

**How wheat genotype drives rhizosphere microbiome assembly**

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## ABSTRACT

Plant health and growth are profoundly affected by interactions with soil microorganisms in both ecological and agricultural contexts. The deposition of photosynthates into the soils surrounding plant roots characterises the rhizosphere environment and provides the basis for the establishment of a plethora of plant-microbe interactions with varying degrees of intimacy. The assembly of the ecological communities of microorganisms inhabiting these microhabitats - the rhizosphere microbiota - can represent the first stages of pathogenesis but may also foster myriad plant-growth-promoting functions. Whilst the microbial determinants of many of these microbiome-mediated functions are increasingly well-defined, the plant traits driving microbiome assembly towards these outcomes remain elusive. Here, *in vivo* detection of novel root exudation traits by luciferase-based biosensor bacteria identified significant sources of variation amongst historical and contemporary wheat germplasm. Whilst not directly attributable to these differences, conserved patterns of rhizobacterial enrichments observed for *Pseudomonas spp.* with particular functional traits reflected the distinct selective pressures imposed on individual soil-dwelling bacterial genomes by different wheat varieties. Subsequent investigation of the genetic basis for variation in the exudation of a group of organic acids from wheat roots thus served as a proof-of-concept for the dissection of traits that *are* responsible for these outcomes. The development of effective screening tools for belowground traits affecting crop microbiomes should enable future studies to decipher signals that might be used to modulate the microbiota for sustainable agriculture goals.

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# Table of Contents

ABSTRACT	3
Table of Contents	4
List of Tables and Figures	6
Acknowledgements	7
Chapter 1	8
INTRODUCTION	8
1.1 Background: The Plant Microbiome	9
1.2 Plant Control of the Rhizosphere Microbiome	11
1.3 Measuring Root Exudation	15
1.5 Wheat ( <i>Triticum aestivum</i> )	18
1.4 <i>Pseudomonas spp.</i>	22
1.6 Conclusions and Research Goals	26
Chapter 2	27
MATERIALS AND METHODS	27
2.1 Plant Material	28
2.2 Bacterial Strains and Growth Conditions	29
2.3 Isolation of Rhizosphere <i>Pseudomonas spp.</i> Populations	30
2.4 Phylogenetic Analysis of Soil-Dwelling Pseudomonads	31
2.5 Phenotypic Characterisation of <i>Pseudomonas spp.</i> Isolates	31
2.6 Bacterial Carbon Source Availability Assessments	32
2.7 Bacterial Luminescence-based Root Exudate Biosensors	33
2.7.1 Statistical Analyses	34
2.8 Quantitative Trait Loci Mapping	35
Chapter 3	36
CHARACTERISING CULTIVAR-SPECIFIC WHEAT RHIZOSPHERE ENVIRONMENTS	36
3.1 Introduction	37
3.2 Results	40
3.2.1 Phylogenetic Analysis of Wheat Root Associated Pseudomonads	40
3.2.2 Characterising Rhizospheric <i>Pseudomonas spp.</i> Populations	42
3.2.3 Dissecting the Role of Wheat Root Exudation	44
3.2.3.1 Bacterial Carbon Source Availability	44
3.2.3.2 Bacterial Luminescence-based Root Exudate Profiling	45
3.3 Discussion	49

Chapter 4	57
RESOLVING THE GENETIC BASIS FOR DIFFERENTIAL WHEAT ROOT EXUDATION	57
4.1 Introduction	58
4.2 Results	60
4.2.1 C4-dicarboxylate root exudation in the primary wheat gene pool	60
4.2.2 Characterising the rhizosphere environment of the Paragon and W440 cultivars	63
4.2.2.1 Bacterial Luminescence-based Root Exudate Profiling	63
4.2.2.2 Bacterial Carbon Source Availability	64
4.2.2.3 Rhizosphere <i>Pseudomonas spp.</i> Populations	65
4.2.3 Mapping Quantitative Trait Loci	67
4.3 Discussion	70
Chapter 5	77
DISCUSSION AND FUTURE PERSPECTIVES	77
List of References	84
Appendix	100

# List of Tables and Figures

## Tables:

Table 1 - Wheat (*Triticum aestivum*) accessions used in this study

Table 2 - Bacterial strains used in this study

Table 3 - Single marker regression analysis for C4-dicarboxylate exudation from wheat roots

## Figures:

Figure 1 - *In vivo* spatiotemporal mapping images of pea (*Pisum sativum*) root colonization with luminescently labelled *R. leguminosarum* biovar *viciae* 3841 (Rlv3841) where Lux expression is controlled by the constitutive neomycin phosphotransferase promoter pNeo (reproduced from Pini *et al.*, 2017)

