistically distinct for both Chevallier (ANOSIM test, *p*-value 0.01) and Tipple (ANOSIM test, *p*-value 0.01). On the other hand, when plant compartment was compared between cultivars, only the rhizosphere bacterial community was significantly different (adonis test, *p*-value 0.01).

In figure 3.5C, the bar chart represents the relative abundance of fungal ASV. Regardless the sample origin, bulk soil, rhizosphere or root endosphere, the most abundant genera were *Saitozyma* and *Apiotrichum* and together they accounted for up 90 % of the ASV abundance in some samples. In addition to these, in the Chevallier rhizosphere, *Goidanichiella* (5.78 %) was also quite abundant; whereas in the roots, *Trichoderma* (7.66 %) was the third most abundant genus after the common members. Regarding the bulk soil, *Candida* (14.78 %), *Fimetariella* (11.40 %) and *Penicillium* (8.17 %) were the most prevalent genera. Lastly, in Tipple rhizosphere a similar profile to the observed in the bulk soil was found, with high abundances of *Candida* (18.39 %) and *Penicillium* (12.01%); in the root endosphere, a dramatic reduction in the number of genera was observed being all practically overtaken by *Apiotrichum* and, to a lesser extent, *Saitozyma.* When Bray-Curtis beta-diversity between samples was studied, the first component was responsible for 42.7 % of the total variance and a clear clustering could be detected for host genotype, i.e., cultivar, and for origin of the material (figure 3.5D), with a tighter clustering between Tipple rhizosphere and bulk soil. Significant differences could be observed for both the host cultivar (ANOSIM test, *p*-value 0.005) and the material origin (adonis test, *p*-value 0.002).

To get a better understanding about the species richness present in each barley cultivar and plant compartment, alpha-diversity expressed as observed species richness, count of unique ASVs, and Shannon index, which considers the number of species but also inequality between species abundance, was calculated for both bacterial (figure 3.6) and fungal communities (figure 3.7). A first observation could be done immediately: the alpha-diversity

and therefore, effectively, the bacterial richness decreases from bulk soil to rhizosphere and lastly, to root endosphere which represent very visually the effect exerted by the plant on its microbial community. First of all, alpha-diversity for bacterial communities was measured between rhizosphere of both cultivars and bulk soil (figure 3.6A). It is interesting to highlight that there were no differences seen in species richness as evident in the observed index. In contrast, significant differences were shown by Shannon index between the rhizospheres of both cultivars and the bulk soil but not between the two cultivars. The rhizosphere compartment of Chevallier and Tipple was examined and as shown before, no differences in alpha-diversity were detected (figure 3.6B). However, when root endosphere was investigated, differences in the observed species richness were shown (ANOVA test, *p*-value 0.045), although not for Shannon index (figure 3.6C). Finally, the plant compartments were compared within each cultivar. Tipple presented a marked difference between the bacterial communities of the rhizosphere and the root endosphere as measured by Shannon index (Krustal test, *p*-value 0.009) but not for the observed richness (figure 3.6D). In the case of Chevallier, both observed richness (Krustal test, *p*-value 0.03) and Shannon index (Krustal test, *p*-value 0.009) were significantly different (figure 3.6E).

Next, I investigated the alpha diversity of the fungal consortia associated with Tipple, Chevallier and bulk soil (figure 3.7). Overall differences between fungal communities in the rhizosphere of the two cultivars and bulk soil were first examined (figure 3.7A). Alpha-diversities of the fungal population were significantly different between the Chevallier rhizosphere and soil for both observed richness (TukeyHSD test, *p*-value 9.6E-5) and Shannon index (Pairwise Wilcox test, *p*-value 0.024) and also between Chevallier and Tipple rhizospheres (observed, TukeyHSD test *p*-value 0.001; Shannon, Pairwise Wilcox test *p*-value 0.024) but not for Tipple in comparison to bulk soil. I then examined the differences in rhizosphere species richness between the two cultivars (figure 3.7B), where both indices were significantly distinct (observed, ANOVA test *p*-value 0.002; Shannon index, ANOVA test *p*-value 0.008). Root endosphere was also compared between cultivars and neither of the indices presented significant differences, although Shannon ANOVA *p*-value was almost significant, 0.056. Finally, the plant compartments were examined within each cultivar. Tipple fungal communities presented a very strong change in alpha-diversity between root population and rhizosphere populations for the two indices studied (observed, ANOVA test *p*-value 0.004; Shannon, ANOVA test *p*-value 0.0002) (figure 3.7D), whereas Chevallier did not present any significant differences (figure 3.7D).

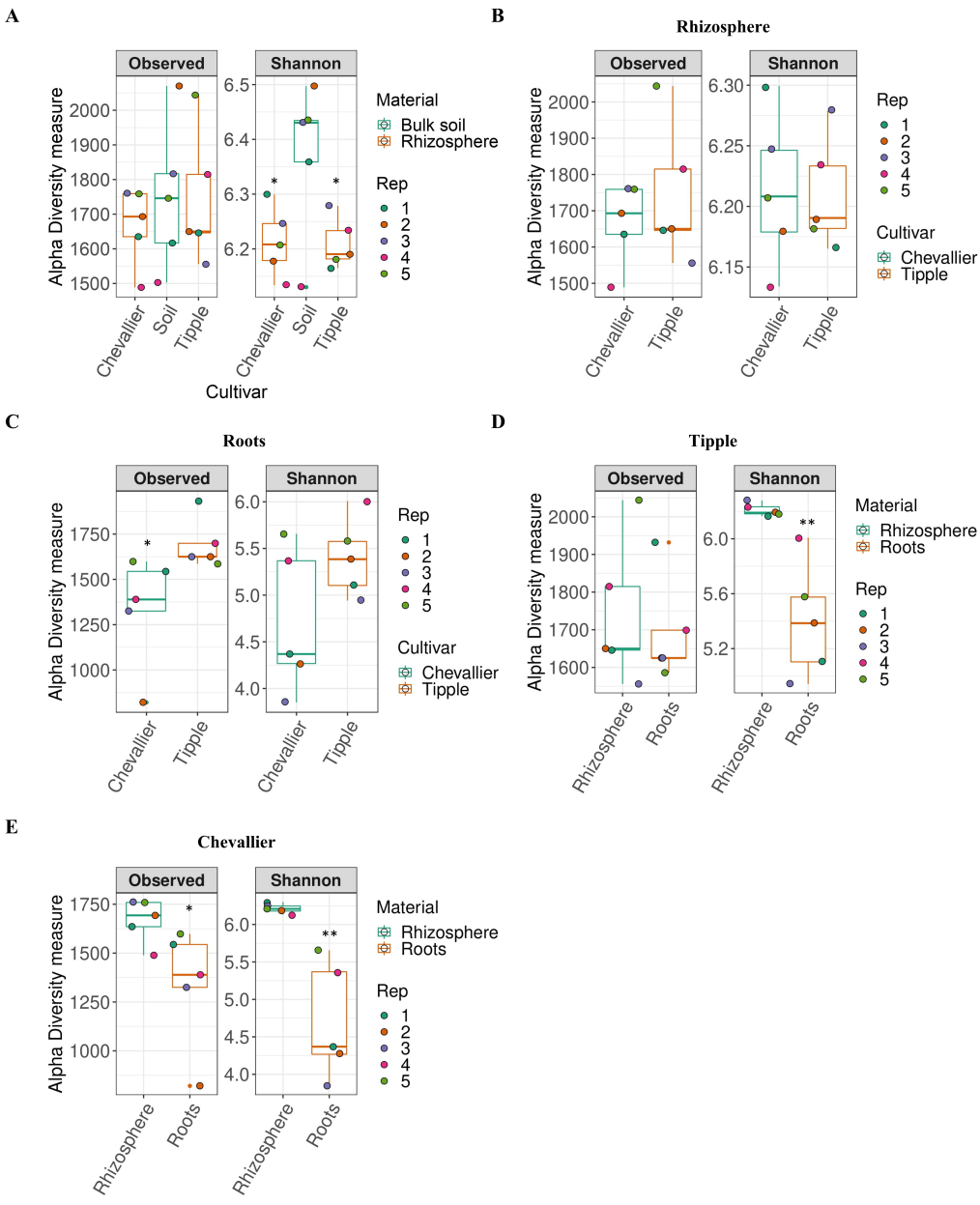

Figure 3.6. Alpha diversity comparison for bacterial communities measured by the observed richness and Shannon diversity. **A.** Overall comparison between the rhizosphere communities of Chevallier and Tipple and the bulk soil. Significant differences found for Shannon index between the cultivars and the bulk soil (TukeyHSD *p*-value Soil-Chevallier 0.05, Soil-Tipple 0.05), but not so between cultivars (TukeyHSD *p*-value 0.19). **B.** Rhizosphere communities comparison between Chevallier and Tipple. No significant differences were found (ANOVA *p*-value Observed 0.47, Shannon 0.92). **C.** Root endosphere communities comparison between Chevallier and Tipple. Differences observed in species richness (ANOVA *p*-value 0.045), but no for Shannon index (ANOVA test *p*-value 0.111). **D.** Comparison of community composition between Tipple compartments. Significant differences found for Shannon index (Krustal test *p*-value 0.009). **E.** Comparison of community composition between Chevallier compartments. Significant differences found for both observed (Krustal test *p*-value 0.03) and Shannon index (Krustal test *p*-value 0.009). Five replicates were used per condition. Asterisks indicate p < 0.05 (*), 0.01 (**) or 0.001(***).

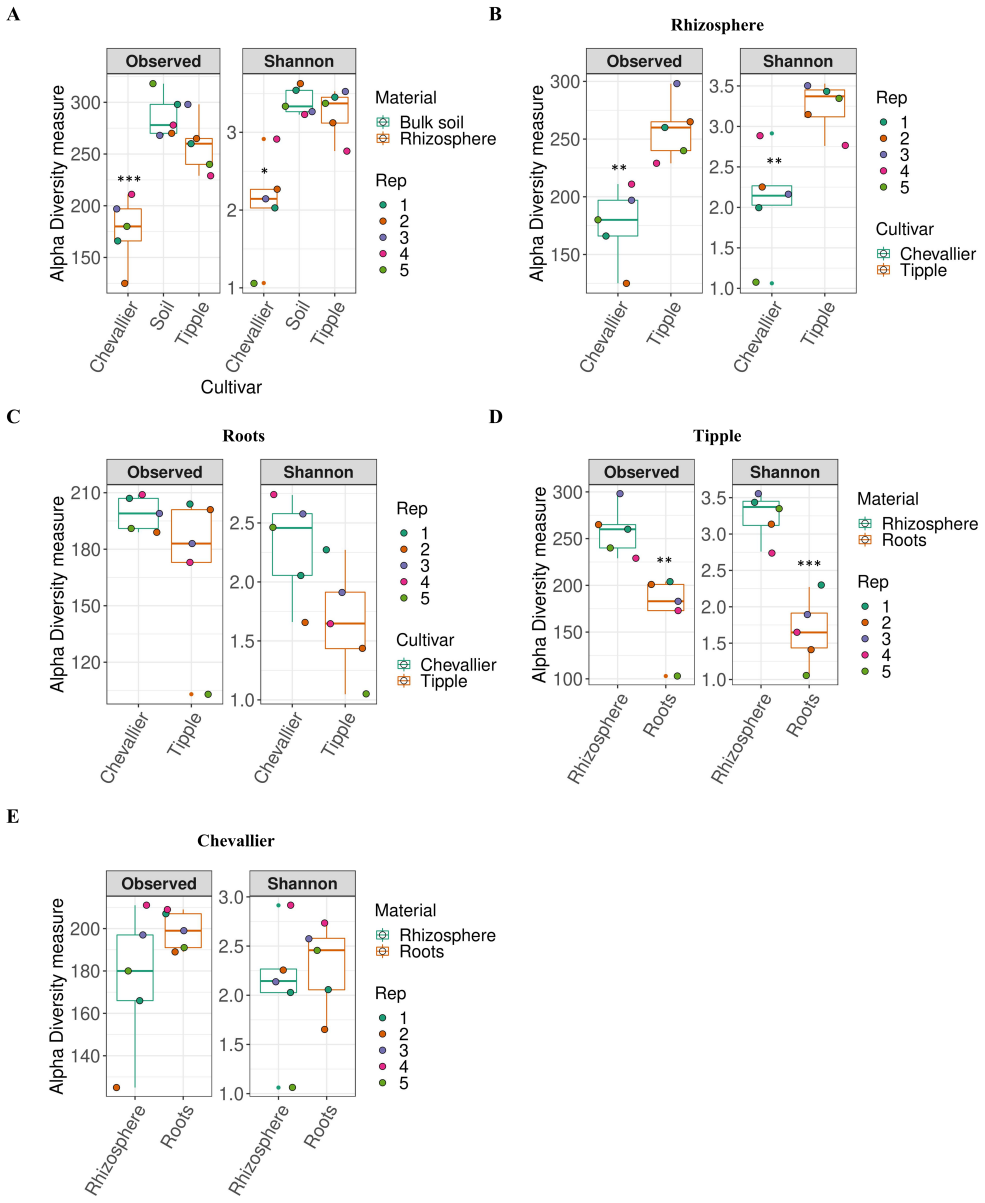

Figure 3.7. Alpha diversity comparison for fungal communities measured by the observed richness and Shannon diversity. **A.** Overall comparison between the rhizosphere communities of Chevallier and Tipple and the bulk soil. Significant differences for both indices, between cultivars and Chevallier and bulk soil (Observed TukeyHSD test p-value Soil-Chevallier 9.6E-5, Chevallier-Tipple 0.0012, Shannon Pairwise Wilcox test p-value Soil-Chevallier 0.024, Chevallier-Tipple 0.024) but not between Tipple and bulk soil (Observed TukeyHSD test p-value Soil-Tipple 0.55, Shannon Pairwise Wilcox test p-value Soil-Tipple 0.55). **B.** Rhizosphere communities comparison between Chevallier and Tipple. Significant differences for both observed (ANOVA test p-value 0.002) and Shannon index (ANOVA test p-value 0.008). **C.** Root endosphere communities comparison between Chevallier and Tipple. No significant differences were found. **D.** Comparison of community composition between Tipple compartments. Both indices were statistically different (observed, ANOVA test p-value 0.004; Shannon ANOVA test p-value 0.0002). **E.** Comparison of community composition between Chevallier compartments. No significant differences observed. Five replicates were used per condition. Asterisks indicate $p < 0.05$ (*), 0.01 (**) or 0.001(***).

All in all, three main conclusions can be inferred from these results: (1) plant compartment or ecological niche plays a fundamental role in bacterial and fungal community assembly and structure, (2) species richness tends to experience a decrease that is dependent on plant compartmentalisation and (3) barley genotype significantly affects the assembly of both bacterial and fungal plant interacting consortia.

In last place, differences in the abundance of certain ASVs between Chevallier and Tipple bacterial populations was investigated (figure 3.8). The two compartments, root endosphere and rhizosphere, were studied. In figure 3.8A the rhizosphere compartment microbiome of Chevallier was compared against Tipple's and a total of 11 ASVs were found to be differentially present between the two cultivars. 4 presented a log2-fold increase in Tipple, whereas 7 were found to be more prevalent in Chevallier. Next, the root endosphere was also assessed and here, only 2 ASVs were found to be differentially abundant between the two cultivars, one in each. However, it is necessary to remark that amplicon sequencing does not provide enough resolution to identify differences at species or strain level (Johnson et al., 2019). Therefore, sequencing of a whole housekeeping gene such as *gyrB* of the isolated rhizosphere strains would give an extra level of resolution.

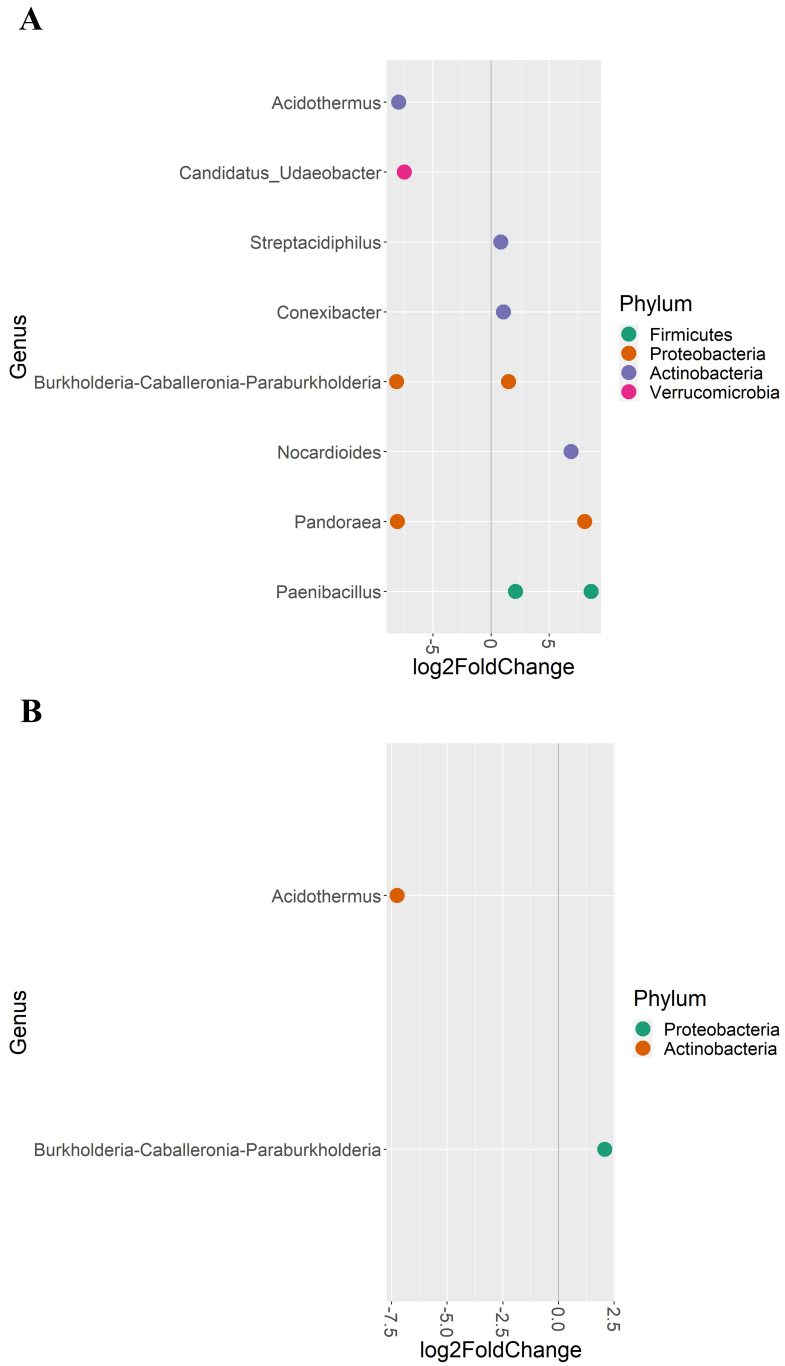

Figure 3.8. Log2-fold change of differentially present ASVs. **A.** In rhizosphere. **B.** In root endosphere. Positive values indicate ASVs more present in Chevallier in comparison to Tipple and negative, the opposite.

3.2.4 Genetic characterisation of Chevallier and Tipple isolates

By analysing the community structure using amplicon sequencing techniques, I could detect interesting differences between the cultivars Chevallier and Tipple, including some differences in *Pseudomonas* abundance. However, this technique usually does not provide enough resolution to capture bacterial strain diversity. Therefore, I decided to study this phenomenon in more detail by sequencing the housekeeping gene *gyrB* of the *Pseudomonas* strains. The genotyping of the strains was based on the sequencing of the DNA gyrase B subunit gene (*gyrB*), which was based on three main criteria previously published (Yamamoto and Harayama, 1995, Yamamoto and Harayama, 1998), (i) it is universally present in bacterial species, (ii) it shows an evolutionary rate higher than 16S rRNA, and lastly, (iii) it provides strain-level resolution.

In this analysis, two totally independent biological sets of bacterial isolates were included in order to maximise the robustness of the study. Therefore, a total of 239 Tipple and Chevallier isolates were selected and their *gyrB* genes were sequenced. 31 bulk soil isolates were randomly selected and included as well as the sequences of 15 relevant type strains. In figure 3.9 an unrooted phylogenetic tree of all the isolates included is shown and two very clear clusters are distinguishable: isolates that are related to the genus *Pseudomonas*, represented in turquoise, and those who belong to other genera, in yellow. In order to study these two groups in more detail, two independent subtrees were created (figure 3.10), both of which are coloured according to the origin of the isolates. The reference strains are in black, bulk soil isolates in purple, Tipple isolates in green and Chevallier strains in blue. The majority of the isolates can be identified as *Pseudomonas* spp. highly related to the *P. fluorescens* complex (figure 3.10A). This phylogenetic tree shows clear patterns of clustering that correspond to a particular plant genotype and interestingly, also according to the two sets of independent bacterial isolates studied, noted in the tree with the suffix a or b. The bulk soil isolates, rather than clustering all together, are spread across the sample. These two observations are not surprising given that the bulk soil acts as the source for the later assembly of the rhizosphere communities and therefore, it will determine the final composition of the *Pseudomonas* communities. 21 isolates were more closely related to other members of the Proteobacteria Phylum (figure 3.10B), such as the Gamma-proteobacteria *Stenotrophomonas rhizophila,* the Betaproteobacteria *Achromobacter xylosoxidans* and *Burkholderia anthina* or the Alphaproteobacteria *Ochrobactrum* sp. which resulted in an almost independent subtree of more phylogenetically distant isolates within the dataset. In

this subtree, the clustering was less evident although still observable in the case of the *S. rhizophila* group for the Chevallier isolates.

Finally, I investigated the overall diversity present in the *Pseudomonas* populations isolated from the rhizosphere of the two cultivars as well as in the bulk soil isolates. To do that, I computed the estimated divergence by Tamura-Nei model (Tamura and Nei, 1993) on the partial *gyrB* sequences alignment used for the construction of the phylogenetic tree. According to this, the general population had a distance index of 0.1371, the bulk soil isolates showed the greatest sequence divergence with a value of 0.1509, followed by Chevallier isolates, 0.1423, and lastly, Tipple with 0.1225. These results indicate that, as expected, the most diverse population is found in the bulk soil and after plant selection, the diversity in the rhizosphere consortia decreases as a consequence of the rhizosphere effect (Berendsen et al., 2012). Interestingly, Tipple *Pseudomonas* population exhibited the lowest diversity of the two cultivars, fact that correlates with the lowest diversity found in the phenotypes displayed by the isolates (figures 3.2 and 3.4)

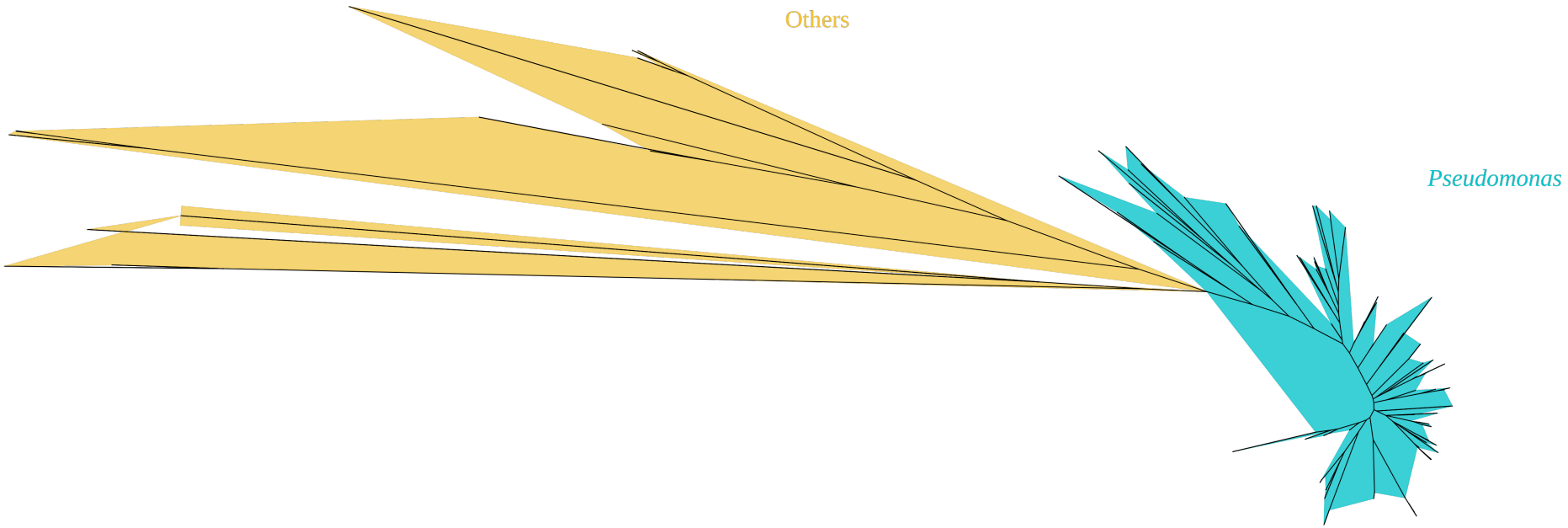

Figure 3.9. Unrooted phylogenetic tree showing the distribution of Chevallier and Tipple rhizosphere isolates. The phylogenetic tree is based on 285 partial sequences of the *gyrB* gene from sequenced and type strains and it was constructed by using the ML method and Tamura-Nei model with 1000 bootstrap value.

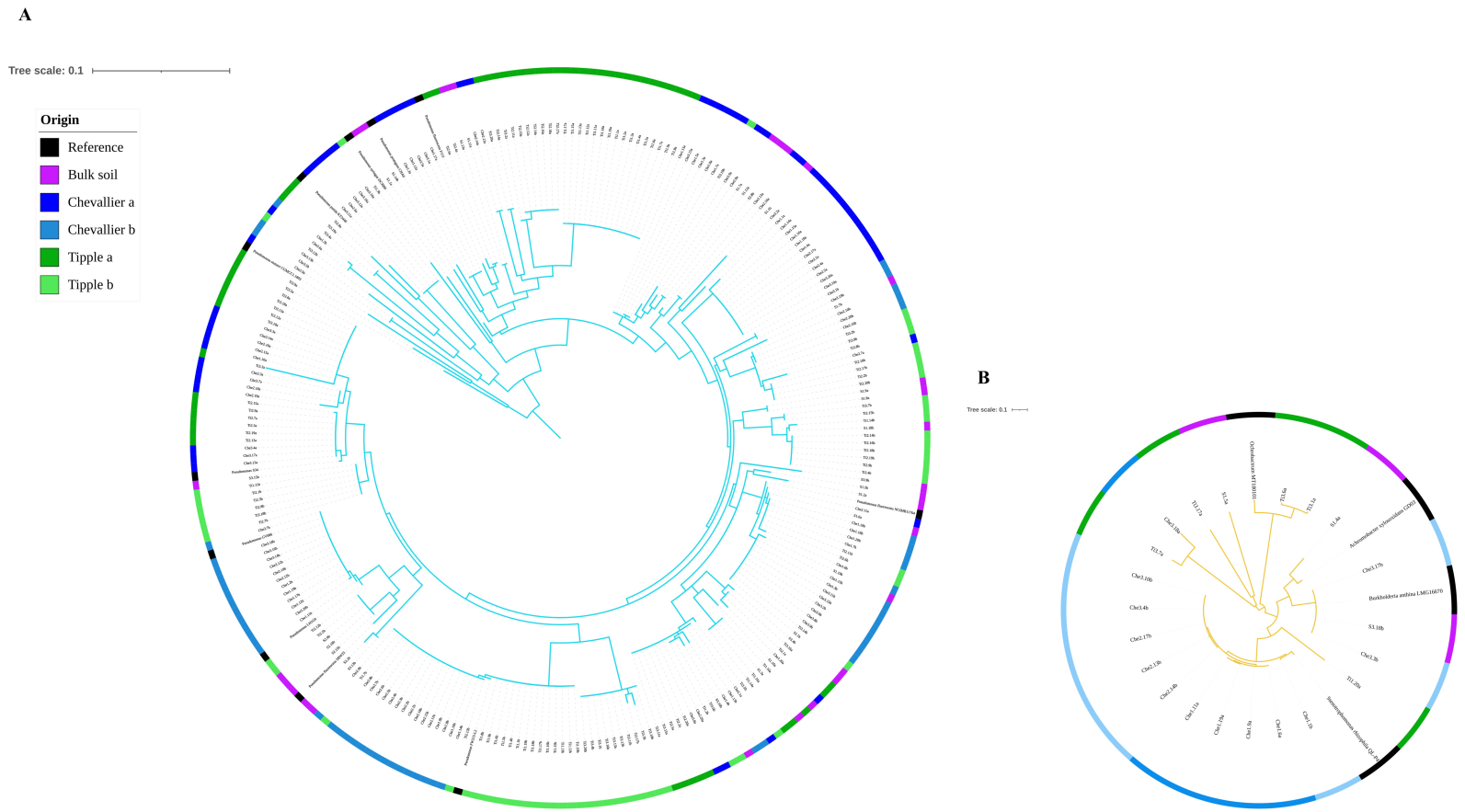

Figure 3.10. *gyrB* phylogeny of the Chevallier and Tipple rhizospheres isolates. **A.** Subtree based on the *Pseudomonas* group only**. B.** Subtree based on the other "rare" taxonomic groups. Two independent datasets, noted as suffixes a and b, were included for the analysis. The phylogenetic tree is based on 285 partial sequences of the *gyrB* gene from sequenced and type strains and it was constructed by using the ML method and Tamura-Nei model with 1000 bootstrap value.