Figure 2 - Geographic origins of the A.E. Watkins landrace collection c. 1930 (reproduced from Winfield *et al.*, 2017)

Figure 3 - Phylogeny of the *Pseudomonas* genus inferred by multi-locus sequence analysis reflecting the diverse lifestyles of the groupings (reproduced from Garrido-Sanz *et al.*, 2016)

Figure 4 - Phylogeny of *Pseudomonas spp.* genotypes based on *gyrB* sequence homology

Figure 5 - The effect of wheat genotype on rhizospheric *Pseudomonas spp.* populations

Figure 6 - Necessity of 'simple' and 'complex' carbon metabolism in rhizobacterial competence

Figure 7 - *In vivo* bioluminescence of a suite of *R. leguminosarum lux* fusion biosensors over time

Figure 8 - *In vivo* bioluminescence of the C4-dicarboxylate biosensor on wheat roots 4 and 7 dpi

Figure 9 - Variation amongst modern wheat cultivars for the C4-dicarboxylate exudation phenotype

Figure 10 - Variation amongst wheat landraces for the C4-dicarboxylate exudation phenotype

Figure 11 - Variation amongst a wheat landrace panel for the C4-dicarboxylate exudation phenotype

Figure 12 - *In vivo* bioluminescence of four *R. leguminosarum* Rlv3841 *lux* fusion biosensors 7 dpi

Figure 13 - Necessity of 'simple' and 'complex' carbon metabolism in rhizobacterial competence

Figure 14 - The effect of wheat genotype on rhizospheric *Pseudomonas spp.* populations

Figure 15 - Frequency distribution of C4-dicarboxylate exudation in the Paragon x W440 population

Figure 16 - Profile plot of loci effecting variation for C4-dicarboxylate exudation from wheat roots

Figure 17 - Locations of three minor QTL conferring C4-dicarboxylate exudation from wheat roots

Figure S1 - Variation for 4 rhizobacterial traits amongst *Pseudomonas spp. in vitro*

Figure S2 - Spatial distribution of luminescent *R. leguminosarum* biosensor bacteria on wheat roots

Figure S3 - Variation amongst four wheat landraces for the C4-dicarboxylate exudation phenotype

Figure S4 - Variation amongst wheat accessions for the C4-dicarboxylate exudation phenotype

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## Chapter 1

### INTRODUCTION

## 1.1 Background: The Plant Microbiome

Microbes are fundamental to the maintenance of life on Earth and are widely acknowledged for their role in the survival and fitness of the many higher organisms with which they associate (Berendsen *et al.*, 2012; Sanchez-Canizares *et al.*, 2017; Hassani *et al.*, 2018). Plant-Microbe interactions are believed to have facilitated the conquest of land itself (Selosse and Le Tacon, 1998), and to this day can profoundly affect the success of plants in both ecological and agricultural contexts (Compant *et al.*, 2010; Vandenkoornhuysen *et al.*, 2015). This is reflected by the increasing prevalence of research into the plant microbiome - that is the dynamic and interactive ecological communities of microorganisms living associatively with all plant tissues along with their theatres of activity, whose distinct physio-chemical properties are defined by the plant. Indeed, the Food and Agriculture Organization of the United Nations has outlined further research and development of plant-growth-promoting microbial associations as a priority for improving sustainable food production (FAO, 2019). The greatest diversity of microorganisms directly interacting with any plant occurs at the interface between its roots and the surrounding soil environment (Reinhold-Hurek *et al.*, 2015). This extremely rich microhabitat was described over 100 years ago by Lorenz Hiltner who coined the term 'rhizosphere' - from the Greek 'rhiza', meaning root, and 'sphere', meaning field of influence - to describe the highly microbially active region that he observed around plant roots (Hiltner, 1904; Hartmann *et al.*, 2008). Indeed, the plant's investment in the rhizosphere is not to be underestimated, with anything between 10% and 44% of the net carbon assimilation of the plant released from the roots (Bais *et al.*, 2006). These so called rhizodeposits - that is the nutrients, border cells, mucilage and other specific exudates supplied by the plant, provide the basis for the establishment of a plethora of plant-microbe interactions with varying degrees of intimacy (Morgan *et al.*, 2005; Turner *et al.*, 2013a).

Whilst plants originate from seeds, the community of microorganisms active in the rhizosphere must assemble from the immense taxonomic diversity of the surrounding bulk soil. In contrast to the elevated microbial density in the rhizosphere, a substantial reduction of

diversity is observed compared with the surrounding soil as a result of the abiotic selection pressures within this niche as well as specific plant-microbe interactions and intense competition between microorganisms for resources (Compant *et al.*, 2010; Philippot *et al.*, 2013). The increased dominance of the Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria and an underrepresentation of Acidobacteria characterise rhizobacterial assemblages when compared with the reservoir of diversity in the surrounding soil (Bulgarelli *et al.*, 2013). These observations are a reflection of a wider trend for the enrichment of so called 'rhizosphere competence' traits within these communities (Compant *et al.*, 2010; Barret *et al.*, 2011). Indeed both the genetic potential for, and functional expression of the genes that control traits such as exudate metabolism, type III secretion, polysaccharide degradation, motility, and chemotaxis correlate well with this rhizospheric enrichment of 'competent' microbes at the community level (Mark *et al.*, 2005; Ofek-Lalzar *et al.*, 2014). Such studies highlight the value of considering the functional significance of selection pressures imposed on the microbiota, especially when many important traits may be represented across a diverse range of taxa with different environmental distribution. This functional redundancy should theoretically ensure the assembly of a 'core functional microbiota' regardless of the edaphic microbial reservoir (Lemanceau *et al.*, 2017). Indeed, Louca *et al.* (2016) describe such a similarity of functional microbial community structures between natural aquatic ecosystems contained within wild bromeliad foliage despite the high variability of the taxonomic composition of individual functional groups. Functional genes are reported as the units of selection in bacterial communities associated with the green macroalga *Ulva australis* (Burke *et al.* 2011), and more recently with the common weed species *Jacobaea vulgaris*, where functional genes, particularly those related to transporters, were consistently and significantly enriched in the rhizosphere (Yan *et al.* 2017).

Although principally determined by the properties of the surrounding soil and the requirement for general competence traits, rhizosphere microbial diversity is at least in part modulated by the identity of the host plant (Lemanceau *et al.*, 1995; Garbeva *et al.*, 2004; Berg and Smalla, 2009; Badri and Vivanco, 2009; Hartmann *et al.*, 2009). Rhizospheric microbial communities

recruited from the same bulk soils have consistently been shown to differ from species to species (Ladygina and Hedlund, 2010; Turner *et al.*, 2013b; Ofek-Lalzar *et al.*, 2014). Furthermore, different accessions of a single species can harbour distinct microbial communities in the rhizosphere (Micallef *et al.*, 2009a; Hardoim *et al.*, 2011; Bouffaud *et al.*, 2012; Inceoğlu *et al.*, 2012; Mahoney *et al.*, 2017), with further modifications coinciding with developmental transitions (Mougel *et al.*, 2006; Micallef *et al.*, 2009b; Chaparro *et al.*, 2013a) and in response to certain stressors (Berendsen *et al.*, 2018) suggesting that plants actively shape the composition of their rhizosphere microbiome. The effect of plant genotype specifically was elegantly demonstrated by Haney *et al.* (2015) who showed that the ability to associate with the root-associated bacterium *Pseudomonas fluorescens* differed substantially across 196 wild accessions of model plant species *Arabidopsis thaliana*. Additionally, Wintermans *et al.* (2016) were able to demonstrate the extent to which *Arabidopsis* genotypes differed in their responsiveness to the plant-growth-promoting activities of well characterised beneficial rhizobacteria. Together these seminal papers demonstrate that plants possess natural genetic variation for both the ability to host particular rhizobacterial populations, and the capacity to profit from their beneficial functions. This knowledge presents an exciting starting point for the development of future crops that are best able to exploit the functions of their root microbiome.

## 1.2 Plant Control of the Rhizosphere Microbiome

The host plant's influence on the rhizosphere environment may be exerted through both its root architecture and the nature of rhizodeposition (Sasse *et al.*, 2018), with the specific chemical profile of root exudates proposed as the principal mechanism controlling microbiome composition (Bais *et al.*, 2006; Badri and Vivanco, 2009; Lareen *et al.*, 2016; Massalha *et al.*, 2017). For the microbes in the rhizosphere, the exudation of primary and more complex secondary metabolites can act as sources of carbon for the growth of microbes able to utilise them or further contribute to the complex network of molecular interactions in the rhizosphere by attracting, deterring or directly antagonising individual

microbial genotypes (Lareen *et al.*, 2016; Massalha *et al.*, 2017; Sasse *et al.*, 2018). The central role of metabolizable exudates in particular was highlighted by a comparative genomics study of over 3,800 bacterial genomes which revealed an increased capacity for carbohydrate metabolism amongst diverse plant-associated bacteria (Levy *et al.*, 2018). Comprehensive investigation of the responses of rhizobacteria to wild oat root exudates has also revealed that bacterial taxa which proliferate during plant growth encode many more organic acid transporters than those with a decreased relative abundance in the rhizosphere environment (Zhalnina *et al.*, 2018). Moreover, by employing an exo-metabolomics approach Zhalnina *et al.* (2018) were able to relate these findings to an increased utilisation of several classes of primary metabolites (including organic acids) from plant-derived exudates *in vitro* - substantiating claims of the central role of exudation in rhizosphere microbiome assembly.