3.3 Discussion

There have been several studies in recent years demonstrating how plants can actively shape their microbiota. This is known as the rhizosphere effect, a phenomenon responsible for the microbial enrichment in the proximities of growing roots when compared to the bulk soil communities (Lundberg et al., 2012, Turner et al., 2013a, Zhalnina et al., 2018, Huang et al., 2019). In this context, various authors have also investigated how the microbial recruitment process is influenced by differences in plant species and genotype (Bodenhausen et al., 2014, Bulgarelli et al., 2015, Muller et al., 2016, Liu et al., 2019).

In this chapter, I aimed to investigate the differences in the rhizospheric *Pseudomonas* communities associated with members of a barley diversity panel, selected to represent a maximal genetic pool, as it is composed mainly by early introduced elite cultivars and landrace cultivars. I then focussed on two specific cultivars to gain a deeper understanding of the microbial communities associated with distinct barley genotypes. I first created a strain collection of root-associated *Pseudomonas*, which were characterised phenotypically and genetically to analyse the differences observed among the cultivars studied. In addition, I applied a culture-independent method to explore the overall bacterial and fungal diversity of the cultivars studied.

The phenotypic characterisation of the strains resulted in a high throughput, fast and cheap general overview of the *Pseudomonas* composition of the different barley cultivars. Interestingly, no obvious clustering was observed according to crop domestication status by the approach used here. This method enabled the identification of specific traits that were enriched in the different rhizosphere communities studied as evident in the microbial IDs created in this study. For example, Chevallier presented a high incidence of siderophore and HCN producers, protease activity and motility whereas Tipple profile was in general quite restricted with only some remarkable numbers of siderophore producers. Given the genetic divergence that these two cultivars present, it was expected that their microbial profiles were distinct, since they present differences for some other agronomic traits such as resistance against pathogens -Fusarium Head Blight (FHB) or powdery mildew-, height or yield (Muhammed, 2012, Goddard et al., 2019). It is plausible to hypothesise that perhaps the resistance profile that Tipple shows against powdery mildew might be partially enhanced by the recruitment of siderophore producers. It has been previously reported that siderophores produced by fluorescent pseudomonads are one of the main determinants in the biocontrol of important pathogens such as *Pythium* sp. (Loper, 1988). The more diverse set of bacterial

phenotypes found in association with Chevallier might have its explanation in the origin of the cultivar. Chevallier is a landrace cultivar that has not been subjected to modern breeding techniques which have been shown to impact the recruitment capability of some crop species (Hetrick et al., 1995, Bouffaud et al., 2012), so potentially it retained the natural ability to sustain a more diverse set of microorganisms.

The phenotyping approach employed here has been used in previous studies (Ayyadurai et al., 2007, Loper et al., 2012, Mauchline et al., 2015) to identify biocontrol agents against phytopathogens, traits involved in the plant-microbe crosstalk process or effects of plant genotype on the incidence of a particular disease. In many studies, classic microbiological techniques and next-generation sequencing are combined in order to provide a more comprehensive picture of the microbial consortia inhabiting the rhizosphere (Bodenhausen et al., 2014, Mauchline et al., 2015, Pfeilmeier et al., 2021). Therefore, in this work a similar approach was taken. With this low-cost screening method, I was able to identify differences between two interesting cultivars that provided a good basis for genotypic and taxonomic characterisation of the associated microbial communities, and a starting point to assess the plant genotype influence on the rhizosphere microbiome.

Targeted amplicon sequencing of the 16S RNA gene and the internal transcribe spacer gave a general overview of the microbiome structure of the rhizosphere and the root endosphere of Chevallier and Tipple. On the one hand, by measuring the beta-diversity between bacterial populations of the two cultivars, differences in the structure of the communities could be detected. Moreover, these were significant for the overall community, between plant niches within the same cultivar and also for the rhizosphere compartment between the two cultivars. On the other hand, alpha-diversity provides information about species richness within the community studied. In that respect, differences in bacterial richness could not be detected between the two cultivars, meaning that similar number of distinct species in a well-structured community were recruited by the two cultivars, with the exception of the root compartment. In this case, Tipple presented a slightly higher observed bacterial richness. Given that the difference between rhizosphere and root compartment in Tipple was not significant for observed richness, i.e., that the number of species recruited by the plant in the rhizosphere and inside the roots is similar, this could explain why Tipple presented a higher observed richness than Chevallier. Regarding the fungal microbiome composition analysis, beta-diversity showed significant differences for the overall structure between cultivars, within compartments of the same cultivar and for the rhizosphere compartment. With reference to alpha-diversities of the fungal population, it is worth mention that Tipple did

not show differences with the bulk soil communities, which could translate as a weaker rhizosphere effect, and also that Chevallier did not show a significant change in neither the observed richness nor the Shannon index between plant niches, meaning that similar fungal species were recruited both in the rhizosphere and the endosphere of the roots. Previous studies have looked at the effect of plant cultivar on the microbial composition and found a variable significant effect mostly depending on the plant species. For example, a much more profound genotype effect has been found in olive rhizosphere (Fernández-González et al., 2019) than in barley (Bulgarelli et al., 2015). However, to what extend this variable contribution of genotype to microbial community affects plant health and fitness remains largely unknown. Differences between plant compartments have already been described as part of the root microbiota differentiation model suggested by Bulgarelli et al. (2013) in which a gradual specialisation of the root microbiota takes place from the surrounding bulk soil to the fine-tuning of the endophyte population. This is especially striking in the case of the fungal communities recruited by Tipple: only two main genera dominated the root compartment, *Saitozyma* and *Apiotrichum*, both basidiomycetous yeast commonly found in soil samples (Moreira and Vale, 2018, Kumla et al., 2020). It is also worth highlighting that the four bacterial genera influenced the most by this gradual specialisation process were *Rhodanobacter, Pseudomonas*, *Massilia* and *Klebsiella*. They experienced a dramatic shift in their abundance from the rhizosphere to the endosphere in both cultivars. *Rhodonobacter* have been found in the rhizosphere of several plant species and it displays an inhibitory effect against certain phytopathogens (De Clercq et al., 2006). Interestingly, in this work their abundance decreased quite drastically from the bulk soil to root endosphere in the two cultivars. The other three genera, *Pseudomonas*, *Massilia* and *Klebsiella*, experienced a huge increase in the root endosphere. They are all well-known members of the plant microbiota of several plant species (Ofek et al., 2012, Mauchline et al., 2015, Gupta et al., 2019), which explains their active and strong selection by the plant host. All in all, the findings of these analyses suggest that (1) barley cultivars Tipple and Chevallier indeed recruited distinct belowground microbial communities that can differ in both their diversity and richness and (2) plant compartment plays a fundamental role in microbial community assembly.

The genotyping of the strains was based on the sequencing of the *gyrB* gene as previously published (Yamamoto and Harayama, 1995, Yamamoto and Harayama, 1998). For this purpose, Tipple and Chevallier strains from two independent experiments were analysed and represented in a phylogenetic tree, together with bulk soil isolates as well as reference strains. I showed how the rhizosphere isolates clustered well according to barley cultivar, which demonstrates the effect that the barley cultivar exerts on the *Pseudomonas* communities.

This observation has been previously reported by some authors. Mauchline et al., for example, reported a clear split of *Pseudomonas* spp. between two wheat cultivars grown in adjacent field plots (Mauchline et al., 2015, Yang et al., 2017). Evidently, there was also a certain level of association between isolates of different origin i.e., between different cultivars and bulk soil. However, this is not surprising: plants from the same species are expected to share a core microbiome that is distinguishable from that of other plant species (Turner et al., 2013b, Bulgarelli et al., 2015, Muller et al., 2016). For example, Lei et al. showed that six plant species -*Ageratum conyzoides, Erigeron annuus, Bidens biternate, Artemisia argyi, Viola japonica* and *Euphorbia hirta*- shared about 74% of bacterial OTUs, and that Proteobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria were the predominant phyla. Interestingly, they also found a highly varied community composition and that some specific bacterial orders were enriched in the rhizosphere of the plant species studied (Lei et al., 2019). Another interesting result from my genotypic experiment was the identification of some "rare" taxonomic groups, such as *S. rhizophila* QL-P4*, A. xylosoxidans* GD03, *B. anthina* LMG16670 and *Ochrobactrum* sp. MT180101. The medium used in this work was selective for *Pseudomonas* isolation, however it is expected that certain members of closely related genera may also be captured by this method. In fact, the genera *Burkholderia* and *Pseudomonas* were considered to comprise a single genus until 1992 (Yabuuchi et al., 1992). The presence of these genera has been previously reported in the plant microbiome (Wolf et al., 2002, Jha and Kumar, 2009, Eberl and Vandamme, 2016, Yu et al., 2017). The best studied example is *Burkholderia* which comprises important plant pathogens as well as a vast number of commensal organisms. In last place, I analysed the sequence divergence of the rhizosphere isolates as a measure of their diversity and found that Chevallier associated *Pseudomonas* showed a higher diversity than those isolated from Tipple rhizosphere. This observation is in agreement with the lower phenotypic diversity displayed by the Tipple isolates. As already mentioned above, perhaps the differences in diversity observed could be due to breeding history of the two cultivars, since modern selection has been demonstrated to impact the root associated communities in some crops (Favela et al., 2021). Interestingly, according to the 16S amplicon sequencing data, the abundance of *Pseudomonas* in Tipple rhizosphere is slightly higher than in Chevallier (4.87 vs 7.55 %), but in light of the divergence results, it is reasonable to hypothesise that the Tipple rhizosphere community might be represented by higher numbers of more closely related strains than the Chevallier root associated microbiome.

This chapter has evidenced the effect that plant cultivar exerts on the soil microbial population. The differences observed could be detected at three different levels: the overall bacterial and fungal population, the genetic differences between strains of the *Pseudomonas* genus and also, the phenotypes that these rhizosphere *Pseudomonas* were able to express under the conditions used. All this points to the idea that community assembly in the plant microbiome occurs at several levels, including strain level and possibly, molecular level specialisation (Mauchline et al., 2015, Balsanelli et al., 2016). The mechanisms responsible for the differences observed will be treated in the next chapters of this thesis.

CHAPTER 4:

Genetic mechanisms of cultivar-specific barley rhizosphere colonisation by *P. fluorescens*

4.1 Introduction

4.1.1 Niche adaptation in beneficial bacteria: genetic determinants in rhizosphere colonisation

The rhizosphere and the roots are hotspots for microbial life as a consequence of the availability of plant-derived nutrients or root exudates. Given that, in general, soil nutrient content is quite low, access to nutrients exacerbates the competition between different microbial species for niche colonisation (Lugtenberg and Kamilova, 2009, Compant et al., 2010). In order to colonise belowground plant organs, bacteria have developed certain mechanisms to improve their competitiveness and to efficiently inhabit the rhizosphere (Zboralski and Filion, 2020). First of all, bacteria need to sense the presence of root exudates and move towards them by a mechanism known as chemotaxis. Chemotaxis is described as the movement towards environments containing higher concentrations of beneficial compounds or lower concentrations of toxic ones (Wadhams and Armitage, 2004, Compant et al., 2010). Root exudates are known to act as chemoattractants, and this can involve specific interactions during host-recognition processes given the variable composition found in root exudates. Root exudates composition can vary with plant species, cultivar, stress status, growth stage and even along the root itself (Lugtenberg and Kamilova, 2009, Compant et al., 2010). Therefore, effectively recognising available nutrients and moving towards them is the first step in rhizosphere colonisation. In *Pseudomonas* spp. three types of chemotaxis driven motility have been described: swimming, swarming and twitching, induced by a diverse array of molecules. The first two depend on flagellar rotations, whereas twitching relies on the extension/retraction movements of type IV pili (Sampedro et al., 2015).

Once in the proximity of the roots, the ability to attach to them is fundamental and for this, biofilm formation and its regulation play a key role. Biofilms are bacterial aggregates glued together by a polymeric matrix made of polysaccharides, secreted proteins and extracellular DNAs that offer protection against biotic and abiotic stresses, such as antibiotics, and provide better access to nutrients. In addition, biofilm formation ensures a critical cell density in a specific location to initiate beneficial or antagonistic interactions with host plants or other microbes, often regulated by quorum-sensing (Danhorn and Fuqua, 2007, Zboralski and Filion, 2020). Biofilm formation follows several steps: reversible attachment, micro-

colony formation and bacterial dispersal. Given the interconnection between flagellar motility and biofilm formation, both processes are usually tightly regulated together (Zboralski and Filion, 2020).

As already mentioned, beneficial bacterial colonisers have to be highly competitive in order to thrive in the rhizosphere. They possess several mechanisms to cope with their neighbours that include the synthesis of secondary metabolites with biocontrol properties against competitors such as antimicrobial compounds, lytic enzymes or siderophores (Compant et al., 2010).

Beneficial bacteria also have to evade the plant immune system, which identifies them as potential threats through the recognition of microbe-associated molecular patterns (MAMPs). This evasion prevents the activation of the plant pattern-triggered immunity (PTI) pathway. In many bacteria this is mediated by a decrease in flagella synthesis (Zboralski and Filion, 2020), as the flagellin peptide flg22 is a key activator of PTI through the receptor FLS2 (Pfeilmeier et al., 2016). In pathogenic *Pseudomonas* such as *P. syringae* pv. *tomato* DC3000, this can be achieved via the extracellular secretion of the alkaline protease AprA. AprA is able to cleave flagellin monomers, disrupting the active epitope flg22 and therefore, abolishing recognition by the plant receptor FLS2. *P. fluorescens* SBW25 also possesses homologues of this gene and therefore a similar role can be inferred in beneficial bacteria (Pel et al., 2014). Another mechanism by which beneficial bacteria can escape PTI is the allelic variation of their flagellin epitopes. In fact, in a recent study by Colaianni et al. (2021), they found that plant-associated communities are enriched in immune evading flg22 epitopes that can result in either receptor antagonism or modulation of the downstream plant immune response. Besides theses mechanisms, in rhizobia-legume interaction, extracellular polysaccharides have been shown to also play an important role in modulating plant host defences for symbiosis establishment (Yu et al., 2019). Such is the case of the exopolysaccharides (EPS) produced by *Sinorhizobium meliloti*. *S. meliloti* EPS are able to chelate calcium ions, block the flg-22-induced calcium spikes and therefore, suppress downstream immune responses, ultimately leading to successful nodulation in *M. truncatula* (Aslam et al., 2008).

Finally, metabolic versatility is also an important trait for efficient rhizosphere and root colonisation. Root exudates offer to the microbial communities inhabiting the rhizosphere a plethora of compounds to grow on, such as sugars, organic acids, fatty acids, amino acids and proteins. However not all the members of the microbiome will uptake and utilise all the

compounds available as efficiently. Therefore, having a diverse metabolic capacity might result in better proliferation and thus, more competitiveness (Lugtenberg and Kamilova, 2009). This is especially true in *Pseudomonas*, a genus capable of colonising a wide variety of different environments in which their versatility is reflected in the large numbers of allelic differences of common genes that facilitate their adaptation to fluctuating environments (Silby et al., 2011).

4.1.2 Metabolism regulation in *Pseudomonas* and its role in plant colonisation

As stated above, metabolic adaptation is a key determinant for efficient proliferation in the rhizosphere and ultimately, for plant colonisation. In an environment as competitive as the soil, adapting either to a wide range of carbon sources or effectively monopolising the most efficient source available can be a key determinant for survival. This metabolic versatility is precisely one of the characteristics of the genus *Pseudomonas*, colonisers of a huge range of environments such as soil, water, plants or animals (Bharwad and Rajkumar, 2019), and the implications for rhizosphere colonisation have been widely studied (Chavarría et al., 2013, Campilongo et al., 2017, Little et al., 2019, Molina et al., 2019). Primary metabolic regulation in pseudomonads relies on the multilevel control of the expression of all the relevant metabolic enzymes. One of the main regulatory networks that allows cells to effectively utilise their preferred compounds in a hierarchical way is called carbon catabolite repression (CCR). In pseudomonads, its control relies on Crc and Hfq regulatory proteins, whose activities are controlled by the small RNAs CrcZ and CrcY (Rojo, 2010, Bharwad and Rajkumar, 2019). This mechanism optimises metabolism by permitting efficient growth with the minimal energetic cost, leading to an improved bacterial ability to outgrow possible competitors (Rojo, 2010).

Another important regulator for carbon primary metabolism is the HexR/RccR regulon. The RpiR regulator HexR is responsible for controlling the uptake and catabolism of glucose through the modulation of genes involved in the glucose phosphorylative and Entner-Doudoroff (ED) pathways. HexR binds to 2-keto-3-deoxy-6-phosphogluconate (KDPG), an exclusive intermediate of the ED pathway, which liberates repression of the target genes and stimulates glucose uptake and metabolism (Daddaoua et al., 2009, Udaondo et al., 2018). RccR, another RpiR regulator, acts as a key regulator of three metabolic pathways: pyruvate metabolism, glyoxylate shunt and gluconeogenesis by binding to two distinct binding sites in the promoter regions of its target genes. The activity of RccR also responds to levels of the ED intermediate KDPG and, as a consequence, RccR increases its affinity for one

binding site over the other facilitating an elegant transcriptional response to a single metabolic intermediate (Campilongo et al., 2017). Together, these two transcription factors control the metabolic reprogramming of the cell in response to nutrient availability in the environment, enabling efficient bacterial proliferation and optimising energy synthesis. Little et al. (2019) recently characterised another possible partner of this regulatory system, the *rccA* gene, which is localised next to *rccR* in the *P. fluorescens* SBW25 genome. RccA is a putative phosphodiesterase that interacts with RccR and is potentially involved in the crosstalk between bacterial lifestyle transition in the rhizosphere and the metabolic state of the cell.

Rhizosphere colonisation is a dynamic and complex process in which host and bacterial factors are both involved and are in constant interaction. In the previous chapter of this work, I showed how two barley cultivars differed in their overall rhizosphere microbiome and moreover, in their recruited *Pseudomonas* populations. Thus, in the following sections, I will focus on investigating what mechanisms are behind this differential recruitment process and how *Pseudomonas* bacteria may adapt to the rhizosphere environment of distinct plant genotypes. Within this framework, this chapter firstly focusses on analysing the relevance of an efficient metabolic adaptation mechanism in *P. fluorescens* SBW25 to thrive in the rhizosphere of two barley cultivars that differ in their root exudation composition. Next, this work aims to characterise the molecular mechanisms responsible for the adaptation of beneficial *Pseudomonas* to plant genotype. To do this, I took two approaches. Firstly, I investigated the genomes of rhizosphere *Pseudomonas* spp. isolated from Chevallier and Tipple barley cultivars seeking putative differentially present genetic signatures between the population of the two cultivars. Secondly, I looked for differences in the gene expression patterns of *P. fluorescens* SBW25 when this was challenged to the rhizospheres of the two cultivars.

4.2 Results

4.2.1 Rhizosphere colonisation competition assay with *P. fluorescens* SBW25 metabolic mutants

As discussed above, several mechanisms are involved in bacterial colonisation success in the plant rhizosphere. In *Pseudomonas*, effective regulation of primary carbon metabolism seems to be crucial for bacterial adaptation to the plant environment (Cole et al., 2017). In my lab, this phenomenon has been widely explored (Little et al., 2016, Campilongo et al., 2017, Little et al., 2019) and therefore, I decided to investigate whether barley cultivar would affect the competitive colonisation efficiency of two previously published metabolic mutants, *P. fluorescens* SBW25Δ*rccR* (Δ*rccR*) and *P. fluorescens* SBW25Δ*hexR* (Δ*hexR*) (Campilongo et al., 2017). RccR is a master regulator of pyruvate metabolism, the glyoxylate shunt and gluconeogenesis, whereas HexR is fundamental for glucose metabolism. By mutating these genes in *P. fluorescens* SBW25, two artificial auxotrophs were designed: Δ*rccR* grows poorly in the presence of carbon sources with six or three molecules of carbon, such as glucose or glycerol, whereas Δ*hexR* presents growth deficiencies with carbon sources of two or four molecules of carbon, for example, acetate or succinate (Campilongo et al., 2017). Given that the two cultivars studied, Chevallier and Tipple, differed in their root exudates composition (see chapter 5, sections 5.2.2 and 5.2.3) and that one of major determinants for microbial shaping in the plant rhizosphere is the composition of root exudates (Zhalnina et al., 2018), it is reasonable to think that bacteria might have metabolically adapted to their host environment.

To test the rhizosphere colonisation fitness of the hexR and rccR mutants in my two barley cultivars, I performed a competitive colonisation assay. Typically, this assay measures the effect of gene mutation on colonisation effectiveness by comparing it against the wild-type (WT) strain. Previous unpublished data suggested that the differential colonisation effect was more striking for the *rccR* mutant and for that reason, a subsequent experiment focused on its performance against WT SBW25 and the *hexR* mutant was performed. In this experiment, Δ*rccR* was compared against WT and Δ*hexR* in order to study their relative ability to colonise the rhizospheres of Chevallier and Tipple (Figure 4.1). In both cultivars, Δ*rccR* colonisation ability was compromised relative to WT, but this deleterious effect was significantly worsened in the Tipple experiment, indicating that in the Tipple rhizosphere the conditions for Δ*rccR* proliferation were less favourable. When Δ*hexR* and Δ*rccR* were compared against each other, Δ*rccR* was significantly overtaken by Δ*hexR* but only in the

Tipple rhizosphere. Interestingly, equal numbers of Δ*hexR* and Δ*rccR* colony forming units were recovered from the Chevallier rhizosphere. These results indicate that the rhizospheres of the two cultivars might be enriched in different carbon sources that ultimately will affect the composition of the bacterial community. Hence, I predict that the Tipple rhizosphere is more abundant in 'complex' carbon sources such as glucose, whereas Chevallier seems to maintain a more balanced composition of organic acids or simple carbon sources, and complex carbon sources.

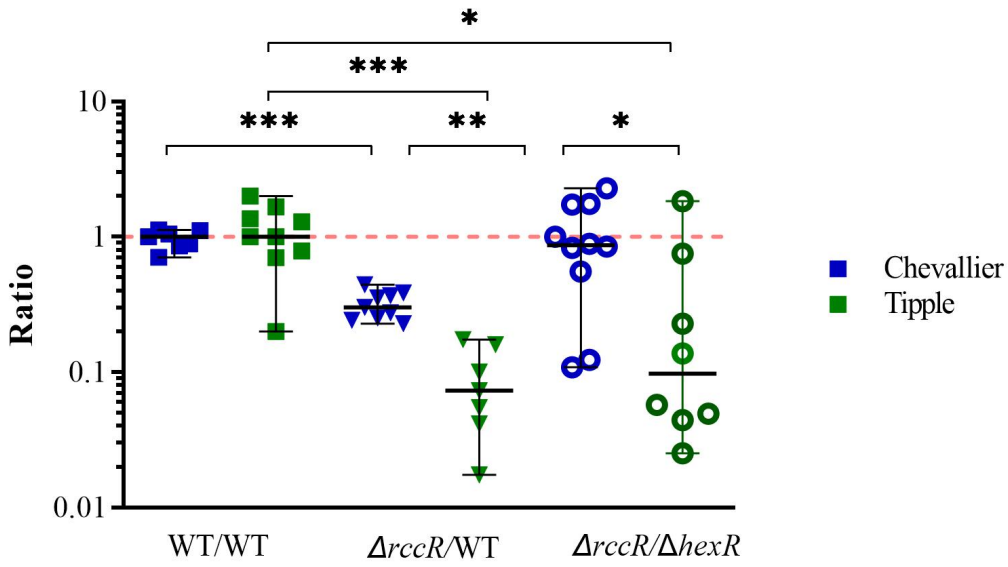

Figure 4.1. Rhizosphere colonisation competition assay. The graph shows the ratios of SBW25 WT and Δ*rccR* to WT-*lacZ* or Δ*rccR*-*lacZ to* Δ*hexR*. CFUs recovered from the rhizospheres of Chevallier (blue) and Tipple (green) barley cultivars at 5 dpi. Each dot represents the ratio of CFUs recovered from an individual plant. 8-10 plants were used per condition and *p*-values were calculated by Mann-Whitney U test, asterisks indicate $p < 0.05$ (*), 0.01 (**) or 0.001(***). Experiment was repeated three times and here a representative graph is shown.

For this reason, I decided to test the growth ability of the *Pseudomonas* collection isolated from Tipple and Chevallier rhizosphere (Chapter 3, section 3.2.1) in two different carbon sources. For this, I chose acetate as a representative of a simple carbon source, and glucose as a complex one. Here, I am using 'complex' to refer to a carbon source that is metabolised via pyruvate dehydrogenase and the ED pathway before entering the Krebs cycle, while 'simple' is defined as those that feed directly into Krebs cycle, with up to two intermediate steps at most. In figure 4.2 the growth curves of all the isolates are represented. These growth curves are vertically plotted, with OD_{600} values stacked in columns from time 0 to 48 h. OD_{600} values were baselined to the smallest value in each dataset to allow comparisons across samples. Figure 4.2A shows the growth of Tipple and Chevallier rhizosphere isolates

in the presence of acetate as the sole carbon source. No obvious pattern could be detected here, although some Chevallier isolates seemed to stand out from the rest. However, when *Pseudomonas* rhizosphere isolates were grown in glucose (figure 4.2B), markedly higher final OD_{600} values were observed for a large number of Tipple isolates, which is in agreement with the trend observed for the metabolic mutant assays, above.

All in all, these results point towards two main phenomena: (1) carbon metabolism regulation seems to play a determining role in enabling bacteria to adapt to different rhizosphere environments, such as plants of different genotypes, and (2) carbon sources available in the rhizosphere might depend on plant genotype. The second phenomenon will be treated in Chapter 5; thus, the rest of this chapter will try to dissect the first phenomenon and shed light on the bacterial genetic mechanisms behind plant rhizosphere colonisation and genotype specialisation.

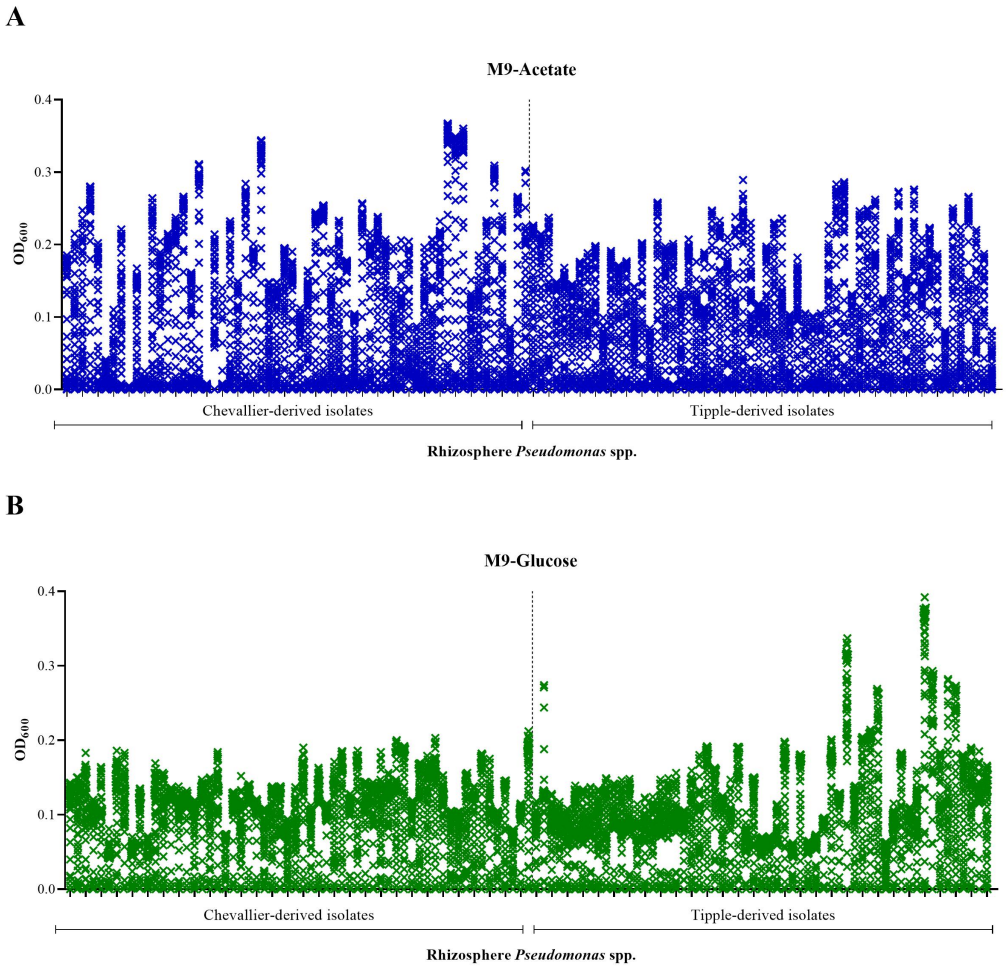

Figure 4.2. Rhizosphere isolates growth at 48 h. **A.** M9 medium supplemented with 0.4 % Acetate. **B.** M9 medium supplemented with 0.4 % Glucose. Each individual cross represents a unique time point, from 0 to 48 h, and the associated OD_{600}. Values were baselined by the lowest of each dataset. Experiment was repeated twice and here a representative graph is shown.

4.2.2 Whole genome analysis of rhizosphere *Pseudomonas*

In order to investigate in more detail the effect that the plant genotype exerts on the root-associated *Pseudomonas* populations at a genetic level, a subset of 42 *Pseudomonas* isolates was selected for whole genome sequencing. 19 of these were selected based on their phylogenetic distribution to ensure maximum diversity and their growth efficiency in different carbon sources. To complete the two subpopulations, 23 additional isolates were selected at random and included to reduce bias.

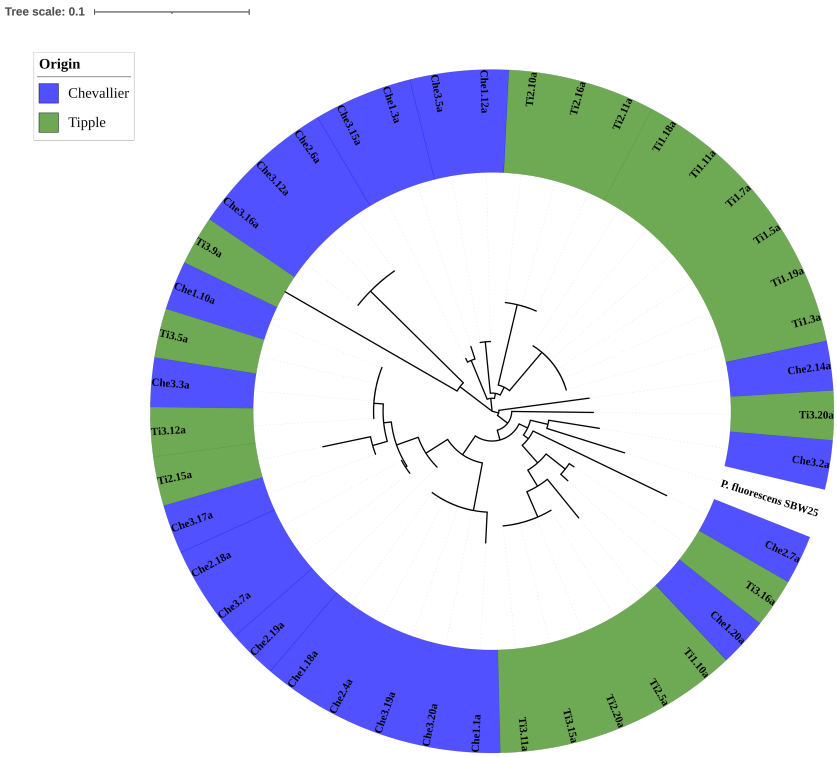

Figure 4.3. *gyrB* phylogeny of the selected subpopulation of Chevallier and Tipple rhizospheres isolates. The phylogenetic tree is based on 43 partial sequences of the *gyrB* gene from sequenced and *P. fluorescens* SBW25 as a type strain. Constructed by the ML method and Tamura-Nei model with 1000 bootstrap value.

In figure 4.3, a phylogenetic tree of the sequenced isolates is shown to visualise the relatedness of all the isolates studied. First of all, I analysed the growth of the isolate subpopulation on fructose and acetate. Fructose was selected to investigate the utilisation efficiency of the rhizosphere isolates of a different complex carbon source, which as glucose is also very abundant in the root exudates of different plant species (Kamilova et al., 2006, Badri and Vivanco, 2009). Figure 4.4 shows the growth curves of the *Pseudomonas* isolates in the presence of acetate (A) or fructose (B) as unique carbon sources. A single curve for all the isolates analysed per cultivar is shown, composed of the mean values of all the isolates

studied at a given time point, to facilitate data visualisation and analysis. SBW25 and the two metabolic mutants, Δ*hexR* and Δ*rccR*, were also included as a control. When isolates from both cultivars were grown in acetate (figure 4.4A), I could not detect any remarkable patterns, besides the slightly higher growth rate than SBW25 that they presented. However, when the isolates grew in fructose (figure 4.4B), as already observed in figure 4.2, a clear split between Tipple and Chevallier isolates was shown: Tipple isolates presented an overall better growth efficiency in glucose than those from Chevallier.

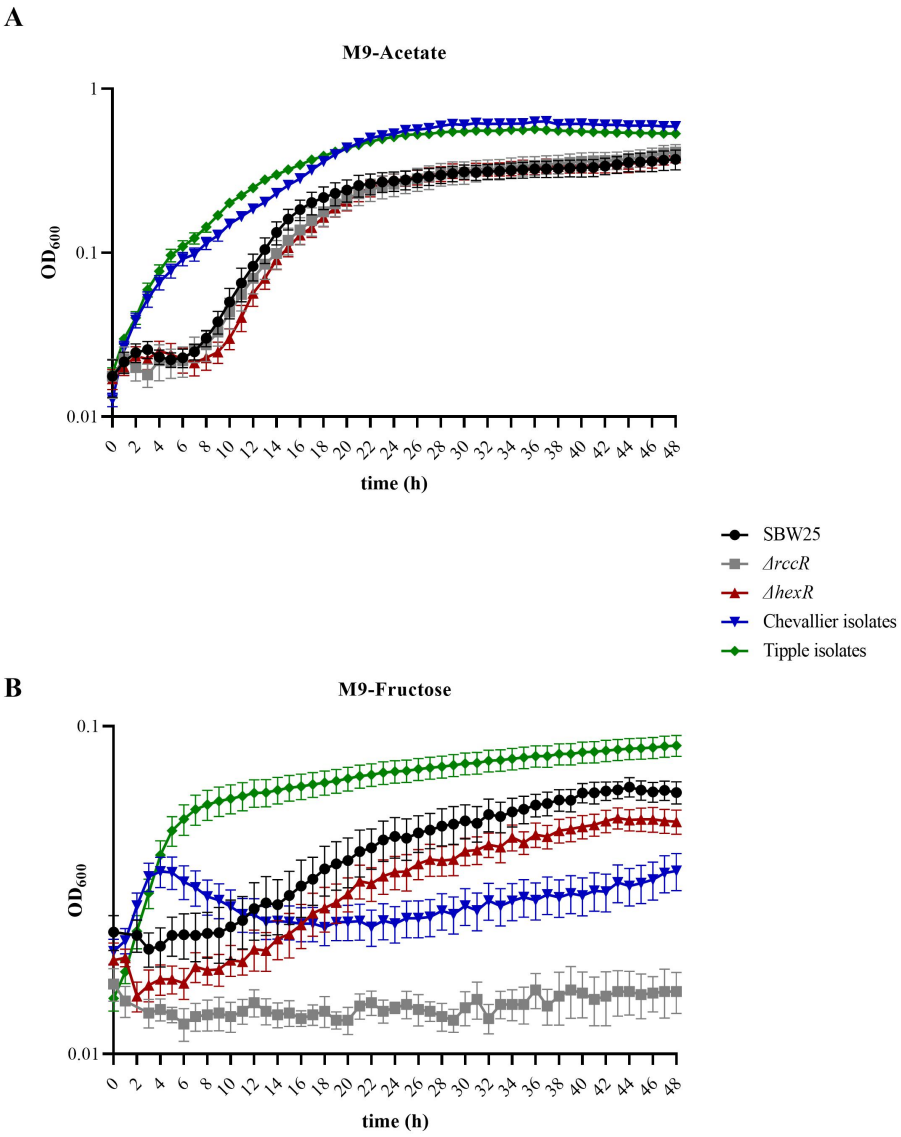

Figure 4.4. Growth of 42 rhizosphere isolates selected for whole genome sequencing at 48 h. **A.** M9 medium supplemented with 0.4 % Acetate. **B.** M9 medium supplemented with 0.4 % Fructose. *P. fluorescens* SBW25 WT is shown in black, Δ*rccR* SBW25 mutant in grey, Δ*hexR* SBW25 mutant in red, Chevallier isolates in blue and Tipple isolates in green. Values for Chevallier and Tipple isolates are represented as the mean values of all the isolates tested. Errors shown as SEM. Experiment was repeated twice and here a representative graph is sh

The genomes of the subpopulations were interrogated with the great help of Dr Govind Chandra (Molecular Microbiology, John Innes Centre). To study the selective pressure that plants apply on rhizosphere bacterial communities, the 42 *Pseudomonas* isolate genomes were searched for genetic markers potentially involved in cultivar-specific plant colonisation using *P. fluorescens* SBW25 as a reference genome. For this purpose, a total of 410 genes were selected as a combination of previously described plant-induced *Pseudomonas* genes, including transporters, biofilm formation regulators, chemotaxis proteins or siderophores (Silby et al., 2009a) together with interesting substrate transporters and transcriptional regulators, two important traits relevant in niche adaptation (Compant et al., 2010, Campilongo et al., 2017). Hits obtained were subjected to two cut-offs: alignment coverage > 66% and sequence identity > 66%, to identify particular genes as the best reciprocal hit. Figure 4.5 shows those genes differentially distributed with a Chi-Square *p*-value < 0.05, a total of 60, between the pseudomonad populations of Chevallier and Tipple. Some of the genes were completely or almost absent in Chevallier rhizosphere subpopulation, for example, *PFLU_1377* or *hscC*, a chaperon, *PFLU_5080*, a hypothetical protein, *PFLU_2583*, a sugar ATP-binding cassette (ABC) transporter, or *PFLU_3086,* a putative phenylacetaldehyde dehydrogenase. In the case of the Chevallier, hits uniquely present among its rhizosphere isolates were not identified by this approach, although some genes were found to be selected at higher rates in Chevallier *Pseudomonas* isolates, such as *PFLU_0315*, a GABA transporter or *PFLU_5743*, a putative decarboxylase. I then assigned molecular functions to all of them based on KEGG orthology (https://www.kegg.jp/) (figure 4.6) and the most abundant categories were signalling and cellular processes, which include metabolism and signal transduction, with approximately 23 % of the selected genes, and transporters with 20 %.

These results indicate that the plant host is capable of selecting for specific bacterial genetic traits and that such bacterial genetic characteristics may reflect the adaptation to the cultivars-dependant root environment undergone by the rhizosphere bacterial consortium. This is likely to contribute a fitness benefit to the bacterial community in order to better colonise the plant rhizosphere and in the following section I will be addressing this question.

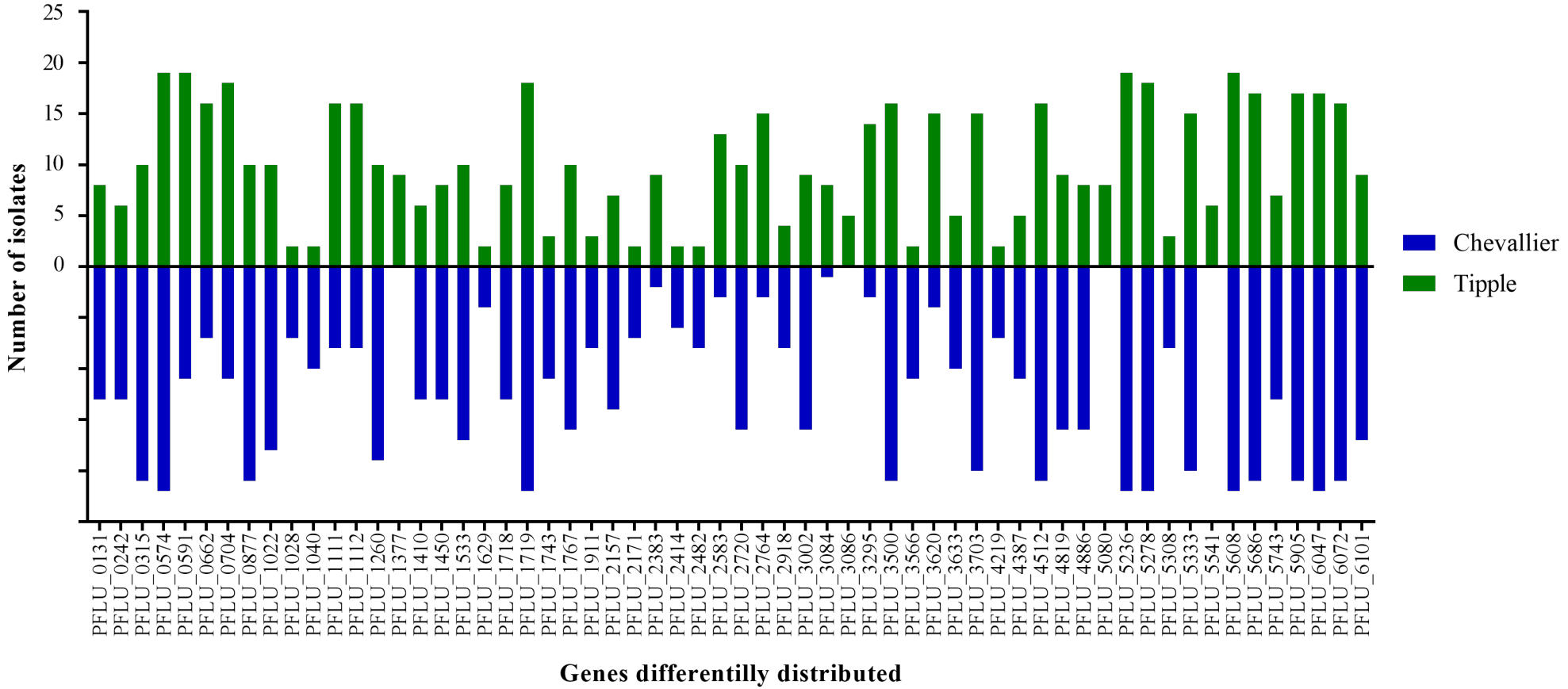

Figure 4.5. Distribution of genes differentially present in the sequenced *Pseudomonas* isolates of Chevallier and Tipple. The bars show the number of strains presenting the SBW25 target gene analysed by reciprocal BLAST. Chevallier isolates are shown in blue and Tipple, in green. All the genes represented were significantly different ($p < 0.05$) in one background from the other according to a Chi-Square test.

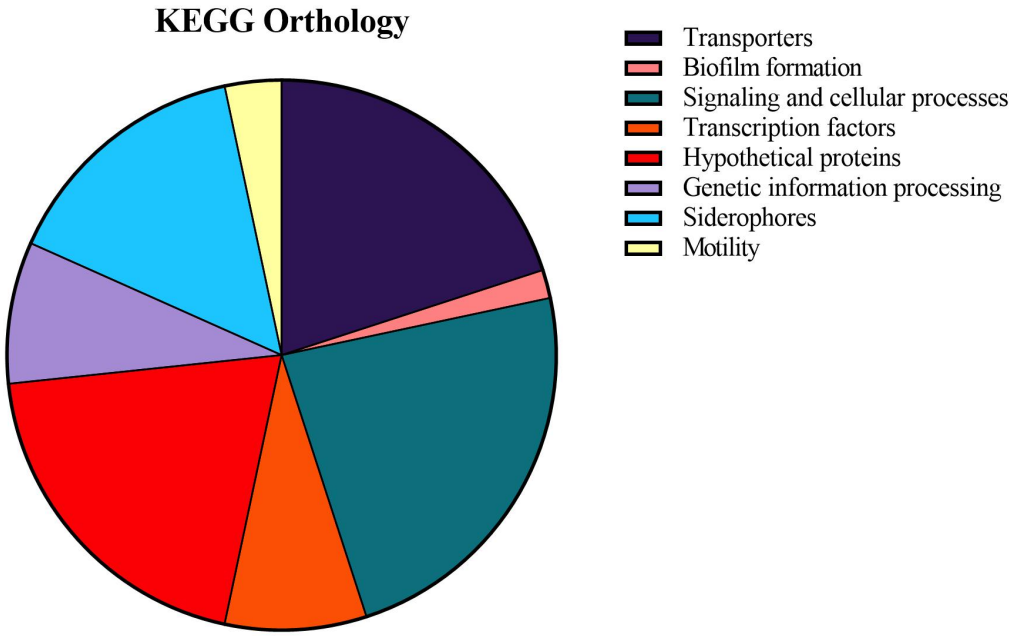

Figure 4.6. KEGG orthology of the differentially distributed genes. Molecular functions assigned according to KEGG database.

4.2.3 Characterisation and testing of genes differentially selected by barley cultivar in *P. fluorescens* SBW25

In the section above, I identified some putative bacterial genes involved in cultivar-specific rhizosphere colonisation. To characterise their role in this process, eight genes were selected to and taken forward to in-depth analysis. These genes were selected based on two criteria, the level of significance of the differential frequency between the two cultivars and their predicted biological role. In figure 4.7, I show a zoom-in to their distribution where it can be observed that, for example, *PFLU_6072* occurs more frequently in Chevallier isolates than in Tipple, with 21 *Pseudomonas* spp. containing an ortholog of this gene versus only 16 in Tipple, *PFLU_3295* was found in 14 Tipple isolates and only in 3 Chevallier ones, or the case of *PFLU_2583*, whose frequency was much higher in Tipple (13), than in Chevallier rhizosphere isolates (3).

In table 4.1, a summary with the functions and the class of the selected genes is shown based on the *Pseudomonas* Genome Database (Winsor et al., 2016). *PFLU_6072* and *PFLU_1533* are classified as LysR-Type family transcriptional regulators (LTTRs). This family of regulators is considered the largest family of transcription factors, with over 40,000 having been described (Reen et al., 2013). LTTRs are universally present in procaryotes, although

they are particularly abundant in Alpha- and Gammaproteobacteria, with *Pseudomonas* spp. considered to contain the greatest abundance (Schell, 1993).

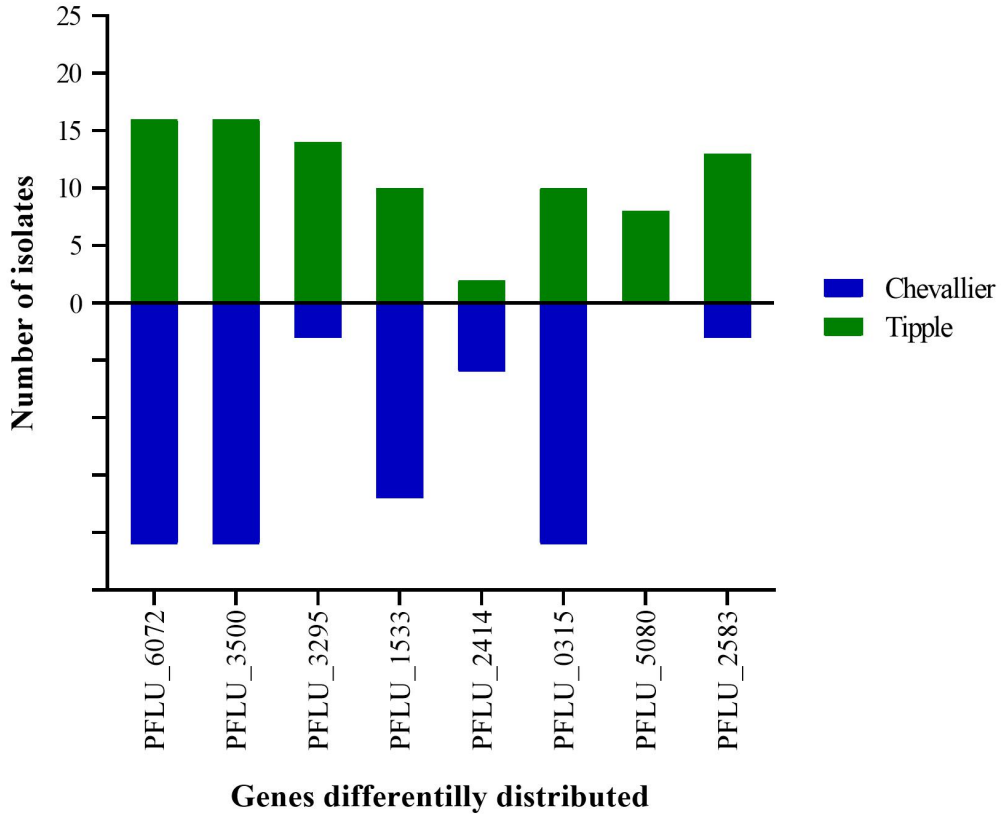

Figure 4.7. Distribution of the selected genes among Chevallier and Tipple rhizosphere *Pseudomonas* population. The bars show the number of strains presenting the SBW25 target gene analysed by reciprocal BLAST. Chevallier isolates are shown in blue and Tipple, in green. All the genes represented were significantly differently abundant ($p < 0.05$) according to Chi-Square test.

This family of regulators are usually characterised by four features: (i) their sizes vary between 276 to 324 aa, (ii) they are able to regulate DNA both in a signal-dependent and signal-independent manner, (iii) they are divergently transcribed from the promoter of the regulated gene which allows simultaneous bidirectional control of transcription of two set of genes and (iv) they usually repress their own transcription. LTTRs are involved in the regulation of a very diverse set of cellular functions, for example, in virulence factors expression, motility, quorum sensing or central metabolism (Schell, 1993, Reen et al., 2013). With regard to *PFLU_6072*, some additional information can be inferred given its genomic context, since it is found next to the previously described genes, *rccR* (*PFLU_6073*) and *rccA* (*PFLU_6074*) (Campilongo et al., 2017, Little et al., 2019). As already stated in previous sections of this chapter, RccR as a key regulatory component in three different metabolic pathways pyruvate metabolism, the glyoxylate shunt and gluconeogenesis,

whereas RccA is theorised to sense the concentration of succinate or another intermediate of the Krebs cycle and regulate activities involved with cell motility or biofilm formation (Campilongo et al., 2017, Little et al., 2019). It is worth mentioning that the adjacent genes *PFLU_6070* or *accD* and *PFLU_6071* or *oadA* also play a role in carbon metabolism, since they encode different subunits of pyruvate carboxylase, crucial for converting pyruvate into oxaloacetate (Lai et al., 2006). Since *PFLU_6072* is flanked by genes involved in carbon metabolism and that bacterial genes tend to cluster by functionality (Ballouz et al., 2010), it is plausible to hypothesise a similar function for this transcription factor.

PFLU_3295 is described as GntR family transcriptional regulator and its orthologue, *vanR*, is described as a repressor of the expression of vanillate demethylase, an enzyme involved in the transport and degradation of vanillate (Morawski et al., 2000). In addition, *PFLU_3294* is described as a putative vanillate O-demethylase oxidoreductase therefore it is highly likely that the putative function of *PFLU_3295* is a regulator of vanillate metabolism.

PFLU_2414 is annotated as a putative iron siderophore-like protein, localised in the periplasm. The predicted function of this protein is as a Fe^{+2}-dicitrate sensor and a FecR-like protein. FecR proteins form part of a complex involved in iron sensing and transport (Ochs et al., 1995) and have been described as anti-sigma factors that are key for signal transduction upon binding of extracellular ferric-complexes to their relevant membrane transporters (Mahren et al., 2002). Therefore, they contribute to extracellular iron complex sensing and transportation. The secretion of citrate and the subsequent uptake of the stable iron complex is key for soil *Pseudomonas* to thrive in the micronutrient-limited environment of the soil (Mimmo et al., 2014). Having an efficient iron acquisition system could be advantageous for competition against other soil bacteria and successful rhizosphere colonisation.

PFLU_3500 has been annotated as a C4-dicarboxylate transporter and has been previously characterised as *dctA* in several other bacterial species, including plant colonisers (Janausch et al., 2002). For example, in *P. aeruginosa* PAO1, C4-dicarboxylate transport is mainly controlled by two transporters: DctA, a low-affinity system, and DctPQM, a high-affinity system (Valentini et al., 2011). In the plant-growth promoting bacterium *P. chlororaphis* O6, the lack of *dctA* has a deleterious effect on tobacco root colonisation (Nam et al., 2006).

PFLU_0315 or *gabP* encodes a permease of the amino acid-polyamine-organocation (APC) superfamily, responsible for uptake of the non-protein amino acid γ-aminobutyric acid (GABA) into the cell (Hu and King, 1998, Schneider et al., 2002). GABA permeases are

found in the genomes of several bacterial taxa, including *Escherichia coli*, in which they were first characterised (Niegemann et al., 1993), *Bacillus* (Planamente et al., 2010), and *Rhizobium* (Hosie et al., 2002). In *Pseudomonas*, *gabP* genes have also been characterised and in some studies have been shown to play a role in plant infection by potentially regulating some virulence factors (McCraw et al., 2016). GABA has been demonstrated to be found in root exudates in this study and by other authors (Badri et al., 2013, Chaparro et al., 2013) and is known to act as a chemoattractant in the plant coloniser *P. putida* KT2440 (Reyes-Darias et al., 2015). Therefore, there is sufficient evidence to hypothesise that the uptake of GABA and its subsequent utilisation by the bacterial community may play an important role in the assembly of the rhizosphere community.

PFLU_5080 is described as a hypothetical protein with no predicted domains that can help elucidate its function. A look to the genomic surroundings gives some insights to a potential role in prophage-like element regulation. *PFLU_5081* is a hypothetical protein with a domain described as prophage regulatory protein and *PFLU_5079* is a putative integrase belonging to the phage integrase family. Analysis with the protein modelling software Phyre2 (Kelley et al., 2015) suggests a putative role as a restriction endonuclease. Therefore, I hypothesise that *PFLU_5080* might be involved in prophage excision.

PFLU_2583 is a putative rhizopine-binding ABC transporter. ABC transporters constitute a large, ubiquitous family whose function is the translocation of many substrates across membranes, both as exporters and importers. Given that (1) rhizopines are rare rhizobia-specialised molecules involved in the legume-*Rhizobium* symbiosis process that are absent in most plant rhizospheres (Geddes et al., 2019), (2) I found an orthologue in some species of *P. chlororaphis*, described as a putative inositol transporter (inositol is a sugar that can act as a rhizopine precursor), (Fry et al., 2001) and (3) modelling with Phyre2 (Kelley et al., 2015) indicates a role as a ribose ABC transporter; it is more likely that the binding substrate of this transporter is a sugar with structural and chemical properties similar to rhizopines.

This set of eight genes were deleted in *P. fluorescens* SBW25 and in order to confirm the fitness of the mutants, I performed growth assays in different media. Two complex media; KB and LB, and several minimal media containing acetate, glucose, glycerol, pyruvate and succinate as carbon sources were tested (figure 4.8). Figure 4.8A and B show the growth curves for the two complex media tested. None of the strains presented a growth penalty in these conditions. When they were grown with acetate or succinate, again no growth deficiencies were observed for any of the mutants (figure 4.8C and D). However, when these

mutants only had pyruvate, glucose or glycerol as the sole carbon source, clearly compromised growth was detected for *Δ6072* (figure 4.8E-G). With this gene being annotated as a LysR regulator, its genomic context and its inability to grow well in certain carbon sources, its role as a potential new regulator involved in carbon metabolism appears highly likely.

Table 4.1. Functional description of the selected genes.

Gene ID	Function	Class	Chi-square *p*-value
PFLU_6072	LysR-family transcriptional regulator	Transcription factors	0.027
PFLU_3295	GntR family transcriptional regulator, *vanR*	Transcription factors	0.002
PFLU_1533	LysR-family transcriptional regulator	Transcription factors	0.014
PFLU_2414	Iron siderophore sensor	Siderophores	0.012
PFLU_0315	GABA permease, *gabP*	Transporters	< 0.001
PFLU_5080	Hypothetical protein	Hypothetical protein	0.005
PFLU_2583	Putative rhizopine-binding	Transporters	0.004

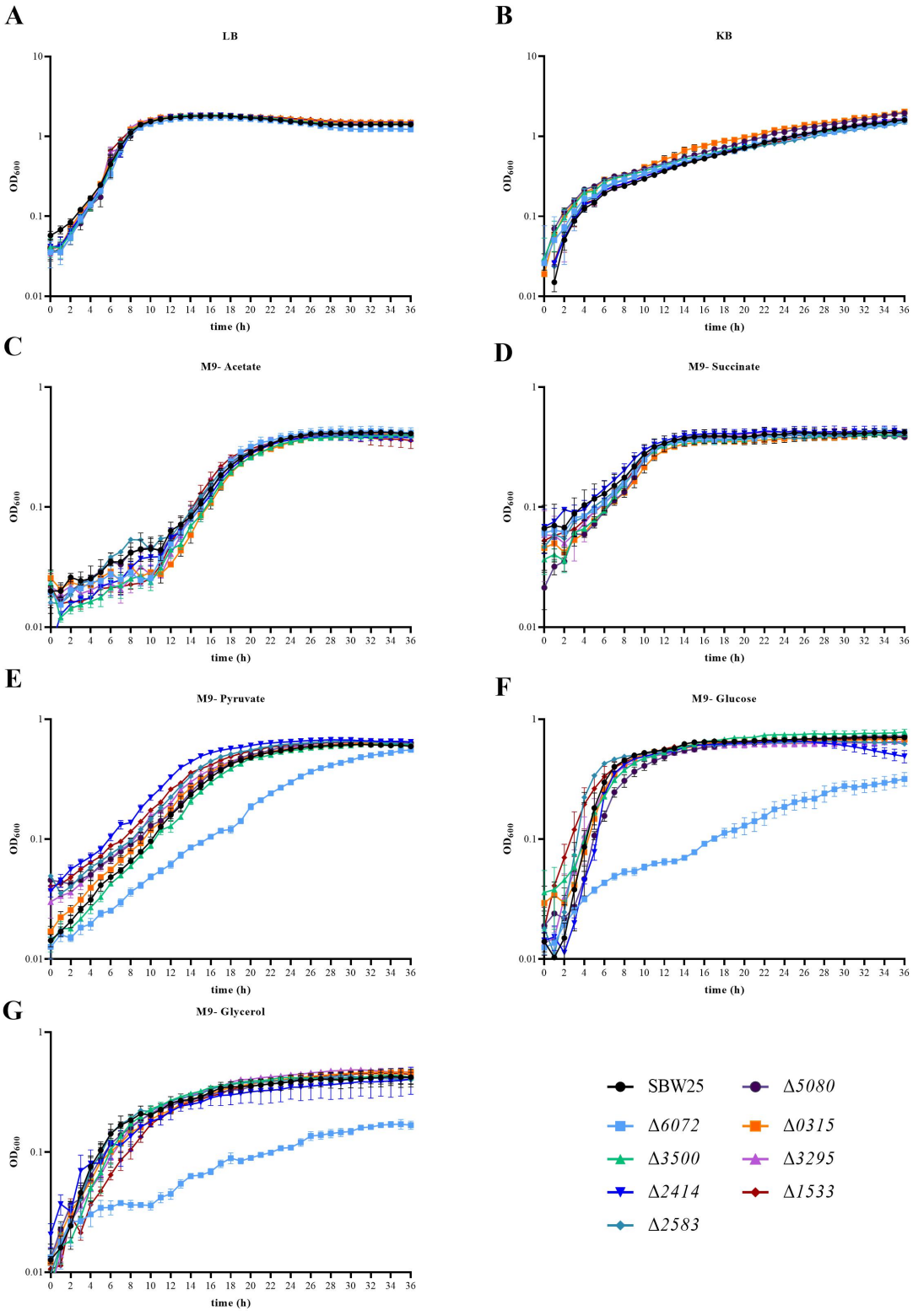

Figure 4.8. Growth curves of deletion mutants in SBW25 of genes differentially distributed between Chevallier and Tipple rhizosphere isolates at 36 h. **A.** LB medium. **B.** KB medium. **C.** M9 minimal medium 0.4% acetate. **D.** M9 minimal medium 0.4% succinate. **E.** M9 minimal medium 0.4% pyruvate. **F.** M9 minimal medium 0.4% glucose. **G.** M9 minimal medium 0.4% Glycerol. 3 biological reps used per strain. Error bars are represented as SEM. Experiment was repeated three times and here a representative graph is shown.

In order to investigate their relevance for barley rhizosphere colonisation and moreover, to assess if their colonisation fitness was affected by plant genotype, I carried out rhizosphere colonisation competition assays with my selected mutants. Each of the mutants was tested against the WT SBW25 and the effectiveness of colonisation was recorded as the ratio of mutant versus WT. The results obtained are summarised in the scatter plot in figure 4.9. Δ*6072* showed an impaired colonisation phenotype in both cultivars, although the differences were more remarkable in the case of Tipple. In light of the growth deficit that the mutant showed before and the results observed here, the hypothesis that in Tipple rhizosphere there is an enrichment in glucose-like molecules gains popularity. This is further supported by the fact that Δ*2583* also exhibited a fitness penalty only in the rhizosphere of Tipple plants. As mentioned above, *PFLU_2583* is a putative sugar transporter and its abundance was found in greater numbers among Tipple-associated isolates than Chevallier ones. However, it is fair to mention that *PFLU_6072* was originally detected to be more frequent among the *Pseudomonas* communities of Chevallier cultivar and therefore, a greater deleterious effect for this cultivar would have been expected. Nevertheless, it is worth mentioning that this gene was quite abundant among the communities of the two cultivars studied, probably indicating an important role in rhizosphere colonisation independently of the plant cultivar. Δ*5080* also displayed a compromised colonisation ability only in Tipple rhizosphere, which indicates that this hypothetical protein might play a role in adaptation to different niches in a plant-genotype dependent manner. The rest of the mutants, however, did not show a decrease in colonisation fitness. This could have its explanation in four phenomena: (1) some of these genes present a functional redundancy within the *P. fluorescens SBW25* genome, (2) the experiment was conducted in a model organism (SBW25) that was not naturally present in the rhizosphere of the two cultivars studied, (3) the nature in which these genes contribute to plant adaptation might not be captured by the method used here and (4) the assay by which these genes were originally identified in first place differs from the one used here.

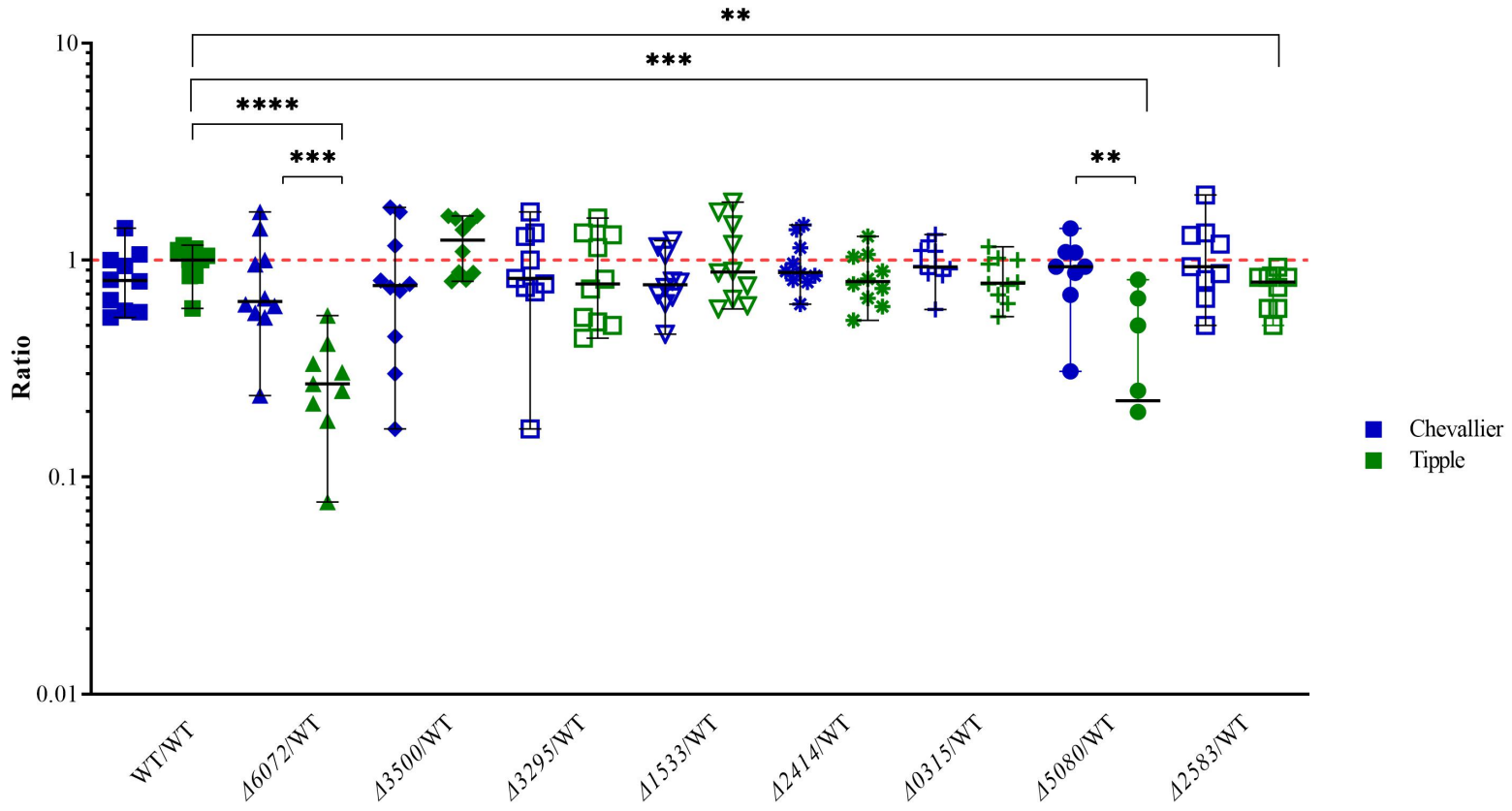

Figure 4.9. Rhizosphere colonisation competition assay. The graph shows the ratios of differentially distributed mutants to SBW25 WT-*lacZ* CFUs recovered from the rhizospheres of Chevallier (blue) and Tipple (green) barley cultivars. Each dot represents the ratio of CFUs recovered from an individual plant. *p*-values were calculated by Mann-Whitney U test and asterisks indicate $p < 0.05$ (*), 0.01 (**), 0.001(***) or 0.0001(****). Experiment was repeated three times and here a representative graph is shown.

4.2.4 RNA-seq study of *P. fluorescens* SBW25 in the rhizosphere of barley

The approach used in the previous section painted a general view of the genetic distribution in a subpopulation of rhizosphere *Pseudomonas,* but to what extent this translates into these or other genes being actively expressed in the rhizosphere of these two cultivars is unknown. For this reason, using as a model organism *P. fluorescens* SBW25, gene expression levels were studied under different conditions by RNA-seq. SBW25 was introduced into the rhizospheres of sterile Tipple and Chevallier plants, its RNA was recovered at one and five dpi and its gene expression levels were compared against a cell-culture control representing the time zero in colonisation, i.e., before SBW25 is in contact with the plant. Having two different time points might allow us to understand the dynamics of SBW25 gene expression throughout plant colonisation, both at the beginning of the interaction and after a degree of stability might have been achieved in the system. This dataset was analysed with the valuable help of Dr Govind Chandra (Molecular Microbiology, John Innes Centre).

Figure 4.10A shows a heat map representing all the SBW25 genes up- or down-regulated in comparison to the control presenting an absolute log2-fold change greater than 1. About 3,034 genes presented a fold change of more or equal to 2 in the plant rhizosphere in comparison to the control, among which some of the genes previously identified could be found, for instance, *PFLU_3500*, *PFLU_0315*, *PFLU_0877*, *PFLU_6072* and *PFLU_1040* among others. Genes that were differentially expressed in the four conditions tested were calculated as those presenting a log2-fold $\leq$ -1 or $\geq$1 and *p*-value $\leq$ 0.05. In figure 4.10B, a summary of genes significantly up or downregulated are shown. A total of 2,158 genes were differentially expressed compared to the cell-culture control in the rhizosphere of Chevallier at 1 dpi, whilst this number increased slightly up to 2,515 at 5 dpi. In both cases, approximately half of the genes were upregulated and the other half, downregulated. In the case of the Tipple rhizosphere, 2,039 presented a differential expression at 1 dpi, of which 950 were upregulated and 1,089, downregulated. At 5 dpi, 2,470 genes presented differential transcription levels, of which nearly half were upregulated and the other half, downregulated. On average, there was a difference in the number of differentially expressed genes at day 5 of 200 genes more than at day 1. Finally, I decided to visually plot the interactions between conditions. To do this, a Venn diagram of the differentially expressed genes in each condition was plotted (figure 4.10C). 1617 SBW25 genes were found to be commonly expressed for all the conditions studied, 97 were unique at the earliest stages of colonisation and 372 seemed to be expressed at later stages of the interaction. Interestingly, some genes seemed

to be specifically linked to one particular plant environment, i.e., plant genotype; thus, a total of 193 genes were found to be exclusively induced in Tipple rhizosphere and 243, only in Chevallier.

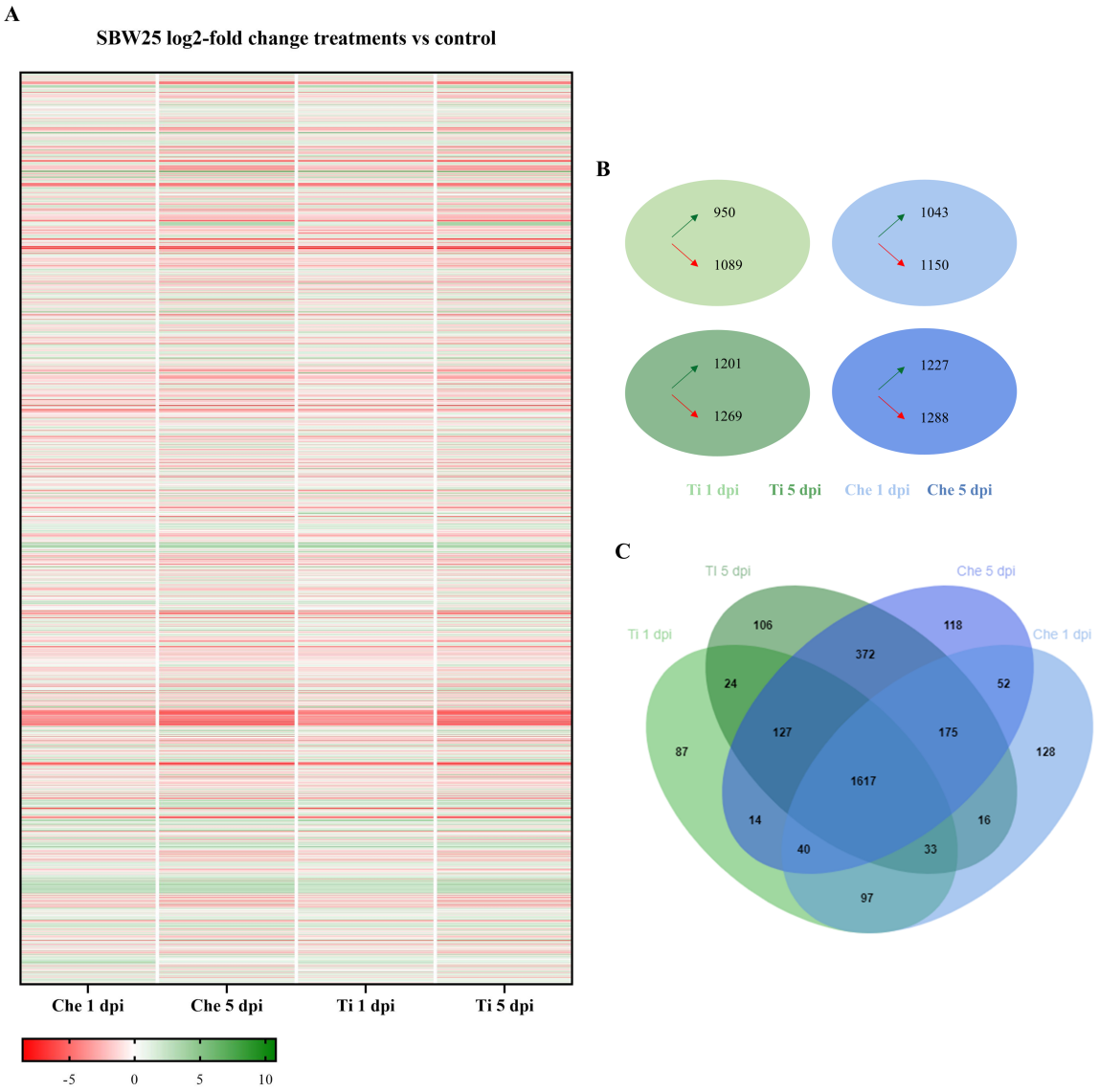

Figure 4.10. Gene expression profile of *P. fluorescens* SBW25 from Chevallier and Tipple rhizospheres. **A.** The heat map shows the pairwise comparison between SBW25 from Chevallier and Tipple rhizospheres at 1 or 5 dpi, relative to the control. Only genes presenting an absolute log2-fold change of 1, 2-fold change, for any of the four conditions are shown in the graph. **B.** Dissection of up- and downregulated genes for every condition tested. **C.** Venn diagram of all the differentially expressed genes in each condition. Genes differentially expressed had $-1 \leq \log2\text{-fold} \leq 1$ and p-value ≤ 0.05. SBW25 challenge to the Tipple rhizosphere is represented in green, to Chevallier, in blue.

In order to study the relevance of these differences and to assess their significance level, pair-wise comparisons between SBW25 from Chevallier and Tipple rhizospheres were carried out. For this purpose, two volcano plots representing the two time points studied were built (figure 4.11A and B). It is worth mentioning the dramatic decrease in differentially expressed genes when the two cultivars were compared in contrast to the controls. At 1 dpi, just 14 genes were indeed differentially expressed between the two cultivars, a number that suffered a sharp decrease at 5 dpi, with only 7 genes presenting a significant fold change. In the heat map (figure 4.11C), the 14 genes significantly up- or down-regulated at day 1 are compared to day 5. It is very noticeable how the transcriptional changes seemed to experience a stabilisation, as less striking fold changes are observed across all the genes studied. The genes that were upregulated in Tipple rhizosphere were *PFLU_3089*, *PFLU_3090*, *PFLU_3091*, *PFLU_3092*, *PFLU_3094*, *PFLU_3096*, *PFLU_3097*, *PFLU_3098*, *PFLU_3735*; whereas *PFLU_3529*, *PFLU_4036*, *PFLU_2454*, *PFLU_4463* and *PFLU_1043* were downregulated.

Table 4.2. Functions of genes differentially expressed in SBW25 from Chevallier and Tipple rhizospheres. Those genes connected by a dashed line are predicted to be part of the same operon.

Gene ID	Function	Description
PFLU_3089	Hypothetical protein with a porin-like domain	Upregulated in Tipple rhizosphere
PFLU_3090	NAD-dependent aldehyde dehydrogenase	Upregulated in Tipple rhizosphere
PFLU_3091	Amino acid permease	Upregulated in Tipple rhizosphere
PFLU_3092	Methyl-accepting chemotaxis protein	Upregulated in Tipple rhizosphere
PFLU_3094	Amino acid permease	Upregulated in Tipple rhizosphere
PFLU_3096	4-aminobutyrate aminotransferase	Upregulated in Tipple rhizosphere
PFLU_3097	NAD-dependent aldehyde dehydrogenase	Upregulated in Tipple rhizosphere
PFLU_3098	Cytochrome b-561 membrane protein	Upregulated in Tipple rhizosphere
PFLU_3735	Hypothetical protein	Upregulated in Tipple rhizosphere
PFLU_3529	Acyl-CoA dehydrogenase	Upregulated in Chevallier rhizosphere
PFLU_4036	4-aminobutyrate aminotransferase	Upregulated in Chevallier rhizosphere
PFLU_2454	Enoyl-CoA hydratase	Upregulated in Chevallier rhizosphere
PFLU_4463	EamA-like transporter family	Upregulated in Chevallier rhizosphere
PFLU_1043	Hypothetical protein	Upregulated in Chevallier rhizosphere

It is interesting that the most upregulated genes appear to be close to each other in the genome and, in fact, some of them are part of the same operon, e.g., *PFLU_3089* and *PFLU_3090* or *PFLU_3097* and *PFLU_3098*. A summary of the functions of these genes can be found in table 4.2. It is interesting to highlight that in the Tipple rhizosphere, amino acid transport seems to be upregulated in SBW25, whereas in the case of Chevallier, no clear pattern can be observed. When the previously identified genes (figure 4.5) were examined in the context of differential expression between the two cultivars, no significant differences could be found. However, about half of them were indeed differentially expressed in the rhizosphere, regardless the cultivar. This is the case of *PFLU_0315*, *PFLU_2583*, *PFLU_3295*, *PFLU_3500* and *PFLU_6072*, among others.

To study the potential role that these genes might have in the cultivar-specific colonisation of the rhizosphere, *PFLU_3091* and *PFLU_4463*, two of the most differentially regulated genes in the rhizospheres of the two cultivars, were selected for further characterisation. *PFLU_3091* is highly upregulated in the Tipple rhizosphere in comparison to Chevallier and it is annotated as amino acid permease. The importance of amino acid metabolism and transport in the rhizosphere has been already discussed throughout this chapter. This has been illustrated by several authors, who have shown the relevance of amino acid release by root exudates and its subsequent metabolism by the rhizosphere microbial community (Badri et al., 2013, Cole et al., 2017, Zhalnina et al., 2018). Since this gene is adjacent to the operon formed by *PFLU_3089* and *3090*, it is worth remarking that these two genes might also have a role in amino acid transport. *PFLU_3090* is highly upregulated in the Tipple rhizosphere in comparison to Chevallier and it is annotated as NAD-dependent aldehyde dehydrogenase. NAD-dependent aldehyde dehydrogenases have been previously described to be critical for the catabolism of lignin by the soil bacterium *Sphingobium* sp.(Kamimura et al., 2017). On the other hand, *PFLU_3089* encodes for a porin-like protein. Altogether this might suggest that these genes have a potential role in substrate uptake and catabolism. *PFLU_4463* was dramatically downregulated in Tipple in comparison to Chevallier, i.e., it was highly upregulated in Chevallier rhizosphere. It is annotated as a hypothetical protein with a EamA domain, a family of transporters in which many are characterised as drug/metabolite transporters. In *Rhizobium,* for example, a protein containing two EamA domains has been shown to be responsible for the uptake of riboflavin (García Angulo et al., 2013).

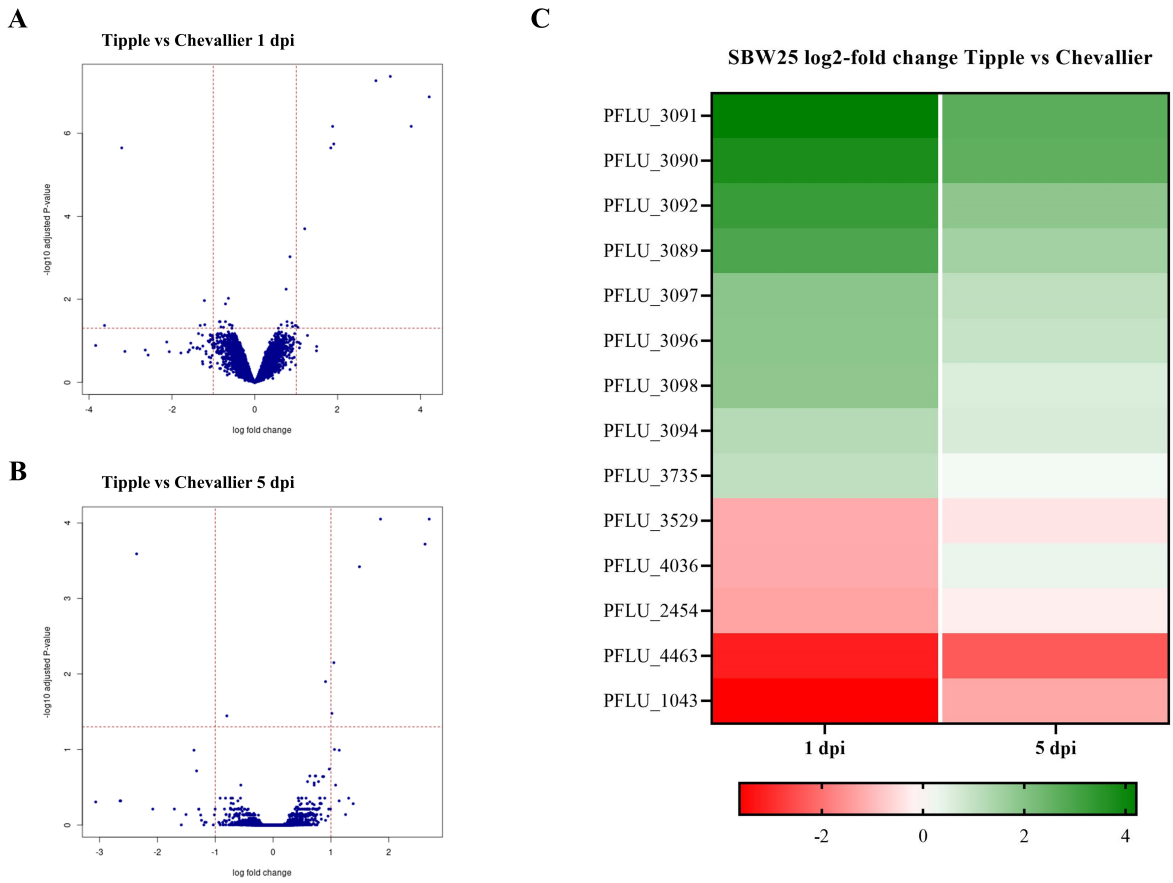

Figure 4.11. Gene expression profiles of *P. fluorescens* SBW25 from Chevallier and Tipple rhizosphere. **A.** Volcano plot represents the pairwise comparison of transcript abundance between SBW25 from Chevallier rhizosphere and from Tipple rhizosphere at 1 dpi. **B.** Volcano plot of the pairwise comparison of transcript abundance between SBW25 from Chevallier rhizosphere and from Tipple rhizosphere at 5 dpi. C. Heat map showing significantly differentially expressed genes (> 2-fold change, *p*-values < 0.05) in SBW25 from Chevallier and Tipple rhizosphere at 1 dpi and 5 dpi.

The two selected genes, *PFLU_3091* and *PFLU_4463*, were deleted in *P. fluorescens* SBW25 and their growth in different media was tested in order to verify whether or not a fitness penalty could be observed under different conditions. To do this, I used LB and KB as complex media and M9 minimal media with different carbon sources (acetate, glucose, glycerol, pyruvate and succinate) (figure 4.12). Neither Δ*3091* nor Δ*4463* presented any growth deficiencies when compared to WT *P. fluorescens* SBW25 in any of the media tested here.

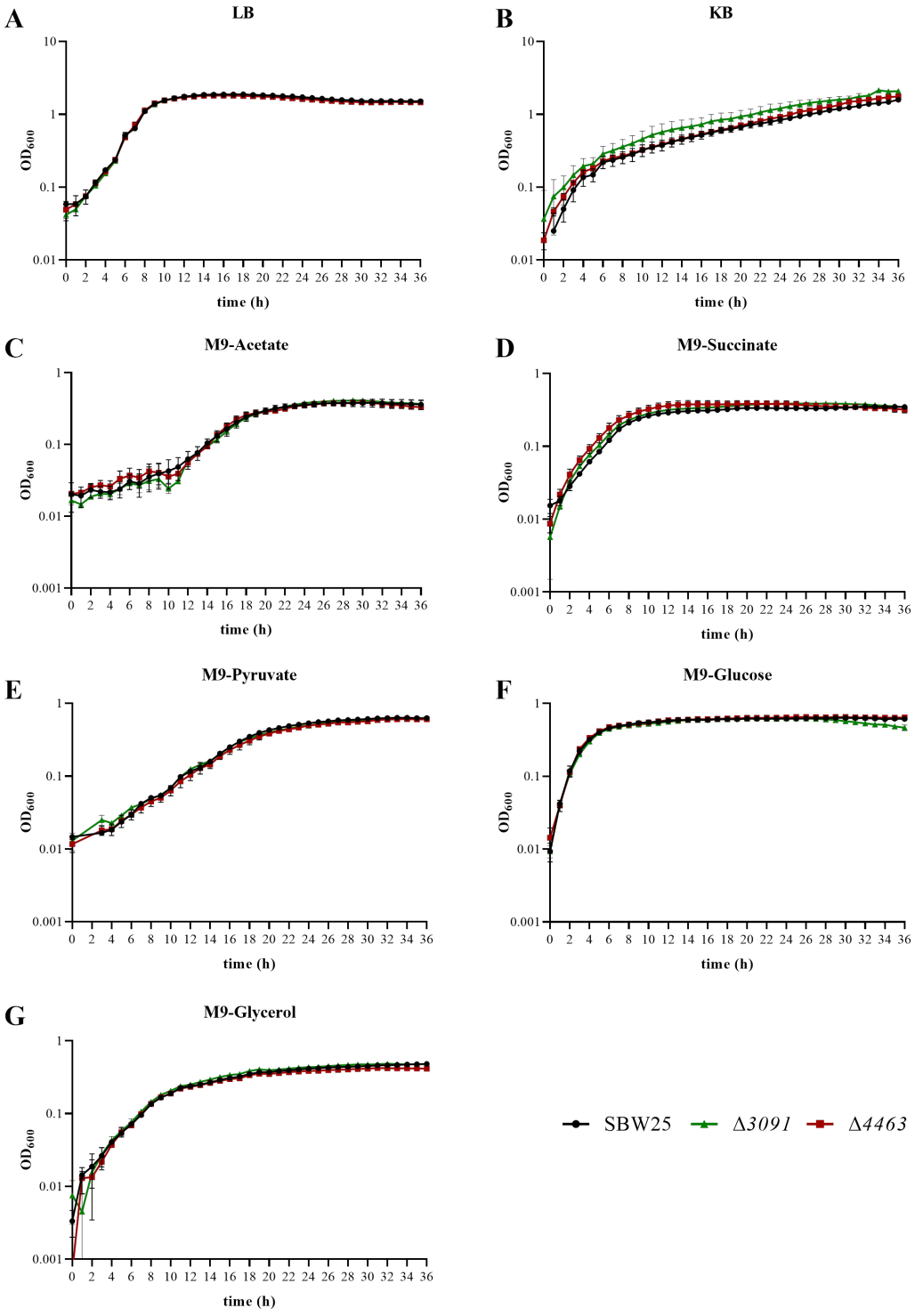

Figure 4.12. Growth curves of deletion mutants in SBW25 of genes differentially expressed in the rhizospheres of Chevallier and Tipple at 36 h**. A.** LB medium. **B.** KB medium. **C.** M9 minimal medium 0.4% acetate. **D.** M9 minimal medium 0.4% succinate. **E.** M9 minimal medium 0.4% pyruvate. **F.** M9 minimal medium 0.4% glucose. **G.** M9 minimal medium 0.4% Glycerol. 3 biological reps used per strain. Error bars are represented as SEM. Experiment was repeated twice and here a representative graph is shown.

Next, I investigated the relevance of *PFLU_3091* and *PFLU_4463* genes in cultivar-specific barley rhizosphere colonisation. This was done by performing a competitive colonisation assay in which the deletion mutants in SBW25 were tested against the WT SBW25 and the effectiveness of colonisation was recorded as the ratio of mutant versus WT. As observed in figure 4.13, both mutants showed an impaired colonisation profile of at least a 30%, however it was only significant for Δ*4463* in Chevallier rhizosphere and for Δ*3091* in Tipple rhizosphere. Interestingly, *PFLU_4463* was about three times more upregulated in Chevallier rhizosphere at 1 dpi than in Tipple's which explains the more compromised colonisation observed for this condition. On the other hand, *PFLU_3091* showed an increase in its expression of approximately 4 log2-fold change in Tipple rhizosphere when compared to Chevallier. Therefore, taken together, these results support the role in cultivar-specific colonisation of *PFLU_3091* and *PFLU_4463* in the interaction between barley and *P. fluorescens.*

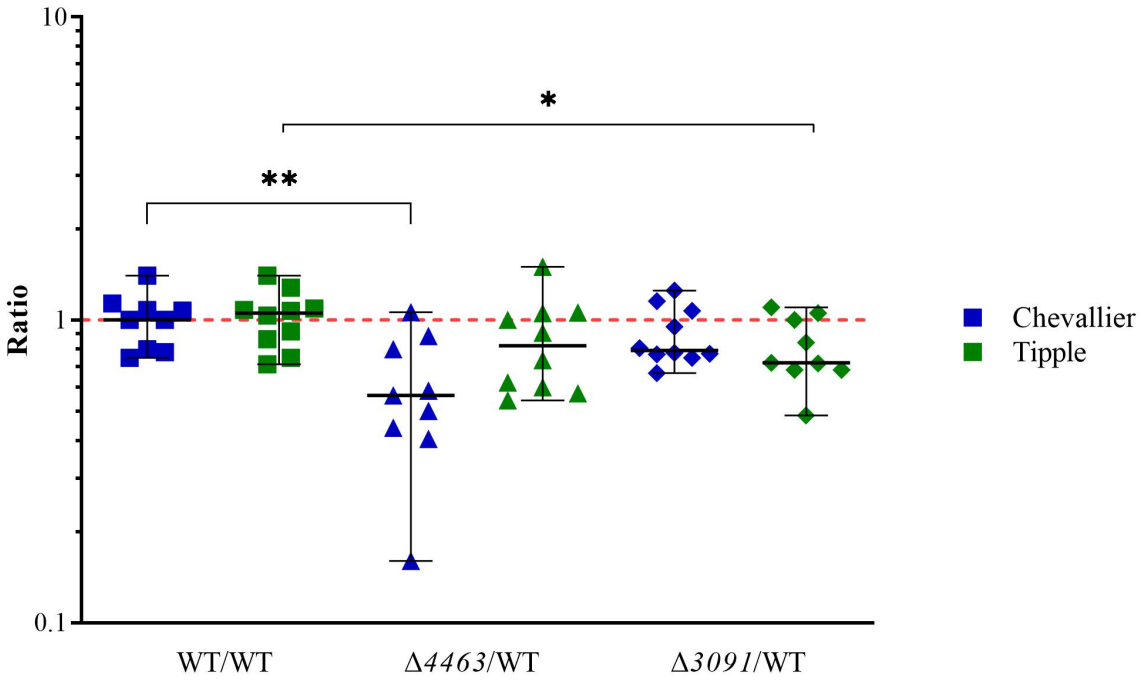

Figure 4.13. Rhizosphere colonisation competition assay with differentially expressed mutants. The graph shows the ratios of Δ*3091* and Δ*4463* to SBW25 WT-lacZ. CFUs recovered from the rhizospheres of Chevallier (blue) and Tipple (green) barley cultivars. Each dot represents the ratio of CFUs recovered from an individual plant. 8-10 plants were used per condition and *p*-values were calculated by Mann-Whitney U test, asterisks indicate $p < 0.05$ (*) and 0.01 (**). Experiment was repeated twice and here a representative graph is shown.

4.3 Discussion

In this chapter, I have aimed to elucidate the genetic mechanisms that are involved in bacterial adaptation to plant host genotype, using for that purpose the model organism *P. fluorescens* SBW25 and two different cultivars of barley, Chevallier and Tipple. First of all, by using the previously characterised metabolic mutants in *P. fluorescens* SBW25 *ΔrccR* and *ΔhexR* (Campilongo et al., 2017), I demonstrated how the ability to utilise specific carbon sources affects colonisation fitness in a plant cultivar-dependent manner. This observation was further supported by the distinct growth rates observed when the native rhizosphere *Pseudomonas* sp. of Chevallier and Tipple were grown in media containing different carbon sources. Tipple isolates displayed a shift towards glucose or fructose utilisation not observed in Chevallier derived strains. To uncover the genomic features behind this phenomenon, the genomes of two *Pseudomonas* subpopulations from Chevallier and Tipple rhizospheres were sequenced and interrogated for the presence or absence of genes potentially involved in plant rhizosphere colonisation. This approach allowed for the identification of about 60 genes whose frequency changed among the isolates of the two cultivars, indicating a putative plant-shaping effect on the soil bacterial communities. By examining individual deletions of a subset of these genes in a *P. fluorescens* SBW25 background, I identified differential carbon-dependent growth and cultivar-specific colonisation defects for some of these genes. I then investigated how the gene expression patterns in SBW25 were affected, both by time post-inoculation and plant cultivar using an RNA-seq approach, which found a set of SBW25 genes that were uniquely expressed in the rhizospheres of the two cultivars studied. Finally, I selected two of the most significantly up/downregulated genes and checked the colonisation ability of strains lacking these genes, and I showed cultivar-dependant differential colonisation rates that correlated with the expression levels observed during the RNA-seq experiment.

Metabolic regulation is a key determinant in bacterial adaptation to different ecological niches, which is especially true in soil microorganisms such as *Pseudomonas* (Cases et al., 2003, Campilongo et al., 2017). In a highly competitive environment such as the soil, the concentration of carbon sources available can define the survival of microorganisms. Hence, bacterial species must adapt to best utilise those carbon sources that enable an efficient proliferation and in order to do that, a well-regulated selection of a specific catabolic pathway over the others is fundamental (Bharwad and Rajkumar, 2019). In this work, I have shown how two *P. fluorescens* SBW25 mutants disturbed in the regulation of central carbon metabolism presented defective rhizosphere colonisation phenotypes that were dependant

on the host plant cultivar. This result confirms the importance of efficient carbon metabolic regulation for niche adaptation (Chavarría et al., 2013) and shows how two plant cultivars can effectively produce different ecological niches for rhizosphere *Pseudomonas* spp., possibly mediated by differences in the carbon content of their root exudates.

Based on the previous premise, I hypothesised that *Pseudomonas* communities adapted to a specific barley cultivar might have been selected for preferential utilisation of certain carbon sources likely to be more abundant in the rhizosphere of one cultivar than in the other. Interestingly, Tipple-derived isolates showed a better growth efficiency in glucose as the sole carbon source that was not observed in isolates from Chevallier, a phenomenon not observed when the isolates were grown with acetate as the unique carbon source. Given the fact that the preferred *Pseudomonas* carbon sources are some organic acids or amino acids (Rojo, 2010), forced adaptation for better utilisation of glucose might be a consequence of a strong selective pressure exerted by the plant host. The huge metabolic versatility present in *Pseudomonas* along with the fact that in plant associated genomes nutrient scavenging and carbohydrate metabolism is overrepresented (Silby et al., 2009a, Levy et al., 2017) make it plausible to think that communities in close contact with plants have evolved to rapidly adapt and best utilise the most abundant carbon sources in the microenvironment. For example, Baran et al. (2015) showed how seven sympatric bacteria of desert biological soil crusts presented a different ability to uptake or release specific compounds. This finding revealed largely non-overlapping substrate preferences for cooccurring soil bacteria that might influence niche compartmentalisation and, ultimately, support soil diversity.

When the genomes of the isolated *Pseudomonas* were investigated for the presence or absence of plant-colonisation related genes, I found 60 unequally distributed genes among the isolates of the two cultivars studied. Within these genes, transport and metabolism/signalling seemed to represent the most abundant categories, as already described by some authors (Silby et al., 2009a, Levy et al., 2017). In the study carried out by Levy et al. (2017), the authors identified genetic traits in bacteria that are associated to plants using a more sophisticated yet similar approach to that used in this study. They sequenced 484 plant associated genomes from several bacterial taxa and included a total of 3,873 bacterial genomes, which were then interrogated to identify plant-associated gene clusters. They showed that in plant associated genomes carbohydrate transport and metabolism were enriched across six out of nine bacteria taxa examined, including *Pseudomonas*. In addition, they empirically validated the relevance of two of the method-captured genes in rice colonisation. In another study on *Rhizobium*-pea interaction, the

authors investigated the adaptations encountered by the bacterium at different stages of the symbiotic process: rhizosphere growth, root colonisation and nodulation. In each of these stages, they identified by insertion sequencing (InSeq) specific genes relevant for bacterial success in these conditions, which were then experimentally validated *in planta* (Wheatley et al., 2020). These approaches, alongside the methodology I use here, represent neat examples of how NGS and classic molecular microbiology techniques can be combined to boost the knowledge in a complex field such as the multilevel interaction of plants and microorganisms.

To further validate the biological role of the identified genes, I selected a set of eight genes and examined single deletion mutants in the model organism *P. fluorescens* SBW25 *in planta.* Three genes, *PFLU_6072*, *PFLU_5080* and *PFLU_2583* presented compromised rhizosphere colonisation at least in one of the cultivars studied. As previously stated, *PFLU_6072* was significantly more compromised in Tipple than in Chevallier, an unexpected result given that this gene was more frequently found in Chevallier rhizosphere isolates. Therefore, a more deleterious effect would have been predicted for deletion of this gene in Chevallier than Tipple. However, it is important to notice that *PFLU6072* was found in pretty high numbers also in Tipple isolates; 21 vs 16, which may explain why its deletion caused deleterious effects in the colonisation of both cultivars. Overall, *PFLU_6072*'s role in remodelling central carbon metabolism in response to substrate availability is supported by three main observations: (i) it is annotated as a transcriptional regulator of the LysR family, (ii) it shows compromise growth in glucose, glycerol and pyruvate and (iii) it is localised in the SBW25 genome flanked by genes known to contribute to carbon metabolism and regulation, *rccR* (PFLU_6073), *rccA* (PFLU_6074), *accD* (PFLU_6070) and *oadA* (PFLU_6071) (Lai et al., 2006, Campilongo et al., 2017, Little et al., 2019). On the other hand, *PFLU_5080* was unique to Tipple rhizosphere *Pseudomonas* isolates and its deletion impaired colonisation only in that cultivar, which confirmed its relevance as a key determinant of cultivar-specific adaptation. The only hint about its putative function comes from a low confidence prediction as a restriction endonuclease and its genetic context, since it is localised flanked by *PFLU_5081*, a protein with a domain described as prophage regulatory protein and *PFLU_5079*, a phage integrase-like protein. Together, this raises the possibility that *PFLU_5080* might play a role in prophage excision, which has been described to impact bacterial host physiology in a wide range of aspects, for example, in *E. coli* it has been shown to impact biofilm formation, motility and growth in a nutrient-dependant way (Wang et al., 2009). Another work in *P. putida* showed that carrying of the prophage Pspu28 negatively impacted its competition in the rhizosphere (Quesada et al.,

2012). By which means *PFLU_5080* might impact colonisation fitness remains unclear and further work is certainly required. In last place, SBW25 lacking *PFLU_2583* showed a compromised colonisation only in the Tipple rhizosphere, environment in which was found to be more abundant. *PFLU_2583* is annotated as a putative sugar transporter, although the exact nature of this carbohydrate is unknown, it is predicted to be either inositol or ribose. This is not surprising as it has been previously described that these two substrate binding proteins may display a certain level of affinity for both substrates and they share both sequence and structural features (Herrou and Crosson, 2013). Together with the previous results observed, this suggests that an efficient sugar transport might be determinant for adaptation to the Tipple rhizosphere. To summarise, these results strengthen the hypothesis that specific genetic traits might determine the establishment and proliferation in the rhizosphere of the bacterial community associated with the plant roots.

The results obtained from the RNA-seq analysis suggest a cellular reprogramming in *P. fluorescens* SBW25, dependent on plant host cultivar, which mostly affects metabolism and transport of certain nutrients, such as amino acids. Genes that were previously identified as unevenly distributed (figure 4.5) were not found to be differentially expressed between the two cultivars. However, about half of them were indeed differentially expressed in the rhizosphere environment of both cultivars, a fact that confirms their potential roles as important genetic determinants for rhizosphere colonisation. It is fundamental to bear in mind that these two approaches were based on different organisms, while the reciprocal BLAST analysis looked at the indigenous *Pseudomonas* spp. population of the two cultivars, the RNA-seq was based on a model organism, *P. fluorescens* SBW25. SBW25 response to the environment provided by the barley cultivars might be not the same as that displayed for the original *Pseudomonas* spp. found in the barley rhizosphere. Therefore, it is not completely surprising that the phenomenon observed at a population level could not be entirely recapitulated in SBW25.

The differential changes in gene expression occur as a consequence of bacterial adaptation to their surrounding environment, a topic that has been extensively studied in the rhizosphere. For example, Nuccio et al. (2020) used metatranscriptomics to investigate microbial functions within the rhizosphere of *Avena fatua* and demonstrated that carbohydrate depolymerisation genes were consistently upregulated. They also observed that the different substrates provided by growing roots, root detritus or aging root material gathered specific microbial groups with specialised metabolic functions. A similar

observation was made in willow rhizosphere, in which genes related to carbon and amino acid uptake and utilisation were upregulated (Yergeau et al., 2014).

Given that a total of 14 *P. fluorescens* SBW25 genes were differentially expressed in the rhizospheres of Chevallier and Tipple barley cultivars, I decided to further characterise their role in adaptation to the specific environment provided by each of the cultivars. To this end, *PFLU_3091* and *PFLU_4463*, two of the most differentially expressed genes, were deleted in SBW25 and their rhizosphere colonisation, assessed. *Δ3091* presented a colonisation penalty in both cultivars, but in comparison to the WT strain, such penalty was only significant in the rhizosphere of Tipple, where it was originally found to be highly expressed. *PFLU_3091* is annotated as an amino acid permease belonging to the APC superfamily. This superfamily can be found in both eukaryotic and prokaryotic cells and includes members that function as solute: cation symporters and solute: solute antiporters. The substrate specificity of the permeases within this family is quite variable as some have a broad specificity, whereas others show affinity for one or a few amino acids or related compounds (Jack et al., 2000). The importance of amino acid uptake in cultivar adaptation has been previously shown in common bean (Mendes et al., 2019). In this work, the authors investigated the rhizosphere microbiome of common beans cultivars varying in their resistance level to *Fusarium oxysporum*. They showed that breeding for resistance against this pathogen unintentionally also selected for changes in the microbiome composition and functions. As a consequence, the resistant cultivar recruited a microbial consortium with a preference for amino acid and amine utilisation (Mendes et al., 2019). The two cultivars studied here also differed in their resistance profile against several fungal diseases, including some *Fusarium* spp. Chevallier has been shown to be more resistant against *F. culmorum*, one of the casual agents of FHB, therefore considering the results seen for Mendes and co-authors, a similar phenomenon would have been expected (Muhammed, 2012, Goddard et al., 2019). Nevertheless, it is important to highlight that the mechanisms of microbial recruitment in the rhizosphere depend hugely on the plant species (Bulgarelli et al., 2013). In addition, given that several FHB QTLs have been identified throughout the barley genome (chromosomes 2, 3, 5, 6 and 7) (Huang et al., 2018) and that Chevallier and Tipple represent different stages in the breeding history (Goddard et al., 2019), it is feasible to hypothesise that co-segregation of loci involved in FHB resistance and some intentionally selected agronomic traits (height, yield, etc.) might have not co-occur in Tipple. The *PFLU_4463* deletion mutant showed an impaired colonisation phenotype highly significant in the rhizosphere of Chevallier, cultivar in which its expression seemed to be more highly upregulated than in Tipple. *PFLU_4463*, is described as a permease with a EamA domain.

As mentioned above, it is considered to belong to the drug/metabolite transporter superfamily which can facilitate export of drugs, nutrient uptake, efflux of nutrients and metabolites and intraorganellar metabolite exchange in eukaryotes (Jack et al., 2001, García Angulo et al., 2013). For example, in the obligate intracellular parasite causal agent of typhus, *Rickettsia*, a EamA transporter is responsible for the direct uptake from the host of the essential metabolite S-adenosylmethionine (Driscoll et al., 2017). This reinforces the idea of *PFLU_4463* role as a transporter, although the nature of the specific substrate remains unknown. Altogether, these results suggest that specialised nutrient transport is a crucial trait for *P. fluorescens* adaptation to the rhizosphere of different barley genotypes.

In conclusion, I have corroborated the importance of *P. fluorescens* SBW25 carbon metabolism regulation for efficient colonisation of the plant rhizosphere, a trait that I have demonstrated to be key in the colonisation of different barley cultivars. By interrogating the genomes of *Pseudomonas* isolated from the rhizospheres of Chevallier and Tipple, I have identified and validated *in planta* potential bacterial genetic signatures that are unique to a specific plant cultivar. This demonstrates that bacterial shaping in the rhizosphere not only occurs at the species or strain level, but also at a molecular level in which specific traits are favoured or selected over others in response to niche adaptation. Finally, by analysing the gene expression levels of *P. fluorescens* SBW25 recovered from the rhizospheres of Tipple and Chevallier, I was able to identify genotype-specific differentially expressed genes. This study provides the foundation to characterise and unravel the molecular mechanisms underpinning specific adaptation of bacteria to plant genotypes.

CHAPTER 5:

Role of root exudates in controlling barley rhizosphere colonisation by *Pseudomonas* spp.

5.1. Introduction

5.1.1 Synthetic communities: a tool to investigate plant-microbe interaction

Over the last few years, the number of studies trying to characterise the microbiome composition has dramatically increased. In order to understand the dynamics behind this process, the design of synthetic bacterial communities or SynComs has gained popularity among the scientific community. By definition, SynComs are consortia of microorganisms artificially designed to model the structure and dynamics of the natural community, albeit with a reduced complexity, capable of providing a broad set of different functions that would not be achievable by a single microorganism (Großkopf and Soyer, 2014, de Souza et al., 2020). The use of SynComs facilitates the study of the interaction between microbes and the plant through the addition, removal or change of specific microorganisms within the community. This makes it possible to address specific questions about the functional roles of individual microorganisms, the effects that they exert on the plant host, and vice versa.

Pfeilmeier et al. (2021) combined a set of key plant immunity mutants and a SynCom of 222 members to study the role of the plant immune system not only in pathogens but in the overall leaf microbiota in *A. thaliana* assembly process. They demonstrated a critical role of an immunity-related NADPH oxidase, RBOHD, for homeostasis maintenance in the leaf microbiota.

Other studies have explored the idea of utilising SynCom to improve specific phenotypic traits in the plant host, for instance, plant growth (Armanhi et al., 2018), regulation of phosphate accumulation (Herrera Paredes et al., 2018) or resistance against certain pathogens (Niu et al., 2017). Synthetic communities have been also used to study the role of plant genotype on bacterial community assembly. When different *A. thaliana* accessions were inoculated with a SynCom formed by seven different bacterial species, it was observed that natural accessions harboured a different community composition compared to the lab accession, Col-0 (Bodenhausen et al., 2014). Another important application of SynComs is to elucidate the role of root exudates in the recruitment of rhizosphere microbial communities (Zhalnina et al., 2018).

5.1.2 Root exudates and its role in microbiome assembly

Many studies have highlighted the relevance of specific plant factors in shaping belowground microbial communities, among which root exudation seems to exert one of the primary effects (Bulgarelli et al., 2013, Philippot et al., 2013). Root exudates refer to an array of substances present in the rhizosphere that are secreted by living roots. This includes both secretions -actively released from the roots-, and diffusates -passively delivered due to osmotic differences (Bais et al., 2006). It is estimated that plants might secrete up to 21 % of their total fixed carbon during photosynthesis, which certainly represents a major cost to the plant (Tkacz and Poole, 2015). Root exudates can be divided into two main classes: low-molecular weight compounds (LMW-C) and high-molecular weight compounds (HMW-C). LMW-C are responsible for a great deal of the diversity observed in root exudates and they include simple sugars, glucose or fructose; amino acids, alanine or glycine; carboxylic acids, citric acid or succinic acid; and other secondary metabolites. On the other hand, HMW-C are less diverse and are mainly represented by mucilage and proteins (Bais et al., 2006).

Plant exudates have an array of different functions. They can participate in plant-plant interactions, modulating these both negatively (allelopathy), and positively, e.g., by inducing herbivore resistance. In addition, they play a fundamental role in modulating plant-microbe interaction processes in the rhizosphere. In this latter category, the role of exudates in stimulating nodulation of legumes by rhizobia and mycorrhizal associations are the two best studied examples. In the first example, flavonoids are recognised by the rhizobial partner, which triggers the signal cascade responsible of nodule formation. In mycorrhization, strigolactones are believed to be the initiation signal (Bais et al., 2006, MacLean et al., 2017).

The exogenous addition of glucose, glycine and citric acid to several soil microcosms has been shown to affect the composition of bacterial communities, especially in Beta-Proteobacteria, Gamma-Proteobacteria and Actinobacteria. Although in this study *in planta* assays were not performed, the authors were able to show a link between molecules commonly present in root exudates and bacterial substrate preference as a driver of the community composition (Eilers et al., 2010).

More recently, a study in *Avena barbata* combined genomics, metabolomics and SynCom experiments to investigate the effect of plant growth on the community composition. First of all, the authors showed how a SynCom of 39 grassland soil isolates was differentially

affected by plant growth, producing a split in the bacterial community based on their response to root growth over time. Positive and negative responders presented functional features in their genomes that were involved in substrate utilisation, an observation that was later corroborated by exometabolomics. Finally, the authors demonstrated how the metabolomic profile of *Avena* root exudates was highly dynamic and depended on developmental stage. Altogether, these results suggest that bacterial communities and plants might synchronise in order to selectively recruit those microorganisms with a certain substrate uptake preference, which ultimately results in differential assembly of the bacterial communities (Zhalnina et al., 2018). Something similar could be observed in a study by Worsley et al. (2021), in which $^{13}CO_2$ DNA stable isotope probing was used to measure the substrate utilisation process in *A. thaliana*. This technique consists in the incubation of the plants with $^{13}CO_2$ which will fix this so-called "heavy" isotope of carbon to later release it as part of the natural root exudation process. Bacteria inhabiting the plant endosphere and rhizosphere will feed on the labelled carbon molecules derived from the plant and as a consequence, they will incorporate the ^{13}C into their DNA. By performing amplicon sequencing on the labelled (heavy) vs non-labelled (light) DNA, they could identify those members of the microbiome being fed by *A. thaliana* exudates. The authors showed that fast-growing Proteobacteria, especially *Pseudomonas*, utilised more efficiently root exudates than other members of the microbiota, such as *Streptomyces*. These results reinforce the hypothesis that uptake of root exudates by the different members of the rhizosphere microbiome is very dynamic and directly impacts the final structure of the microbial consortium (Worsley et al., 2021).

5.1.3 Quantitative trait loci (QTL) analysis in plant studies

Many important agronomic traits are quantitatively inherited, which means that their expression is controlled by hundreds or thousands of QTL with a relative individual small effect on the trait. QTL analysis is a statistical method that links phenotypic data for one trait measured, such as yield, height or disease resistance, and genotypic data, normally molecular markers, with the goal of identifying the genetic basis of variation in complex traits. In order to perform this analysis, lines presenting differences in the trait studied are required (Miles and Wayne, 2008, Würschum, 2012). Typically, these parental lines will be crossed to obtain a suitable mapping population. The most commonly used type of segregating population is one formed by recombinant inbred lines (RILs). This population is achieved by repeated self-crossing from a F_1 hybrid until fully homozygous individuals are achieved, typically

reaching a F_5 at least. The individual lines will contain approximately a 50% of each parental genome in different combinations (Dekkers and Hospital, 2002, Pollard, 2012).

The development of genomic tools and the explosion in next generation sequencing (NGS) have made possible the implementation of marker-assisted selection (MAS). MAS is an indirect selection process of a specific trait using molecular markers linked to the trait of interest (Würschum, 2012). The choice of the most suitable molecular marker is an important part of the breeding process as it will depend on the trait to be studied and the conditions of the experiment. In the case of barley, the most used molecular markers in the two last decades have been restricted fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), expressed sequence tags (ESTs), transposon-based markers (IRAP, iPBS) and single-nucleotide polymorphisms (SNPs). Although SNPs are still the most commonly used type of marker, NGS has facilitated the design of markers based on sequence, rather than being based on fragment length (Bevan et al., 2017, Gozukirmizi and Karlik, 2017).

In this chapter I aimed to answer two main questions. Firstly, I hypothesised that given the differential recruitment of bacterial communities (refer to Chapter 3, sections 3.2.2 to 3.2.4), differential growth promotion effects on the two cultivars studied would be observed when inoculated with their non-native microbial communities. In order to test this, I performed rhizosphere and *Pseudomonas* SynCom swaps in Chevallier and Tipple barley cultivars and measured plant dry weight as a growth promotion output. Secondly, I hypothesised that barley cultivars would exert an influence on root exudates composition, and this would impact on microbial community shaping. To investigate this, I conducted *in planta* screening using luminescence bacterial biosensors and *in vitro* GC-MS analysis. Finally, given the importance of specific metabolites to microbial community dynamics, and given that the genetic resources for QTL mapping were available, I investigated the genetic determinants of microbiome-shaping sugar secretion in barley.

5.2. Results

5.2.1. Cross inoculation assay

In order to determine the specificity of the interactions between microbial communities and plants and to measure the plant growth promoting effects of the rhizosphere communities inhabiting the roots of the two cultivars studied, a cross inoculation experiment was designed. I tested the effect on two different cultivars of two distinct microbial communities: the overall rhizosphere consortium and a *Pseudomonas* SynCom, formed from the previously isolated barley rhizosphere *Pseudomonas* strains (see Chapter 3 for details). Briefly, both cultivars were axenically inoculated with either a Chevallier rhizosphere microbiome extract (CRh) or a Tipple rhizosphere extract (TRh) and with two SynComs of Chevallier-derived *Pseudomonas* (CPs) or Tipple-derived *Pseudomonas* (TPs). After three weeks, plants were harvested, and their dry weights (DW) were recorded. In figure 5.1 two replicates of this experiment are shown. The bar charts show the different combinations of the conditions tested and the results are summarised as the DW of the whole plant relative to an uninoculated control. First of all, Chevallier and Tipple plants were challenged with the rhizosphere microbiome extracts, both endogenous and exogenous. When Tipple plants were inoculated with CRh (TxCRh), an average growth deficit of 7 % was observed. However, Tipple plants inoculated with their native rhizosphere community (TxTRh) showed a slight increase in growth in comparison to a Tipple uninoculated control, of about 12% on average. On the other hand, Chevallier plants showed a better response to the rhizosphere extracts, which seemed to be independent of the inoculum origin. When Chevallier plants were inoculated with CRh and TRh, average increases in DW of approximately 45% and 30%, respectively, were observed.

Secondly, the two cultivars were inoculated with the two rhizosphere *Pseudomonas* SynComs to test whether *Pseudomonas* isolates alone were able to recapitulate the effects seen for the overall rhizosphere microbiota. Whereas Chevallier inoculated with either CPs or TPs showed a growth increase in both cases, 15 % and 20% in average, Tipple's response to the *Pseudomonas* SynComs was negative in both cases, even with its own SynCom, TPs.

Together, these results suggest that Tipple is less capable of coping with a set of microorganisms not adapted to its own rhizosphere and that *Pseudomonas* SynComs seem to promote plant host growth in Chevallier cultivar, but not so in Tipple. This cultivar might

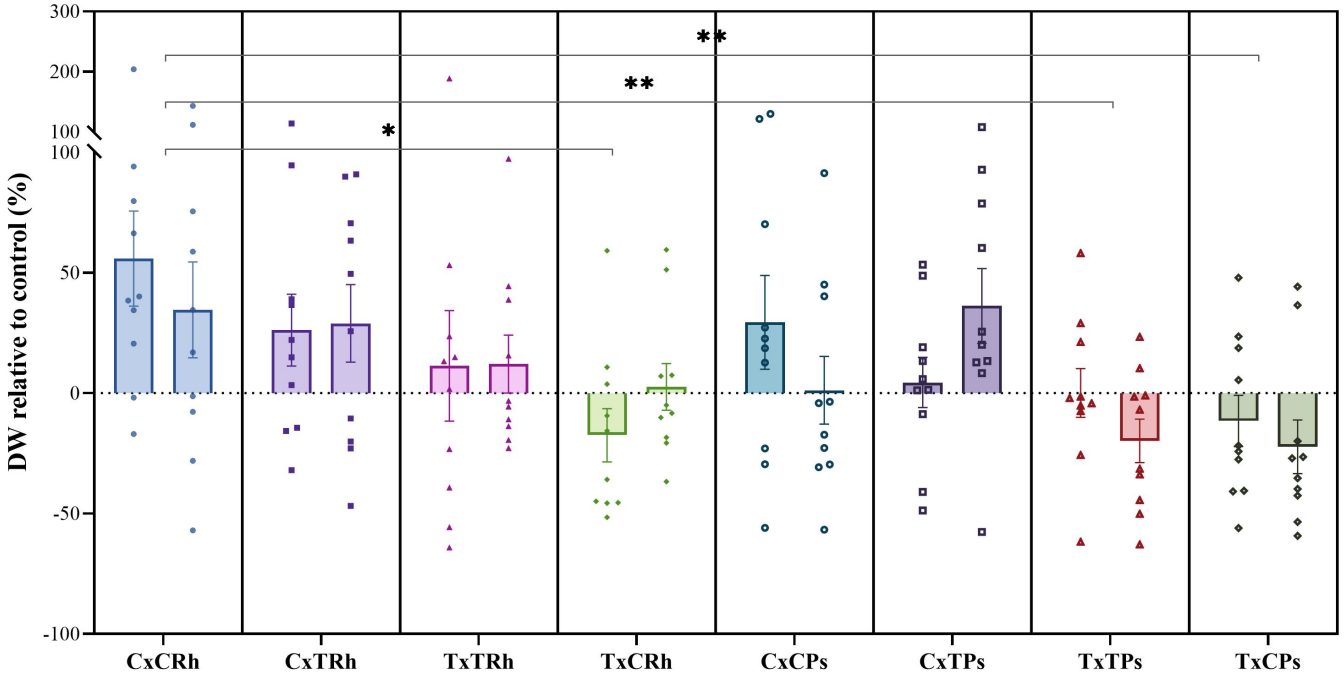

Figure 5.1. Cross-inoculation assay between Chevallier and Tipple. Chevallier (C) or Tipple (T) seedlings were inoculated under controlled conditions either with a rhizosphere extract (CRh/TRh) or with a SynCom of rhizosphere *Pseudomonas* (CPs/TPs). The graph summarises their dry weights (DW) at 3 weeks post-inoculation relative to the uninoculated controls. Two biologically independent repetitions of the experiment are represented side by side and up to 10 plants were used per condition. Data are presented as mean +/- std error. *p*-values were calculated by Tukey's multiple comparison test and asterisks indicate $p < 0.05$ (*), 0.01 (**).

provide a more hostile rhizosphere environment for non-adapted communities to establish and in general, it seems to be less responsive to microbial stimuli.

Given the fact that these responses seemed to be very distinct from one another, I wanted to investigate which plant determinants were responsible for the differential behaviour observed. Many studies have pointed to the role of root exudates composition as an important factor in shaping rhizosphere microbial communities (Sasse et al., 2018, Zhalnina et al., 2018). Therefore, in the following sections of this chapter the root exudates composition of Chevallier and Tipple will be dissected.

5.2.2. Bioluminescence assisted screening of root exudates *in vivo*

As an initial attempt to understand the nature of the root exudates of Tipple and Chevallier, an *in vivo* temporal mapping was performed using luminescent biosensors. These biosensors were published by Pini et al. (2017) and Rubia et al. (2020), and in these works, the authors developed a set of *Rhizobium leguminosarum* bv. viciae 3841 (Rlv3841) based biosensors able to sense specific solutes *in planta*. These biosensors were constructed by transcriptional fusions of the promoter regions upstream of genes known to be induced in the rhizosphere to the *lux*CDABE operon in a plasmid-based system (Frederix et al., 2014). The biosensors can be grouped by classification of inducer: (1) sugars and polyols, (2) organic acids, (3) amino acids, and (4) flavonoids. In this work, the flavonoid-induced biosensors were not utilised since these are specific to the nodulation process, which does not take place in barley. In total, 14 biosensors were used here: xylose, fructose, sucrose, erythritol, mannitol, *myo*-inositol, formate, malonate, C4-dicarboxylates, tartrate, salicylic acid, phenylalanine, proline and GABA.

Chevallier and Tipple seedlings were inoculated with the different *lux* biosensors (Rlv3841_*lux*) as described in the material and methods section 2.12 and images were acquired and analysed at 2, 5 and 7 days post-inoculation (dpi). In figure 5.2 results grouped by cultivar and time point are shown. One of the most obvious observations is the signal reduction over time for most of the biosensors used, which is probably due to the growth system used. Both plant and bacteria can suffer nutrient limitation and ultimately, this might affect the root exudation pattern over extended experimental times. At 2 dpi, Chevallier exudates showed greater amounts of C4-dicarboxylates, formate and tartrate; whereas for Tipple, the most abundant metabolites were fructose, sucrose and GABA. At 5 dpi, the most abundant metabolites in Chevallier exudates were C4-dicarboxylates, formate and fructose;

and in Tipple, fructose, C4-dicarboxylates, tartrate and sucrose. Finally at 7 dpi, the most abundant Chevallier exudates were C4-dicarboxylates, fructose and tartrate; in the case of Tipple, proline, fructose and sucrose were the most predominant molecules. Since this experiment was performed as a rapid initial screening of the general exudation profile of the two cultivars, only 2 plants per condition were used and therefore, more robustness in the data is necessary. Hence, a subset of Rlv3841_*lux* biosensors was selected and the experiment was repeated.

Fructose, C4-dicarboxylates, sucrose and phenylalanine biosensors were selected based on two criteria: (1) their previously observed abundance in the rhizospheres of Chevallier and Tipple and (2) to represent the groups of molecules often found in the rhizosphere; sugars, organic acids and amino acids (Eilers et al., 2010). Again, plants were evaluated at 2, 5 and 7 dpi and a summary of the results can be found in figure 5.3. The fructose biosensor was the most differentially expressed among the whole dataset, especially in Tipple at 2 dpi. For the rest of the biosensors, I could not detect significant differences. Nonetheless, it is interesting to highlight that the Tipple rhizosphere might be enriched in sugar-like compounds, such as fructose or sucrose and in contrast, Chevallier exudates may contain a higher concentration of C4-dicarboxylates and perhaps a more balanced overall exudate composition. These results are consistent with the microbial colonisation patterns observed in Chapter 4 (section 4.2.1), where two *P. fluorescens* SBW25 metabolic mutants showed a distinct colonisation pattern for Chevallier and Tipple. These mutants present growth deficiencies that are dependent on the carbon source available and therefore, the hypothesis that the differences in colonisation fitness might be due to unequal concentrations of some metabolites is strengthened by these results.

Although the *in planta* screening provided an efficient method to detect differences in root-derived molecules, it is constrained by the availability of a particular biosensor, making it impossible to detect molecules not included in the initial experimental design. Thus, I decided to explore the root exudates diversity in more detail by gas chromatography–mass spectrometry (GC-MS).

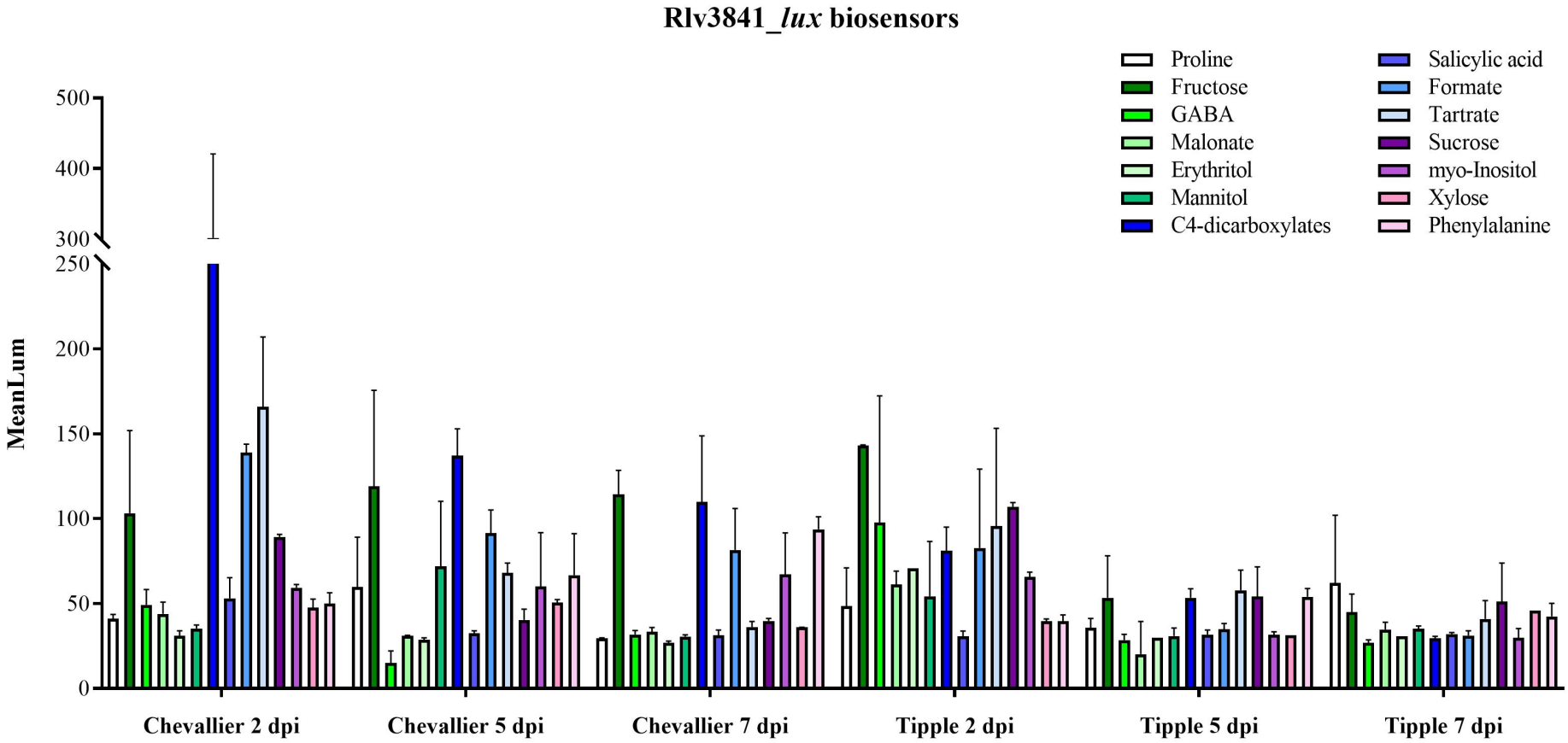

Figure 5.2. *In planta* temporal screening of root metabolites produced by Chevallier and Tipple with luminescent biosensors. Luminescent biosensors based on WT Rlv3841 (Pini et al., 2017) were inoculated in the roots of 2 days old Chevallier and Tipple seedlings. Images were acquired at 2, 5 and 7 dpi and 2 plants were used per condition. Mean values of luminescence intensity are shown in the graph. Error bars are represented by SEM. *p*-values not calculated due to the small sample size (2). Experiment was repeated twice and here a representative graph is shown.

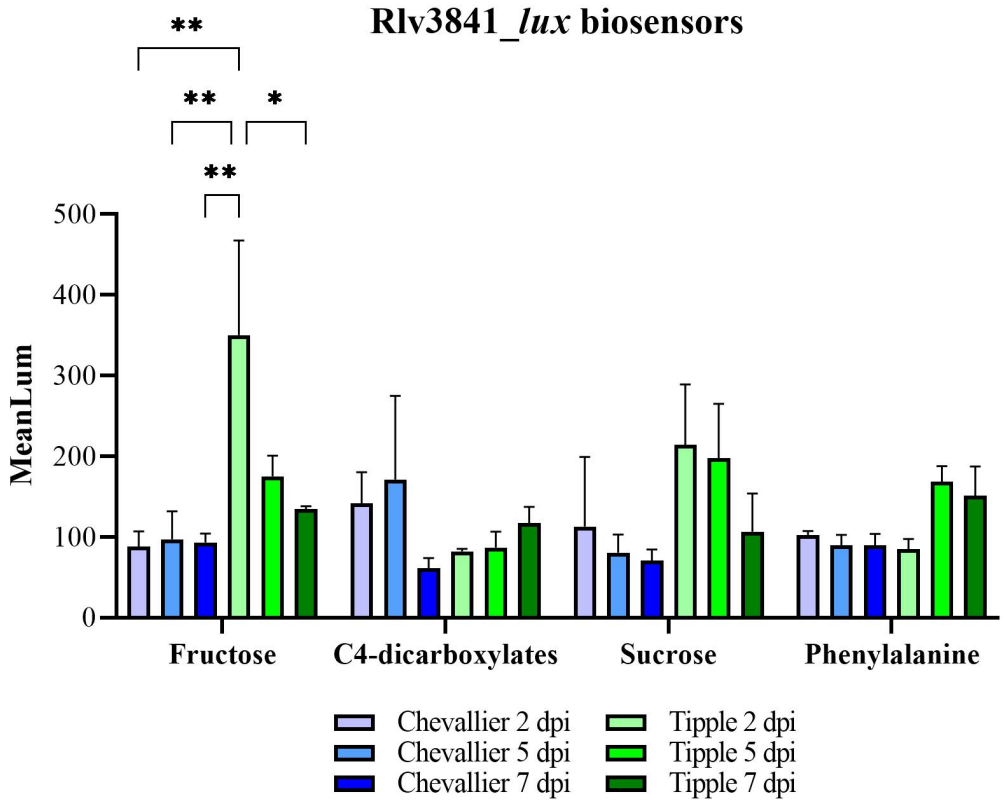

Figure 5.3. *In planta* temporal screening of root metabolites produced by Chevallier and Tipple with luminescent biosensors. Fructose, C4-dicarboxylates, sucrose and phenylalanine biosensors based on WT Rlv3841 (Pini et al., 2017) were inoculated in the roots of 2 days old Chevallier and Tipple seedlings. Images were acquired at 2, 5 and 7 dpi and 3 plants were used per condition. Mean values of luminescence intensity are shown in the graph. Error bars are represented by SEM. 3 plants were used per conditions, p-values were calculated by Tukey's multiple comparison test and asterisks indicate $p < 0.05$ (*), 0.01 (**). Experiment was repeated three times and here a representative graph is shown.

5.2.3. Root exudates analysis by GC-MS

GC-MS is a powerful analytical method that enables the identification of different substances present in a given sample. It is recommended to detect small volatile and semi-volatile compounds and it is especially suited for untargeted metabolite identification, which makes it suitable for the purposes of this study.

Chevallier and Tipple barley plants were grown for 3 weeks under axenic conditions and their root exudates were extracted and analysed by GC-MS. Between three and four biologically independent repetitions were used, which were each based on a pool of four plants to decrease sample variability and to increase sample concentration. The analysis was repeated four times and a representative example is shown here (figure 5.4, figure S2). In order to reduce the variability between the replicates and to make appropriate comparisons, the dataset was normalised and the median of all samples was established as a baseline. The data shown here is the log2 normalised abundance in comparison to this median. Only those samples with a log2-fold change of more than two were included to avoid noise background. By this approach, a total of 105 entities could be detected (Tables S1 and S2). In figure 5.4A the overall exudates composition is represented by a heat map. Using this heat map, it is easy to observe at first glance how distinct the two profiles are for some of the compounds studied. For example, Chevallier root exudates seemed to have a greater concentration of compounds 52 to 105, the range in which the majority of the unidentified metabolites were found. Although the heat map is a very visual tool to represent changes in abundance, I also decided to represent this dataset in a linear profile plot in order to individually visualise the abundance differences between the root exudates of Tipple and Chevallier (figure 5.4B). The most striking difference between the two cultivars was a derivative of phosphoric acid massively present in Chevallier, 16.474 and not in Tipple, -12.613. Although less impressive, it is worth mentioning the abundance difference found for D-glucose, whose log2 abundance was 4.389 in Tipple, the second most abundant metabolite, and -2.405 in Chevallier.

To look at the individual metabolites and analyse separately those identified by the libraries used from the unidentified ones, two graphs were created (figure 5.5A and B). Figure 5.5A represents those compounds (a total of 59) that showed a match with any of the libraries used, classified by categories: carbohydrates, organic acids, amino acids and others. In Tipple root exudates, the most abundant sugars were D-glucose and an erythrose derivative,

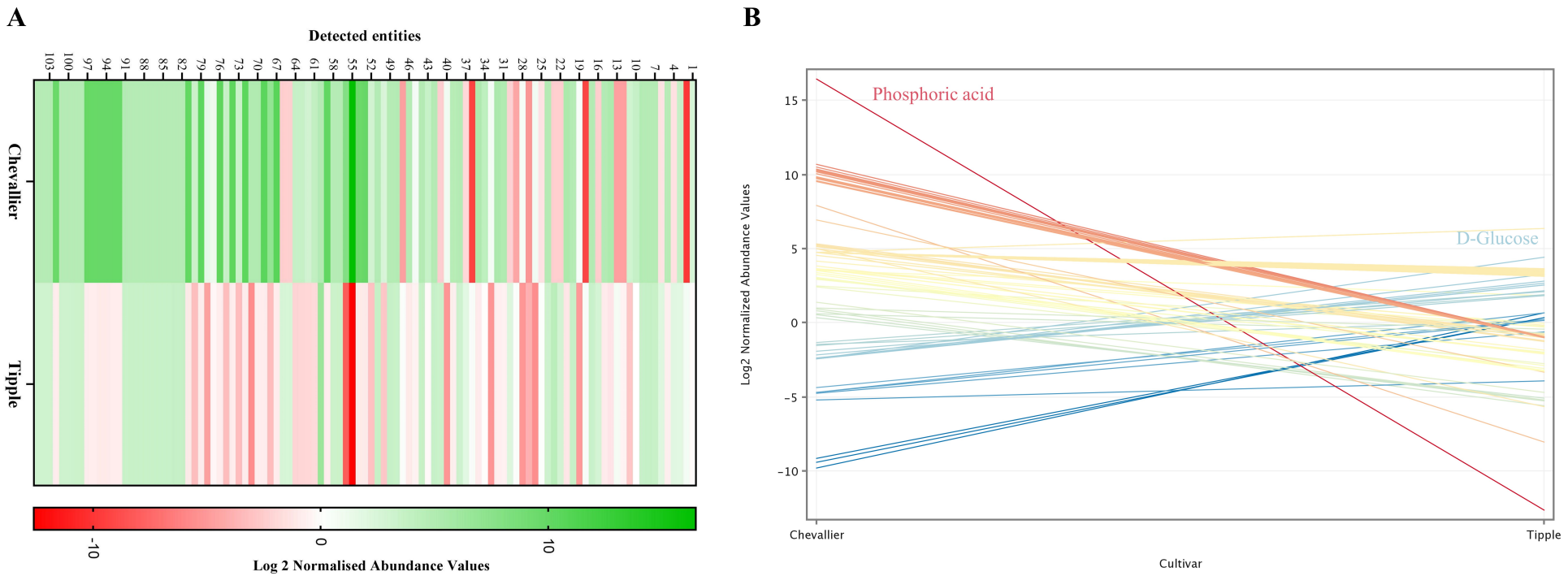

Figure 5.4. Chevallier and Tipple root exudates entities detected by GC-MS. A. Heat map of the overall root exudates composition presenting a log2-fold change ≥| 2 in at least one cultivar. B. Linear profile plot of the compounds presenting log2-fold change ≥ 2 in at least one cultivar. A total of 105 compounds were detected. Normalisation of the samples was done by the 75th percentile shift method to take the medians of only reliable intensity values and reduce the noise caused by sample variation and instrument response. The abundance for each compound was baselined to the median of all samples. Sample per condition, n=3.

D-erythrose-4-phosphate; 6-hydroxycaproic acid and gluconic acid were the most prevalent organic acids; the most common amino acid was a threonine derivative, L-allothreonine; and lastly, among the compounds labelled as “others”, pipecolic acid and a nitrogenated compound, propanamine, were notably present. In Chevallier exudates, the predominant carbohydrates were sedoheptulose and D-sphingosine; regarding amino acids, L-glutamic acid 2, a derivative of glutamate, and beta-alanine stood out. The most abundant organic acids were a phosphoric acid-like molecule, phosphoric acid 2, and pelargonic acid; and within the other metabolites, a benzoic acid, m-toluic acid, and an isocyanate, tert-butyl isocyanate presented the greatest abundances.

Figure 5.5B shows those metabolites that could not be matched with any molecule present in the libraries used, 46 in total. As mentioned above, one of the most remarkable observations is the overall greater abundance of these metabolites in the root exudates of Chevallier, for example compounds 8, 10 or 13 were present almost exclusively in this cultivar. Likewise, there were a smaller group of compounds that were more abundant in Tipple than in Chevallier exudates, for example compounds 6 and 7.

This analysis provides a good general overview of root exudates composition of the two cultivars studied, Tipple and Chevallier. Considering the results showed previously, it is plausible to think that these two cultivars differ in the secretion of certain molecules, especially in sugars and organic acids, which might lead to a differential recruitment of root-associated microorganisms, including *Pseudomonas* spp

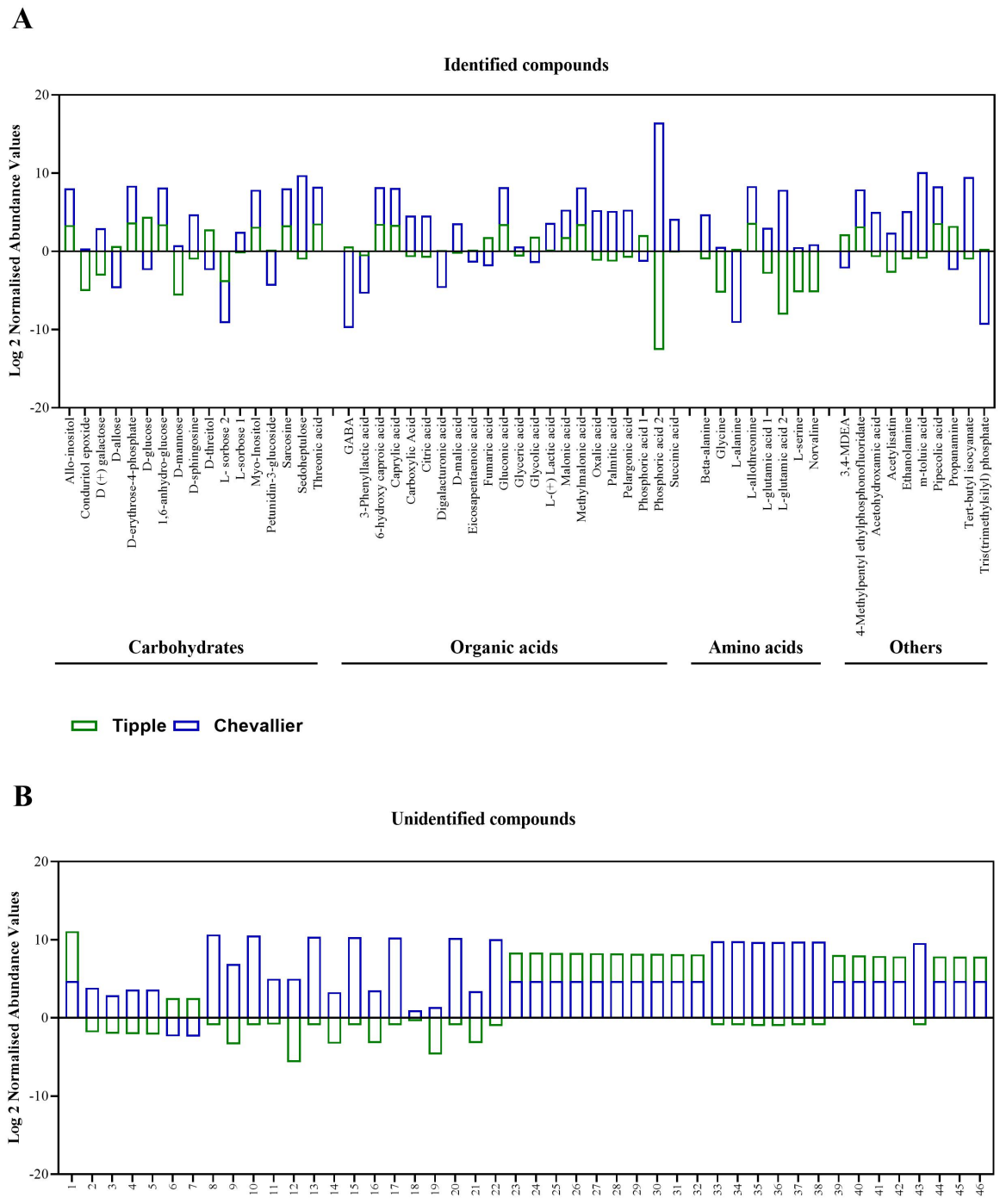

Figure 5.5. Differential abundance of detected compounds in the root exudates. A. Compounds showing a match in the library. B. Compounds without a match in the library. Entities detected in Chevallier roots exudates are represented in blue and Tipple, in green. The two graphs are only based on those compounds showing a log2-fold change ≥ 2 in at least one cultivar. Normalisation of the samples was done by 75th percentile shift method to take the medians of only reliable intensity values and reduce the noise caused by sample variation and instrument response. The abundance for each compound was baselined to the median of all samples. Sample per condition, n= 3.

5.2.4. QTL mapping of CxT RIL F_7 for sugars exudation

The fructose luminescent biosensor (Rlv3841_*lux*::fructose) presented a markedly different signal when inoculated on Chevallier and Tipple roots, and its variability seemed to be smaller than other biosensors used. In addition, this biosensor responded to a broader range of inducers making it able to detect the secretion not only of fructose but also lactulose, mannitol, mannose, sorbitol and sucrose. These features made it a good candidate to be utilised as a screening tool for QTL mapping of interesting traits linked to microbial shaping in the rhizosphere.

In order to find the genetic mechanisms of the differences in fructose-like molecules between the two cultivars studied, a previously published and genotyped mapping population was used (Goddard et al., 2019). The Chevallier and Tipple crossing population (CxT) is a F_7 RIL population formed by 188 individual lines, from which 110 were analysed in this work.

CxT lines were divided into batches of 20 plants, with the exception of the first one that contained 10, and three seedlings per line were used. Luminescence emitted by the fructose biosensor was evaluated at 4 dpi, as the variation at this time point seemed to be smaller and the differences observed were stronger based on previous analyses (figure 5.4). In figure 5.6, the six batches of CxT lines are represented separately to provide a general overview of the differences observed. Lines studied presented a variation in fructose secretion that oscillates from almost nothing -lines 12, 519 or 642- to high -lines 140, 484 or 646. This data set was normalised (Material and Methods, section 2.12) and the mean luminescence values used for the QTL analysis are shown in table 5.1.

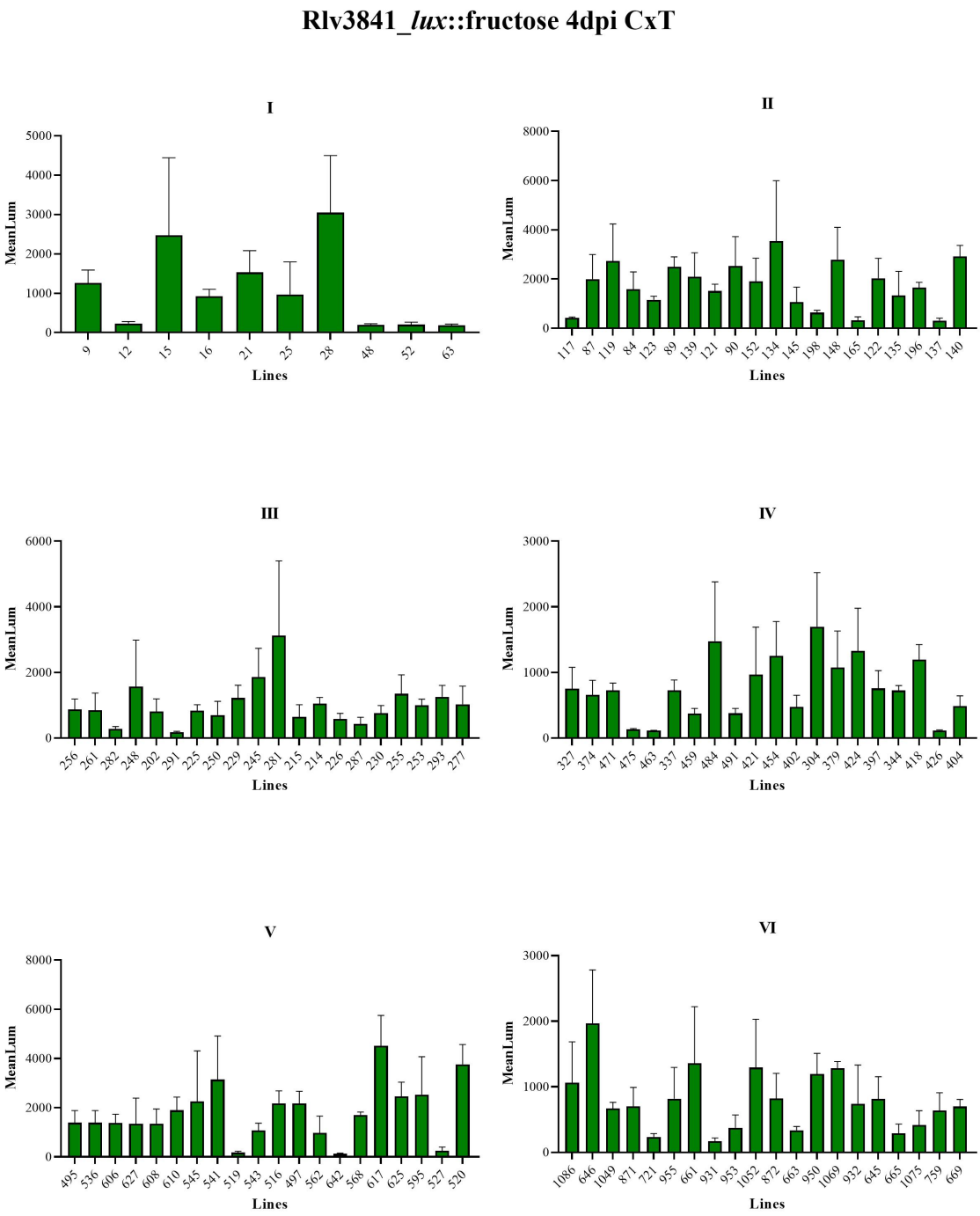

Figure 5.6. Screening of a CxT mapping population (RIL, F_7) with Rlv3841_*lux*::fructose biosensor. A fructose biosensor based on WT Rlv3841 (Pini et al., 2017) was inoculated onto the roots of 2 day old seedlings. Images were acquired at 4 dpi and 3 plants were used per condition. Mean values of luminescence intensity are shown in the graph. Error bars are represented by SEM. 110 lines of the CxT population were studied in batches of 20 lines, with the exception of the first 10 lines. The six batches are shown as separate graphs.

Table 5.1 Normalised mean values of Rlv3841_*lux*::fructose luminescence intensity of all the CxT lines tested. Error is represented as SEM. See material and methods 2.12 for normalisation method.

Line	Normalised mean		
9	0.407	±	0.120
12	0.030	±	0.018
15	0.851	±	0.720
16	0.283	±	0.065
21	0.506	±	0.202
25	0.300	±	0.303
28	1.061	±	0.532
48	0.018	±	0.009
52	0.020	±	0.023
63	0.014	±	0.010
117	0.100	±	0.012
87	0.675	±	0.367
119	0.945	±	0.552
84	0.525	±	0.257
123	0.367	±	0.054
89	0.859	±	0.149
139	0.712	±	0.357
121	0.498	±	0.102
90	0.871	±	0.439
152	0.643	±	0.344
134	1.244	±	0.898
145	0.335	±	0.224
198	0.178	±	0.034
148	0.967	±	0.481
165	0.062	±	0.054
122	0.688	±	0.299
135	0.434	±	0.358
196	0.550	±	0.078
137	0.060	±	0.035
140	1.014	±	0.165
256	0.265	±	0.117
261	0.257	±	0.191
282	0.050	±	0.025
248	0.520	±	0.520
202	0.243	±	0.140
291	0.013	±	0.008
225	0.250	±	0.068

Line	Normalised mean		
250	0.203	±	0.153
229	0.394	±	0.142
245	0.628	±	0.318
281	1.090	±	0.834
215	0.182	±	0.137
214	0.329	±	0.070
226	0.161	±	0.061
287	0.104	±	0.074
230	0.225	±	0.084
255	0.440	±	0.084
253	0.311	±	0.212
293	0.406	±	0.069
277	0.323	±	0.128
327	0.221	±	0.203
374	0.187	±	0.119
471	0.212	±	0.081
475	-0.005	±	0.040
463	-0.012	±	0.004
337	0.212	±	0.002
459	0.082	±	0.058
484	0.487	±	0.029
491	0.084	±	0.331
421	0.299	±	0.028
454	0.404	±	0.266
402	0.120	±	0.192
304	0.567	±	0.064
379	0.340	±	0.302
424	0.432	±	0.204
397	0.223	±	0.238
344	0.212	±	0.099
418	0.384	±	0.027
426	-0.011	±	0.084
404	0.125	±	0.003
495	0.454	±	0.057
536	0.454	±	0.181
606	0.452	±	0.181
627	0.438	±	0.129

Line	Normalised mean		
608	0.441	±	0.381
610	0.638	±	0.217
545	0.771	±	0.197
541	1.097	±	0.752
519	0.009	±	0.648
543	0.339	±	0.021
516	0.743	±	0.109
497	0.740	±	0.185
562	0.300	±	0.180
642	-0.009	±	0.254
568	0.570	±	0.009
617	1.597	±	0.043
625	0.845	±	0.454
595	0.872	±	0.212
527	0.035	±	0.564
520	1.323	±	0.056
1086	0.335	±	0.295
646	0.666	±	0.228
1049	0.191	±	0.299
871	0.203	±	0.034
721	0.032	±	0.106
955	0.245	±	0.019
661	0.444	±	0.175
931	0.008	±	0.316
953	0.083	±	0.019
1052	0.262	±	0.072
872	0.246	±	0.222
663	0.069	±	0.140
950	0.383	±	0.021
1069	0.415	±	0.116
932	0.218	±	0.038
645	0.245	±	0.216
665	0.053	±	0.123
1075	0.099	±	0.051
759	0.179	±	0.080
669	0.202	±	0.099

With help of Dr Rachel Goddard and Dr Paul Nicholson (Crop Genetics, John Innes Centre, UK), the data was analysed for correlation with the SNPs markers present in the CxT population lines. Figure 5.7 shows the schematic representation of the QTL analysis performed. Two genomic regions correlated with fructose exudation, one in chromosome 3 and another in chromosome 6. The region found in chromosome 3 was located between position 0 and 37.66 cM, with a consensus position at 17.97 cM and was responsible for 9.37 % of the variance. On the other hand, the chromosome 6 consensus position was found at 101.75 cM, between 78.53 and 124.97 cM, and accounted for 8.44 % of the variance. Together, these two regions explained 17.81 % of the variance in the dataset. In barley, it can be estimated that the average correlation between kb and cM is about 50 (Sandhu et al., 2001, Stephens et al., 2004), although this ratio varies across the chromosome. Taking this as an approximation, it can be estimated that the region in chromosome 3 is about 1900 kb, whereas for chromosome 6, it would be around 2300 kb. Since these two regions were rather large, an additional step of definition was required. I started by narrowing down the region in chromosome 6 and to do that, 12 recombinant lines in the targeted region were selected and the number of repetitions was increased in an attempt to reduce the variation and hence to improve the resolution. In figure 5.8, I show a comparison between the values obtained for the refining subset and the general initial screening. As shown in the graph, a certain level of variation is observed across the two repetitions of the analysis, which is most obvious for lines 519, 541 or 661. When the data obtained for the subset was subjected to QTL analysis, no further definition could be achieved and due to this, narrowing down of the second peak found was not carried out. I reasoned that in order to decrease experimental variability and achieve a deeper level of resolution, a more robust technique needs to be designed and optimised.

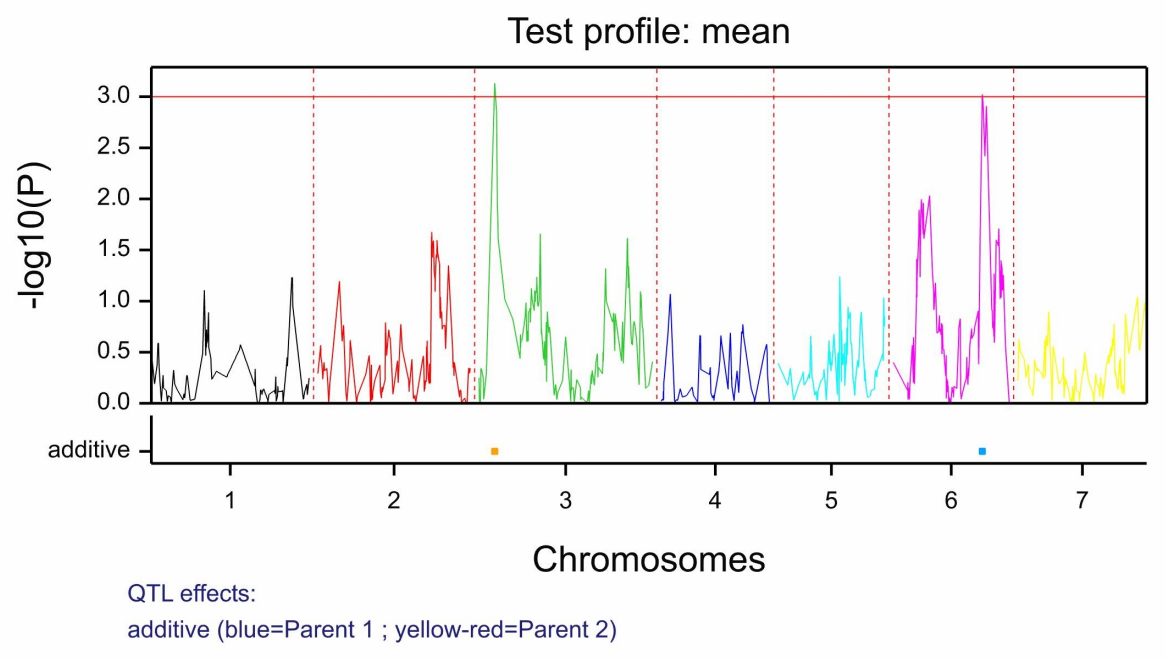

Figure 5.7. QTL analysis plot. -log10(P) values are based on the fructose secretion screening results of a CxT RIL F_7 population. Total lines analysed=110.

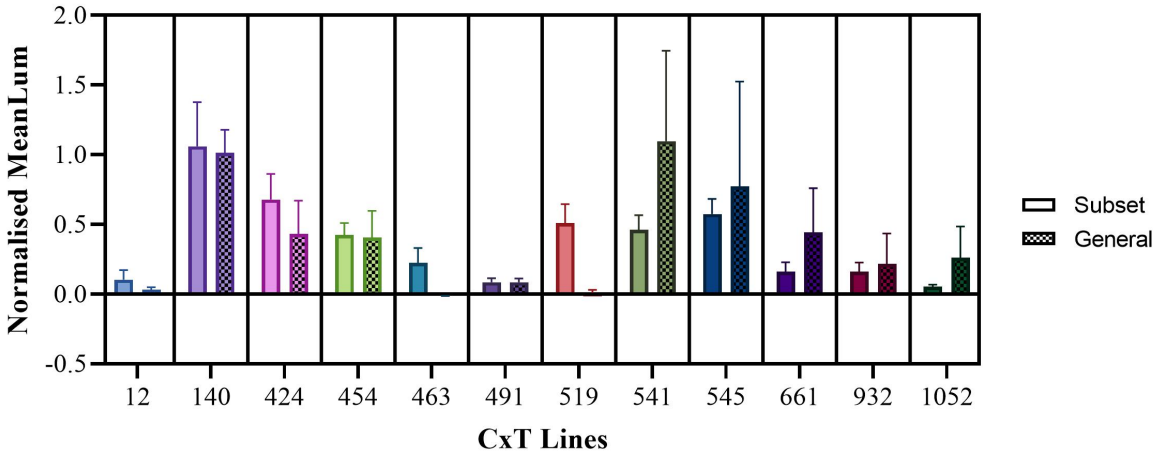

Figure 5.8. Comparison of the refining subset vs the general screening. Plot showing normalised mean luminescence values. This subset was based on up to 12 plants and general screening on 3. Error bars are represented as SEM.

5.3. Discussion

In this chapter, I demonstrated how Chevallier and Tipple cultivars differed in their ability to interact with endogenous and exogenous microbial communities by performing a cross-inoculation assay. I then investigated what plant determinants might be involved in driving this phenomenon by studying *in vivo* and *in vitro* the composition of root exudates, one of the key determinants in plant-microbe interaction (Haichar et al., 2012, Sanchez-Canizares et al., 2017). This allowed the identification of a set of molecules likely to play a role in this process, i.e., fructose-like molecules. Next, using a bacterial biosensor, Rlv3841_*lux*::fructose, I screened a mapping population of Chevallier and Tipple for fructose secretion. Two large QTL regions were identified in chromosomes 3 and 6 of barley, however individual loci could not be determined.

Host preference in microbial communities and effects on host fitness have just begun to be studied. In the work of Wippel et al. (2021), it was shown how two SynComs created from the roots of *Lotus japonicus* and *A. thaliana* had an adaptative advantage when inoculated in their original host. They also studied the effects at a whole transcriptome level of the SynComs on the two plant species and showed SynCom specific gene expression patterns that, in the case of the native strains, involved components of the host immune system. In this study, with a cross-inoculation experiment, I showed a differential effect on plant host fitness measured as DW increase, that was remarkable in the case of Tipple. This cultivar seemed to possess a substantially less flexible environment than Chevallier for exogenous communities to adapt which, in light of this experiment, translated into growth deficiencies for the plant host. When *Pseudomonas* SynComs were inoculated onto the two genotypes studied, only Chevallier showed a positive response. Tipple showed either a slight growth deficit or a neutral effect for both its native and non-native *Pseudomonas* SynCom. This result may be a consequence of the ratio of strains used for this experiment, which did not take into consideration the original abundances present in the rhizosphere. It is reasonable to think that the relative abundances of individual strains might impact the plant host fitness and that such an effect could be exacerbated in a cultivar like Tipple, which was more sensitive to a previously adapted community. Potentially, in longer experimental times with sufficient time for the communities to re-assemble, other effects would have been observed. In addition, taking into consideration the findings of previous experiments and the root exudates GC-MS profiling data, it is plausible to hypothesise that the lower general abundance of root-derived metabolites might be one of one of the factors underpinning the minor effects observed in Tipple. On the other hand, Chevallier has shown to be more

flexible to accommodate an exogenous microbial community, which makes it capable of benefiting more from its microbiome, at least in terms of plant growth promotion.

Rlv3841-based luminescence biosensors provided a powerful tool to screen *in vivo* and in real time root-derived molecules that are likely to be utilised by the rhizosphere microbial members. These biosensors were firstly published in 2017 by Phil Poole's lab (Oxford, UK) and they demonstrated their utility in analysing the importance of certain compounds during the nodulation process both in pea and vetch, as well as defining when and where such compounds were secreted from the roots. Later on, a proline biosensor, based on the same system, was published (Rubia et al., 2020). In this paper, Rubia and co-workers studied the influence of proline in the nodulation process under osmotic stress and provided a useful tool to monitor proline in the root environment. Given that bacteria of the genus *Rhizobium* are well studied members of the microbiota of several plant species (Berendsen et al., 2012, Tkacz and Poole, 2015) and their colonisation ability of barley roots has been previously shown (Yang et al., 2017, Geddes et al., 2019), I used this set of biosensors as a proxy to measure root exudation differences between Chevallier and Tipple. The most abundant compounds found were dicarboxylates, C4 and tartrate, and sugars, sucrose and fructose; an observation also made by the original authors in pea roots. Pini and co-workers could show that dicarboxylates and sucrose were the main carbon sources secreted by the pea roots and metabolised by *Rhizobium* (Pini et al., 2017). It is fair to mention that those molecules either not targeted in the original reporter design or not metabolised by the microorganism used, i.e., *Rhizobium*, will not be captured by this method and therefore making use of unbiased techniques becomes necessary for identification of the overall metabolomic profile. For this reason, I performed GC-MS analysis in an attempt to capture the diversity of metabolites present in the root exudates of my barley cultivars.

Mass spectrometry-based techniques have been broadly used in the identification of compounds present in root exudates and their role in plant microbial community assembly. For example, a very elegant study by Huang et al. (2019) identified and elucidated the biosynthetic network of root-derived triterpenes in *A. thaliana*. This novel network had the potential to synthesise more than 50 unknown root metabolites, from which three were further characterised: thalianin, thalianyl fatty acid esters and arabidin. Huang and co-workers were then able to prove the link between the secretion of these compounds and the microbiota assembly process in the rhizosphere. When *A. thaliana* was disrupted in any of these three pathways, an enrichment of *Bacteroidetes* and a depletion for Delta-proteobacteria could be shown. Furthermore, they also showed how particular bacterial

strains, for example *Pseudomonas* sp. A15, capable of cleaving thalianyl fatty acid esters and utilising the resulting products as a carbon source, were selectively affected by the presence of these molecules. In another study on tomato, Korenblum and co-workers identified a set of metabolites, mostly acyl sugars, which were differentially induced depending on the diversity of the soil community. When plants were grown in a split-root hydroponic system and inoculated with soil extracts varying in diversity, the authors could detect changes in the metabolomic profile not only locally, but also systemically. They demonstrated how some metabolites were induced upon contact with different microbial communities and how, although the microbial stimuli might occur locally in a determined part of the root system, the signal can be transduced systemically, leading to an overall metabolomic reprogramming (Korenblum et al., 2020). In my work, I have studied the root exudates composition of two barley cultivars with the primary objective of identifying the mechanisms responsible for the recruitment of beneficial *Pseudomonas*. Since previous studies, as illustrated above, have identified the presence of particular metabolites and their effect on root microbiota assembly, a similar approach was taken here for Chevallier vs. Tipple. I used GC-MS to detect a total of 105 differentially exudated metabolites of which about half were matched to a previously described molecule and classified as carbohydrates/sugars, organic acids, amino acids or others. The other half contained compounds that could not be aligned to any pre-identified entity. Within the compounds categorised as "others", it is worth mentioning the presence of pipecolic acid, an important regulator of immunity in plants that is associated with systemic acquired resistance (SAR) (Wang et al., 2018), and the benzoic acid identified as m-toluic acid, an important structural element for several plant primary and specialised metabolites - plant hormones, defence compounds or attractants for pollinators (Widhalm and Dudareva, 2015). The most interesting differences between Chevallier and Tipple root exudates composition were observed in two phenomena: (1) the differential secretion of sugars and organic acids and (2) the generally lower abundance of all exudates in Tipple. Chevallier and Tipple showed a distinct profile of sugars exuded, for instance in Tipple the most abundant sugar was a D-glucose-like molecule whereas in Chevallier the most abundant molecule was sedoheptulose. The presence of higher contents of sugars like fructose in root exudates has been previously reported in modern cultivars (Calvo et al., 2019). The authors studied the effect of global warming indexes -elevated CO_2 and drought- on the root exudation profile of two barley cultivars, Bambina, that was released in 2009, and Golden Promise, in 1966. Among the differential responses found, they could observe that the more modern cultivar Bambina exuded on average 53% more fructose than Golden Promise. Regarding the second

phenomenon, it is possible to speculate that the reduction in both diversity and abundance of root-derived metabolites found in Tipple could be a secondary effect of the intense breeding process to which modern cultivars have been subjected. In a recent study in tetraploid wheat (*Triticum turgidum* L.), the authors showed differences in the root exudation profiles of a panel of 10 wheat genotypes corresponding to the key steps in the domestication process (Iannucci et al., 2017). Preece and Peñuelas reviewed this topic in 2020, and highlighted the effects that intense breeding processes have had on traits such as mycorrhizal association, pest resistance or resistance to abiotic stresses. Plants can secrete an average of 15-20 % of their photosynthates, a trait that can be expensive to maintain. Allocation of energy to other parts of the plant to increase crop yield, together with the application of synthetic fertilisers, might have impacted the ability of modern cultivars to recruit and maintain beneficial microbial communities (Bulgarelli et al., 2013, Bulgarelli et al., 2015, Martín-Robles et al., 2018, Preece and Penuelas, 2020).

Finally, I decided to study the genetic mechanisms controlling the differential sugar exudation profile found in the two cultivars by performing QTL mapping on a CxT RIL F_7 population. I decided to use the Rlv3841-based fructose biosensor to conduct this screen given its higher consistency and its potential to detect not only fructose but also other sugars such as lactulose, mannitol, mannose, sorbitol and sucrose (and probably others not included in the original inducer screening). In this analysis, two large QTLs were identified, one in chromosome 3 at 17.97 cM and a second one in chromosome 6 at 101.75 cM. Since the regions discovered were incredibly large, individual genes could not be identified. As previously described by some authors, some plant ABC transporters involved in sugar and phenolic compounds excretion have an important effect in shaping microbial communities (Badri et al., 2009) which makes it feasible to hypothesise that a similar mechanism may underpin this phenomenon.

All in all, the results in this chapter show that fitness effects on the plant host are specific in a cultivar dependent manner, in which root exudates composition is highly likely to play a fundamental role. I demonstrated that Chevallier and Tipple differed in the composition of root exudates by two independent techniques, *in vivo* an *in vitro*, with noticeable differences emerging for certain sugars and organic acids as well as for some unidentified compounds. Lastly, I used a previously published CxT population to screen for QTLs in the barley genome responsible for the differential exudation of one particular sugar, fructose, and found two large genomic regions linked to this trait. These loci might plausibly be involved in the recruitment process of rhizosphere *Pseudomonas*.

CHAPTER 6:

General discussion

6.1 Introduction

Feeding an increasing global population has become one of the biggest challenges of this century. Moreover, increased food demand must be met without increasing arable land use and by employing agronomic practices that are more sustainable and respectful to the environment. Therefore, new avenues need to be explored in order to change the current production model into a more productive yet sustainable agricultural system, an approach that has been named sustainable intensification (Struik and Kuyper, 2017). One possible way to achieve this is by exploiting the natural capacity of plants to produce more via engineering the plant microbiota, especially those microorganisms inhabiting the rhizosphere (Tkacz and Poole, 2015). In the rhizosphere, the microbial diversity is incredibly large and given that these bugs are in close contact with the plant, they can have a huge influence on plant growth, health or stress resistance. Hence, understanding the mechanisms that control the assembly of the rhizosphere microbiome is vital to improve the plant recruitment of PGPR and plant yield in a more sustainable manner (Turner et al., 2013a, Sasse et al., 2018).

The central aim of this thesis was to investigate the genetic mechanisms that are responsible for *P. fluorescens* rhizosphere colonisation in barley of different genotypes. To this end, I have combined microbial high-throughput isolation and characterisation, NGS, microbial genetics and plant assays to shed light on the mechanisms responsible for the barley cultivar-specific colonisation by *P. fluorescens.*

6.2 Effect of plant genotype on the rhizosphere microbiome structure

Over the past few years, several authors have investigated the microbiomes of distinct plant species, identifying unique characteristics linked to soil type, plant species and development stage. At the same time, these studies all demonstrated the existence of a core microbiome intrinsically associated to a specific plant species that is shared even across different continents (Lundberg et al., 2012, Chaparro et al., 2014, Wagner et al., 2016, Zhou et al., 2020, Stopnisek and Shade, 2021).

In chapter 3, I examined the effect that barley cultivar has on the rhizosphere microbial community. I was able to show that plant effects on the microbial consortia are consistently evident at different organisational levels, from total population structure all the way to

microbial genotypic features and molecular mechanisms. First of all, I phenotypically analysed *Pseudomonas* populations isolated from the rhizospheres of a diversity panel consisting of 12 different barley cultivars. To do this, I developed protocols for high-throughput phenotypic screening. This screening was used as a proxy to evaluate previously described traits involved in the rhizosphere colonisation process, which are also commonly found in those rhizobacteria with plant-growing promoting characteristics (Lugtenberg and Kamilova, 2009). At the same time, the evaluation of the phenotypic traits found in the rhizosphere isolates partially acts as an indicator of the general diversity of the *Pseudomonas* population found in the rhizosphere. Here I showed that a wider range of phenotypic traits was observed in the Chevallier derived *Pseudomonas* isolates which then correlated with a higher divergence in their *gyrB* sequences. Hence, indicating more diversity within the Chevallier rhizosphere isolates than those associated with Tipple.

Plants secrete around 20 % of their fixed carbon, even up to 40% according to some authors, which constitutes one of the major carbon sources in the soil (Badri and Vivanco, 2009, Lugtenberg and Kamilova, 2009). In order to colonise the plant and, ultimately, to obtain food, bacteria must be able to move towards this food source. Therefore, motility is a fundamental trait for effective rhizosphere colonisation (Compant et al., 2010). Once in the root surroundings, microbes need to be able to effectively colonise and attach to the root surface (Compant et al., 2010) for which the ability to form a stable biofilm is a key determinant. This has been measured here by the ability to bind Congo Red, a method widely published elsewhere for this purpose (Mauchline et al., 2015, Jones and Wozniak, 2017). Finally, in a competitive, nutrient-poor environment such as the bulk soil, possessing a diverse arsenal of pathways that enhances bacterial chances to monopolise scarce resources is critical (Compant et al., 2010). Bacterial competitive capability against other members of the soil community is also very important for a successful proliferation in the rhizosphere (Lugtenberg and Kamilova, 2009). This broad category was assessed via four approaches: (1) production of siderophores, (2) secretion of proteases, (3) emission of toxic volatiles and (4) suppression of other members of the soil consortia.

The cultivars Chevallier and Tipple were selected following the phenotypic screening experiment, during which I observed a distinct pattern of bacterial phenotypes linked to these two plant genotypes. These two cultivars have been extensively studied, good genetic resources are available and their origins and breeding history are quite distinct (Goddard et al., 2019). These characteristics made Chevallier and Tipple excellent candidates for a more detailed study of the determinants of bacterial recruitment in the barley rhizosphere. When

the total microbial communities were assessed by amplicon sequencing, significant differences for both bacterial and fungal members could be found between the two cultivars, as well as between the different plant compartments (root endosphere and rhizosphere).

To this date, several studies have shown the effect of plant genotype and compartment over the composition of the rhizosphere microbiota (Bodenhausen et al., 2014, Bulgarelli et al., 2015, Liu et al., 2019). According to the genotyping of the rhizosphere *Pseudomonas* isolates performed here, it seems that the striking changes in the bacterial population also occur at species or strain level in addition to differences observed between higher taxa. There are a few examples in the literature in which this same phenomenon has been described in plants and these are normally restricted to genus or family level differences. For instance, in a study on the wild perennial *Boechera stricta*, the effect of genotype, year of harvesting, environment and age of the plants were investigated. The authors could identify differential abundances for some OTUs rather than absence or presence or specific taxa. For example, they could observe an average fold change in OTUs attributed to plant genotype of 2.0 in roots and almost 4.0 in leaves (Wagner et al., 2016). In tobacco seeds, a similar observation was made where members of the family *Enterobacteriaceae* accounted for the most OTUs with a differential abundance between cultivars, followed by *Pseudomonas*. Interestingly, the authors identified a few OTUs that were only present in some of the tobacco cultivars studied but not in the others. Moreover, they were able to see a higher level of shared OTUs in those cultivars whose parental lines were common, a fact that reinforces the idea that the plant microbiome is, at least partially, vertically inherited (Chen et al., 2020). In this context, a study by Matsumoto et al. (2021) showed the prevalence of *Sphingomonas* as a core member of rice seeds endosphere microbiome which was maintained at high rates over three successive generations. The presence of a particular endophytic strain, *S. melonis*, was linked to rice cultivar resistance against *Burkholderia plantarii* and it could be artificially transferred to disease-sensible cultivars to confer resistance. This study showed the impact that the indigenous microbiome has in preserving homeostasis in the plant holobiont.

In the studies mentioned above, 16S amplicon sequencing was used and hence the resolution achieved is insufficient to characterise specific interactions down to species or strain level. It has been shown that none of the variable regions usually covered by partial short-reads of the 16S are able to capture the diversity found when the full gene is sequenced (Johnson et al., 2019). For this reason, in this work I decided to combine culture-based methods with NGS in an attempt to study in more detail the mechanisms behind cultivar-specific colonisation. I focussed exclusively on a well-known member of the plant microbiome, the

Gamma-proteobacterial genus *Pseudomonas* (Muller et al., 2016). When *Pseudomonas* spp. isolated from the rhizospheres of Chevallier and Tipple barley cultivars were *gyrB*-genotyped, they displayed a clear clustering according to their origin. Some groups of genotypes comprised a mix of isolates from the two cultivars and the bulk soil isolates. This is not surprising, given that the soil acts as a reservoir for the subsequent microbial recruitment and, as mentioned above, the same plant species would be expected to recruit a core microbiome that will persist regardless the soil properties, the plant cultivar and even, the continent (Stopnisek and Shade, 2021). Consequently, the following analysis of these bacterial communities can help to understand how the plant shapes the microbial community and to elucidate the genetic determinants responsible for the bacterial adaptation process to the plant rhizosphere. This analysis will help to identify bacterial loci relevant for plant rhizosphere colonisation and at the same time, to determine which of those plant colonisation traits might be also relevant for cultivar-specific adaptation.

6.3 Adaptation mechanisms of *Pseudomonas* to the rhizosphere

Plants play a key role as drivers of carbon uptake from the atmosphere into the soil. However, soil dwelling microorganisms also contribute hugely to the balance of carbon in the soil. By acting as plant symbionts, pathogens or by decomposing soil organic matter, they can change the dynamics of carbon storage in the soil and influence its availability (Trivedi et al., 2013, Lladó et al., 2017). They also impact greatly the nitrogen cycle acting at different steps of the process: atmospheric nitrogen fixation, nitrification, nitrate reduction, ammonium oxidation or denitrification. Finally, soil microorganisms help to mineralise organic phosphorous into inorganic phosphates and to solubilise it when it is immobilised in the soil complex (Aislabie et al., 2013). All this makes the soil an incredibly diverse and complex environment in which multilevel interactions play a fundamental role in the correct function and balance of the organisms inhabiting it.

In the complex habitat of the soil, bacterial communities must rely to a great extent on plant exudates to survive. Root exudates composition varies with plant species, cultivar, age and developmental stage. Thus, it is indispensable for bacterial survival to efficiently adapt to the plethora of compounds available in the belowground environment (Turner et al., 2013a). In chapter 4, I examined how the rhizosphere adaptation process occurs in the *Pseudomonas* population. To do this, I used two barley cultivars, Chevallier and Tipple, which differed in their rhizosphere microbial population and their root exudation profiles, alongside their

native *Pseudomonas* root-associated isolates and the model organism *P. fluorescens* SBW25.

Carbon metabolic regulation has been identified as a major determinant of effective adaptation to the plant environment (Campilongo et al., 2017, Little et al., 2019). Here, confirmed the importance of this process during microbial colonisation of different barley genotypes. By using previously published biomarker strains (Campilongo et al., 2017), I observed a differential colonisation phenomenon with a *P. fluorescens* SBW25 deletion mutant, Δ*rccR.* This mutant lacks the ability to swich between different pathways involved in carbon metabolism, such as pyruvate metabolism, the glyoxylate shunt and gluconeogenesis, impairing to a great extent its growth on sugars like glucose or glycerol. This mutant showed differentially reduced colonisation fitness in the barley cultivar Tipple, suggesting that rapid, flexible control of carbon metabolism is a key factor in bacterial adaptation to available food sources. This finding was further supported by the better growth performance seen for the Tipple native rhizosphere strains growing in the presence of glucose as the sole carbon source. These results also showed how two plant genotypes can differ in their root exudates composition for certain type of molecules, such as sugars, and the impact that this phenotype might have on microbial shaping, at least for *Pseudomonas* populations.

Analysis of the genomic features present in plant-associated bacteria is a powerful tool to investigate the mechanisms responsible for plant colonisation (Levy et al., 2017). Here, this approach has been used to identify bacterial genes with relevant roles in genotype specialisation within a representative subpopulation of rhizosphere *Pseudomonas* spp.. By analysing previously described plant-association genes in *P. fluorescens* SBW25, I observed that the distribution frequency of certain genetic traits differed between Chevallier and Tipple derived isolates. This phenomenon points towards a possible adaptation of the *Pseudomonas* community to the specific niche provided by the barley cultivar. Although functional characterisation of these genes in the original strains would have provided a more robust foundation for this study, the deletion and further *in planta* examination was carried out in the model organism *P. fluorescens* SBW25, in order to bypass the difficulties that environmental isolates often showed for genetic manipulation. Moreover, deletion of the target genes in *P. fluorescens* SBW25 allowed me to compare the effect of the individual deletions with one another in a uniform genetic background. With this approach, three new barley cultivar-discrimination genes were identified: *PFLU_6072*, a transcription factor likely to be involved in regulation of carbon metabolism; *PFLU_5080*, an unknown protein

with a putative role in prophage excision; and *PFLU_2583*, a sugar transporter. As discussed in chapter 4, carbon metabolic adaptation appears to be crucial for adaptation to the plant environment (Loper et al., 2012, Campilongo et al., 2017). Thus, the finding of *PFLU_6072* and *PFLU_2583* as selected traits among the *Pseudomonas* isolates is congruent with this fact. On the other hand, *PFLU_5080* is likely to have phage-related functions. It is known that phage-mediated regulation of the bacterial host behaviour can be very relevant in some environments. For example, in *P. aeruginosa* it has been demonstrated that these prophages play an important role in controlling biofilm formation and bacterial densities at the infection site. Another example of prophage relevance in environmental adaptation, it is found in *E. coli* where it was proved that phage-infected bacteria grew better than those non-infected under limiting carbon conditions (Argov et al., 2017). Therefore, it is possible that *PFLU_5080* has an analogous role here at modulating the bacterial host response to environmental changes. These findings demonstrate the efficacy of the approach used here to identify cultivar-specific colonisation genes in *Pseudomonas*. At this stage, it is fair to mention that more resolution may have been achieved by conducting a complete genome comparison between the isolates. However, this approach was not practical to utilise for this study, due to the nature of the sequencing data obtained.

There are very few examples in the literature showing bacterial responses to different plant genotypes. In one of them, the authors investigated the impact on symbiosis of the interaction between different *Medicago*-*Sinorhizobium* partners. In this study, the interaction between the plant and bacterial genotypes showed an influence on the transcriptional rewiring during nodulation that ultimately impacted the outcome of rhizobium-legume symbiosis (Fagorzi et al., 2021). Here, I analysed by RNA-seq the gene expression levels of *P. fluorescens* SBW25 growing in the rhizospheres of the selected cultivars, Tipple and Chevallier. I identified an average of 1000 plant-induced genes as well as a subset of genotype-specific differentially expressed genes. Interestingly, very few genotype-specific genes emerged from this study: only 14, from which 9 were upregulated in the Tipple rhizosphere and 5 in Chevallier. *PFLU_3091*, an amino acid permease upregulated in the Tipple rhizosphere, and *PFLU_4463,* a drug/metabolite transporter upregulated in Chevallier, were deleted in *P. fluorescens* SBW25 to test their contributions to rhizosphere colonisation fitness *in planta.* This experiment validated the importance of both genes for cultivar adaptation, as they showed differential fitness in agreement to the expression dataset. Therefore, this work proved how the plant genotype impacts not only the distribution of certain bacterial genotypes and their genetic features in the rhizosphere, but also how it can directly modulate

cellular reprograming in an individual bacterial strain. These findings will help to further elucidate the basis of the crosstalk between the plant and its microbiome.

In the first section of chapter 5, I could link the relevance of the differential recruitment of rhizosphere *Pseudomonas* with the plant host performance under lab conditions. In general lines, the most striking output obtained from this experiment was that Tipple cultivar did not benefit from application of microorganisms not adapted to its own rhizosphere. Tipple might exert higher selective pressure, via root exudation, for a narrower subset of genotypes than Chevallier and might depend more on these than Chevallier for subsequent growth and health. Specifically in the case of rhizosphere *Pseudomonas* community, this phenomenon translates in a lower *gyrB* diversity for the Tipple rhizosphere isolates. Interestingly, when the overall rhizosphere microbiome was analysed by 16S amplicon sequencing, differences in alpha-diversity were not identified between the two cultivars. In fact, a higher percentage of *Pseudomonas* ASV was found in Tipple rhizosphere (7.55 %) than in Chevallier (4.87 %), but in light if the *gyrB* genotyping results, it appears that Tipple *Pseudomonas* rhizosphere consortium might be less diverse. For these reasons, I hypothesised that Tipple rhizosphere might present an environment less flexible for non-adapted microbes to proliferate. In *A. thaliana*, Haney et al. (2015) demonstrated that the plant host could limit the growth of specific genotype incompatible species of *P. fluorescens*. When the incompatible *Pseudomonas* strains were inoculated into the different *A. thaliana* genotypes, a plant growth defect was observed. They also showed that the ability of the plant to support certain *Pseudomonas* isolates was linked to the presence of pathogens. Consequentially, it is crucial to mention that the benefits offered by the plant microbiome are not limited to plant growth (output measured in this study), and therefore they might become relevant under conditions not tested here, for example, an enhance resistance against certain phytopathogens or a higher tolerance to draught (Berendsen et al., 2012).

6.4 Role of root exudates in microbial recruitment

The importance of root exudates in rhizosphere microbiome assembly has been widely studied by various authors (Haichar et al., 2008, Chaparro et al., 2013, Huang et al., 2019). Fundamentally, they are composed of sugars, amino acids, carboxylic acids, phenolic compounds and some secondary metabolites, such as hormones (Badri and Vivanco, 2009). Exudation profiles vary with plant developmental stage, age, plant species and genotype and they can influence interactions with other plants and microbial partners (Vives-Peris et al., 2020). In barley, the role of root exudates has been investigated for their allelopathic

properties in different cultivars against weeds such as *Bromus diandrus*. In this study, a compound with an inhibitory potential was found: saponarin, a flavonoid not previously characterised (Bouhaouel et al., 2019). However, as far as I am aware, there has been no reference to the particular role of barley root exudates in influencing microbiome assembly.

In chapter 5, I characterised the root exudates composition of Chevallier and Tipple, with the aim to understand the potential drivers behind the differential recruitment process in the rhizosphere microbiome. By combining bacterial *in vivo* screening, *in vitro* exudates extraction and further characterisation by GC-MS, I identified differences in the abundance of several metabolites, such as sugars or carboxylates. Moreover, I was able to demonstrate that in general, Tipple exudates seemed to comprise a less diverse array of compounds than Chevallier. Together, these two findings further supported two main hypotheses of this work: (1) the Tipple rhizosphere is enriched in sugars like glucose, fructose or sucrose and (2) the less diverse root exudates with a higher prevalence of specific compounds might explain the higher specialisation observed among the *Pseudomonas* isolates derived from Tipple rhizosphere. These two facts led me to reason that differences in the exudation rates of a sugar such as fructose might impact the subsequent establishment of the microbial communities, especially in the case of *Pseudomonas*. Therefore, using a Rlv3841 based fructose biosensor, I screened a CxT RIL population in order to identify genomic regions (QTLs) responsible for controlling this trait in barley. Two genomic regions were identified by this approach; however, identification of individual loci was not possible given that they each comprised about 2000 kb. In previous studies, several mechanisms have been described by which plants secrete metabolites. Typically, their transport can be passive (diffusion, ion channels or vesicles) or active (ABC or MATE transporters). The chemical nature of the compound in each case will determine the secretion mechanism through which they are exuded. For example, low weight molecules will be release by diffusion, whereas phenolic compounds will be translocated through MATE transporters (Vives-Peris et al., 2020). Interestingly, in *A. thaliana* the deletion of the ABC transporter Atabcg30 resulted in the translocation of more phenolic compounds and less sugars, which was showed to modify the rhizosphere microbiota (Badri et al., 2009).

All in all, the approaches taken here demonstrated the power of combining *in planta* and *in vitro* techniques in order to characterise root exudates. Moreover, the combination of these two methods was shown to be sufficiently precise to capture the differences present between two cultivars of the same plant species, i.e., barley in this study, which can help to shed light on the genetic control of differential root exudation in distinct genotypes.

6.5 Concluding remarks and applications

During the colonisation of different barley genotypes, which effectively represent distinct, but related, rhizosphere environments, *Pseudomonas* communities must adapt to the prevailing conditions in order to proliferate and thrive in the rhizosphere. Having a diverse carbon metabolism that enables the efficient utilisation of the different carbon sources available appears to be a key determinant of the genotype adaptation process. This adaptation translates into an active selection process by the plant genotype for those soil isolates that encode genes responsible for the efficient transport and metabolism of the metabolites more abundant in a given genotype. This finding was supported both by the composition of the *Pseudomonas* populations isolated from the rhizospheres of Chevallier and Tipple, and also in the differential gene expression patterns observed in the model organism *P. fluorescens* SBW25. Consequently, this points towards the existence of putative bacterial genetic signatures unique to a specific barley cultivar, and suggests that the bacterial shaping seen for the whole rhizosphere community in response to niche adaptation also happens at the molecular level.

Differences in root exudation for the two cultivars studied were indirectly supported by the fitness penalty observed in the metabolic *P. fluorescens* SBW25 mutants. The hypothesis of differential sugars and organic acids secretion between the two cultivars was corroborated by bacterial sensors and GC-MS analysis. In summary, this study shows that the Tipple rhizosphere is richer in glucose-like compounds and the array of molecules present in Tipple exudates is less diverse. This appears to lead to a microbial community dominated by a relatively lower diversity of species capable of more efficiently utilising these molecules and more susceptible to a change in the community structure. On the other hand, the most abundant sugar in the Chevallier rhizosphere was sedoheptulose and a greater overall abundance of metabolites was detected. This phenomenon may explain why Chevallier genotype is better at coping with a non-adapted microbial community.

Given that differences in root exudation, especially sugars secretion, may play a determinant role at shaping *Pseudomonas* communities in the rhizosphere, QTL mapping for fructose secretion was performed. This showed two large QTLs in the barley genome, one in chromosome 3 and another in chromosome 6, that might be involved in *Pseudomonas* recruitment process in the barley rhizosphere.

The work showed in this thesis opens an avenue for downstream studies and applications. First of all, the barley-associated bacterial collections can be exploited with completely different objectives to those described here. For example, they may be useful for the selection of new biocontrol agents, identification of novel biosynthetic gene clusters encoding antibiotics or other interesting natural products or, lastly, the assembly of synthetic communities for plant colonisation experiments. Secondly, the *P. fluorescens* SBW25 genes identified as colonisation determinants in a cultivar-specific manner require further characterisation to validate their functional role. Several exciting molecular microbiology projects lead directly from the findings of this study. Third, the individual roles of the root exudates compounds detected by GC-MS analysis, currently an enigma, invites further analysis. For those compounds with a match in the library used, the process would certainly be easier. An approach similar to that taken by Eilers et al. (2010) could be used to identify how specific molecules may impact the microbial community structure. The case of the unknown components of the root exudates undoubtedly requires a much more extensive work, in which several purification steps and follow-on testing will be necessary. Finally, refining the QTL regions identified here will benefit from the creation of new recombinant lines in these genomic regions or the development of a different, perhaps more refined, screening approach. For instance, performing RNA-seq analysis on the two cultivars' root tissue in the presence and absence of the *Pseudomonas* population or the model *P. fluorescens* SBW25 could help to elucidate the specific loci that are involved in the differential recruitment process.

Given that the world population will significantly increase during this century and the consequences of climate change will exacerbate current agricultural challenges (Tkacz and Poole, 2015), finding alternative ways to improve plant production is crucial. In the context of a more sustainable agriculture, it is necessary that the productivity boost is achieved without a massive application of agrochemicals, in a more respectful way to the environment. It has been proposed that engineering of the microbiota represents a sensible way to achieve such goal (Orozco-Mosqueda et al., 2018). During my thesis, I have been able to show how barley genotype influences the rhizosphere microbiome composition at different levels, from the general structure for both bacterial and fungal communities to differential gene expression in a single microorganism. The study shown here will help to address the current food and environmental challenges by understanding the mechanisms involved in the rhizosphere microbiome assembly process. Investigating the mechanisms controlling the crosstalk between both partners, *P. fluorescens* and barley, can establish the foundation to engineer the rhizosphere microbiome by (1) developing bioinoculants that are

best adapted to the plant rhizosphere and (2) utilising plant breeding to select for barley cultivars, or other crops, with QTLs associated with the differential recruitment of beneficial bacteria.

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Appendix

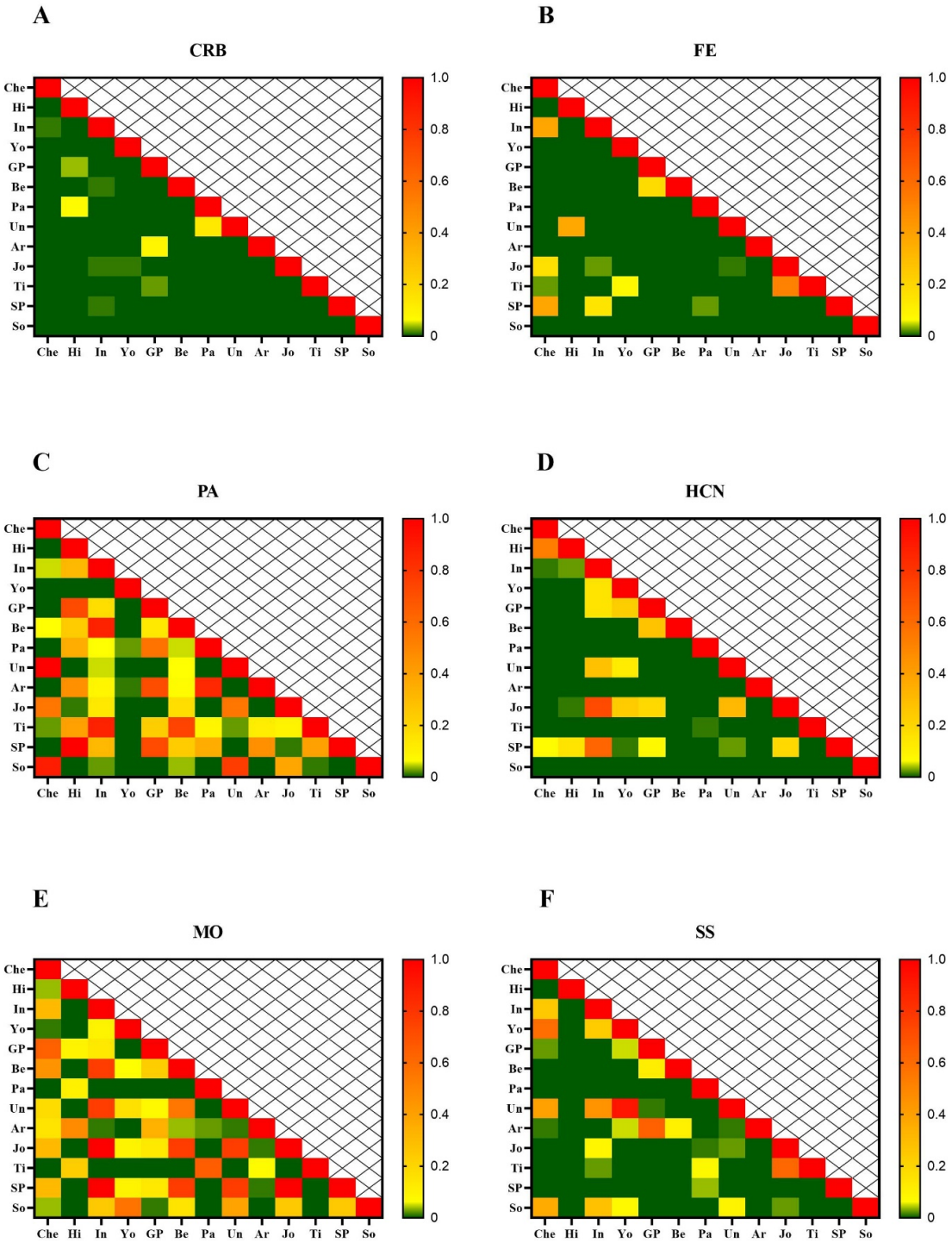

Figure S1. Chi-Square comparisons of all the phenotypic traits examined. **A.** Congo Red binding (CRB). **B.** Fluorescence emission (FE). **C.** Protease activity (PA**). D.** Hydrogen cyanide production (HCN). **E.** Motility (MO). **F.** Streptomyces suppression (SS).

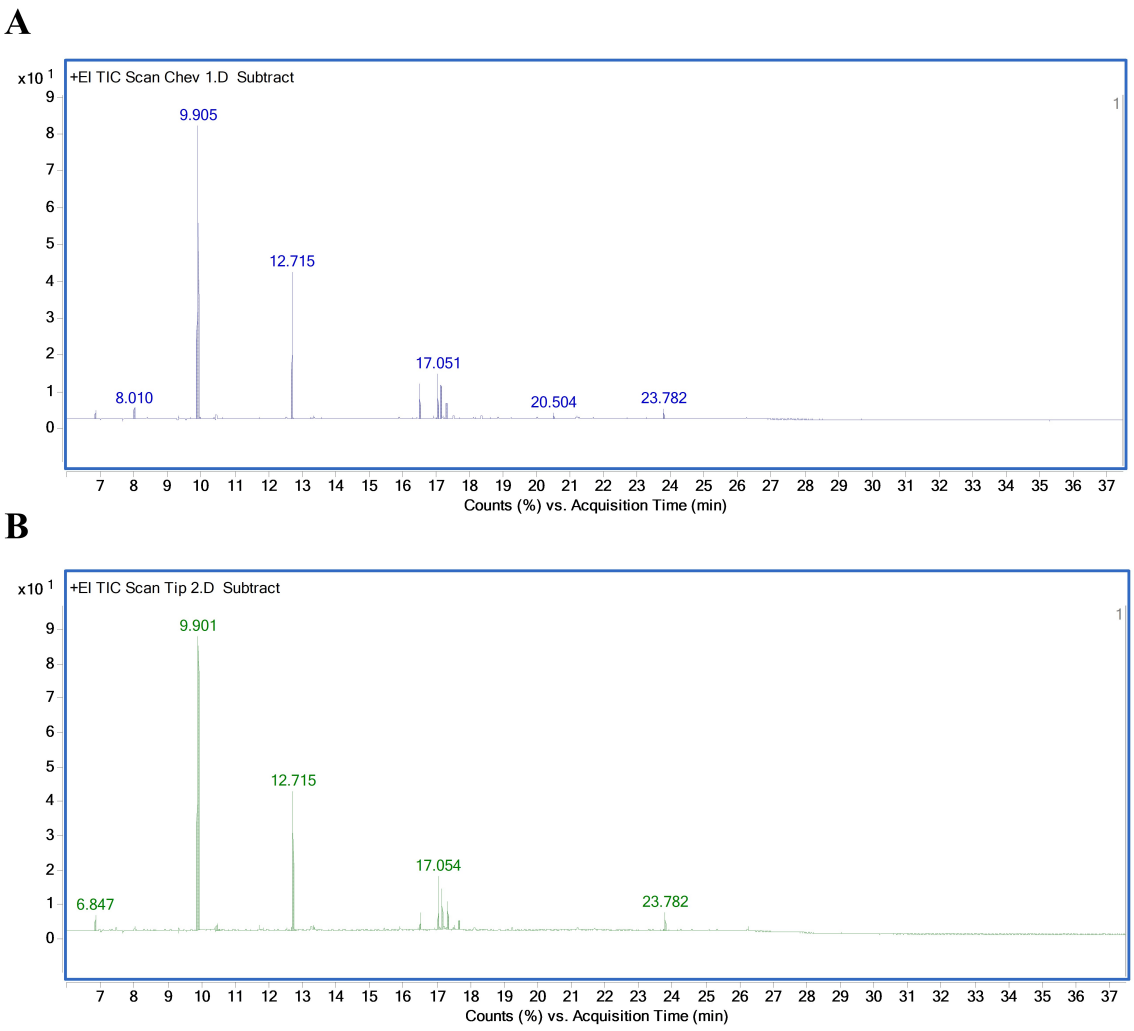

Figure S2. Chromatogram of barley root exudates. **A.** Root exudates of Chevallier cultivar. **B.** Root exudates of Tipple.

Table S1. Log2 normalised abundances of the identified compound detected in the root exudates. Values represented as the mean of 3 values ± SEM.

Compound	Chevallier		Tipple		Retention time
L-sorbose 1	2.499 ±	2.626	-0.232 ±	0.234	17.070
GABA	-9.815 ±	4.717	0.641 ±	0.137	13.264
Malonic acid	3.508 ±	9.978	1.817 ±	4.821	7.781
Glycolic acid	-1.504 ±	4.717	1.874 ±	4.385	7.083
Citric acid	4.568 ±	3.786	-0.797 ±	0.225	16.513
Phosphoric acid 1	-1.340 ±	4.717	2.073 ±	4.412	20.154
Threonic acid	4.717 ±	4.717	3.539 ±	4.556	13.575
L-allothreonine	4.717 ±	4.717	3.623 ±	4.640	10.199
1,6-anhydro-glucose	4.717 ±	4.717	3.444 ±	4.461	18.160
L-(+) Lactic acid	3.623 ±	3.853	-0.016 ±	0.193	6.849
L-glutamic acid 1	3.029 ±	4.903	-2.867 ±	4.820	13.159
3-Phenyllactic acid	-4.770 ±	4.951	-0.635 ±	1.231	26.257
D-allose	-4.713 ±	4.713	0.685 ±	0.658	17.215
Palmitic acid	5.171 ±	4.554	-1.300 ±	0.394	18.851
Beta-alanine	4.723 ±	4.294	-1.013 ±	0.417	10.401
Ribitol	-2.405 ±	4.717	2.678 ±	5.248	15.431
Arabitol	4.717 ±	4.717	3.366 ±	4.383	24.252
L-alanine	-9.160 ±	4.717	0.333 ±	0.333	7.466
Conduritol epoxide	0.354 ±	0.356	-5.081 ±	4.947	19.240
Gluconic acid	4.717 ±	4.717	3.475 ±	4.492	18.357
Oxalic acid	5.272 ±	4.610	-1.202 ±	0.423	8.008
D-threitol	-2.405 ±	4.717	2.816 ±	5.343	15.301
D-glucose	-2.405 ±	4.717	4.389 ±	6.105	17.419
Myo-Inositol	4.717 ±	4.717	3.153 ±	4.170	22.062
Eicosapentaenoic acid	-1.437 ±	0.975	0.183 ±	0.017	23.783
D-mannose	0.776 ±	1.112	-5.627 ±	5.683	17.352
L- sorbose 2	-5.235 ±	5.757	-3.935 ±	6.594	17.150
L-serine	0.535 ±	0.121	-5.233 ±	4.635	9.688
Petunidin-3-glucoside	-4.394 ±	4.563	0.235 ±	0.299	24.719
3,4-MDEA	-2.187 ±	4.717	2.174 ±	4.878	8.494
Acetohydroxamic acid	5.029 ±	4.886	-0.729 ±	0.346	7.658
Pelargonic acid	5.325 ±	4.797	-0.804 ±	0.258	11.104
Glycine	0.552 ±	0.000	-5.265 ±	4.506	10.356
Succinic acid	4.158 ±	4.336	-0.149 ±	0.197	10.452
Ethanolamine	5.145 ±	4.750	-1.024 ±	0.374	9.311
Tris(trimethylsilyl) phosphate	-9.382 ±	6.762	0.331 ±	0.381	9.910
Fumaric acid	-1.913 ±	4.717	1.803 ±	4.893	10.944

4-Methylpentyl ethylphosphonofluoridate	4.717	±	4.717	3.194	±	4.211	21.217
Carboxylic Acid	4.572	±	4.431	-0.747	±	0.350	8.389
Norvaline	0.901	±	8.620	-5.236	±	5.249	8.495
Propanamine	-2.405	±	4.717	3.245	±	5.521	13.336
Methylmalonic acid	4.717	±	4.717	3.465	±	4.481	8.063
D-malic acid	3.598	±	3.652	-0.301	±	0.324	12.716
6-hydroxy caproic acid	4.717	±	4.717	3.501	±	4.517	14.888
Glyceric acid	0.618	±	0.258	-0.664	±	0.279	10.647
D-sphingosine	4.743	±	4.269	-1.040	±	0.376	19.402
Digalacturonic acid	-4.684	±	4.684	0.166	±	0.162	21.686
Caprylic acid	4.717	±	4.717	3.384	±	4.722	16.310
D-erythrose-4-phosphate	4.717	±	4.717	3.674	±	5.011	15.898
Acetylisatin	2.393	±	4.636	-2.739	±	5.568	11.715
Pipecolic acid	4.717	±	4.717	3.600	±	4.937	13.158
D (+) galactose	2.971	±	4.881	-3.080	±	4.945	17.384
Sedoheptulose	9.739	±	4.677	-1.042	±	0.374	18.161
Tert-butyl isocyanate	9.515	±	4.570	-1.042	±	0.374	13.295
Phosphoric acid 2	16.474	±	4.462	-12.613	±	0.374	9.893
L-glutamic acid 2	7.877	±	0.261	-8.070	±	0.374	13.293
Sarcosine	4.717	±	4.717	3.330	±	4.995	9.693
Allo-inositol	4.717	±	4.717	3.340	±	5.004	19.237
m-toluic acid	10.144	±	4.937	-0.948	±	0.374	26.257