Importantly, the role of root exudates in structuring the rhizosphere microbiome has been verified in the absence of the plant itself, establishing causality for the response of diverse soil microbial communities to natural blends of *A. thaliana* root exudates (Badri *et al.*, 2013). The sheer complexity of this process is perfectly exemplified by the findings of Huang *et al.* (2019) who demonstrated that the purified products of a recently discovered specialized metabolic network capable of synthesizing more than 50 previously unknown *A. thaliana* root metabolites selectively modulate the growth of taxonomically diverse rhizosphere microbes, with examples of both positive and negative modulation in evidence. Excitingly the authors were also able to confirm that *A. thaliana* mutants impaired in the biosynthesis of these compounds exhibited shifts in the overall diversity and composition of their root microbiome compared with those of the wildtype - specifically disrupting the portion of the microbiota specific to *A. thaliana* when compared with other plant species (Huang *et al.*, 2019). This highlights the importance of specific root exudation phenotypes in determining the structure and function of the microbial communities associated with particular plant accessions.

Indeed the influence of host genotype has long been investigated (Neal *et al.*, 1970), with a number of studies employing transgenic exudate synthesis pathways (Oger *et al.*, 1997;

Narasimhan *et al.*, 2003) amongst the first to highlight the role of root exudation in promoting the growth of individual strains of interest (Savka and Farrand, 1997; Narasimhan *et al.*, 2003). These studies represented the beginnings of ongoing efforts to 'engineer' the rhizosphere by altering root exudation such that it becomes 'biased' towards the success of desirable microbial partners (O'Connell *et al.*, 1996). However, whilst these approaches are highly promising for the development of synthetic signalling pathways that could induce novel bacterial functions such as symbiotic nitrogen fixation (Geddes *et al.*, 2019) and pollutant removal (Narasimhan *et al.*, 2003), it is easy to imagine that mechanisms promoting existing beneficial functions will already be present in plants and simply remain to be elucidated. Presently, mechanistic evidence of plants naturally recruiting specific microbes to the rhizosphere microbiome abound, seminal examples of which include the elegant description of selective stimulation of the beneficial rhizobacterium *Bacillus subtilis* FB17 mediated by pathogen-induced malic acid exudation from *A. thaliana* roots (Rudrappa *et al.*, 2008), and the increased accumulation of *Pseudomonas putida* KT2440 on maize roots exuding benzoxazinoids (Neal *et al.*, 2012).

Clearly, the release of exudates does not just impact single microbial species, but rather influences many organisms with effects that ripple throughout the community shaping the rhizosphere microbiome composition (Oger *et al.*, 2004; Badri *et al.*, 2009). The cascading effects of root exudation on the rhizosphere microbiota have since been the subject of increasingly detailed studies of microbial community composition enabled by next-generation sequencing technologies. Such investigations have helped reveal the influential role of root exudation in curating highly specific microbiomes in response to many factors. Interestingly, several studies that have considered exudation alongside analyses of the rhizosphere microbiota appear to confirm that the effects of host plant genotype (Micallef *et al.*, 2009a) as well as developmental stage (Chaparro *et al.*, 2013b; Zhalnina *et al.*, 2018) can be attributed to differences in root exudate chemistry that modulate the microbiome. Indeed, it is now believed that significant genetic variation exists, even between accessions of a single species (Mönchgesang *et al.*, 2016a) such that it may be exploited with a view to promoting

beneficial relationships with the rhizosphere community (Bakker *et al.*, 2012; Peiffer *et al.*, 2013; Philippot *et al.*, 2013). This approach could represent a new era in plant breeding where the activities of the microbes naturally recruited by the plant are considered alongside the plants own traits (Ryan *et al.*, 2009; Wissuwa *et al.*, 2009; Dessaux *et al.*, 2016; Wei and Jousset, 2017). However, in order for this vision to be realised, an intimate understanding of the relationship between the microbial function that a plant attempts to recruit and the precise nature of the exudate chemistry needed to recruit it must be achieved (Oyserman *et al.*, 2018).

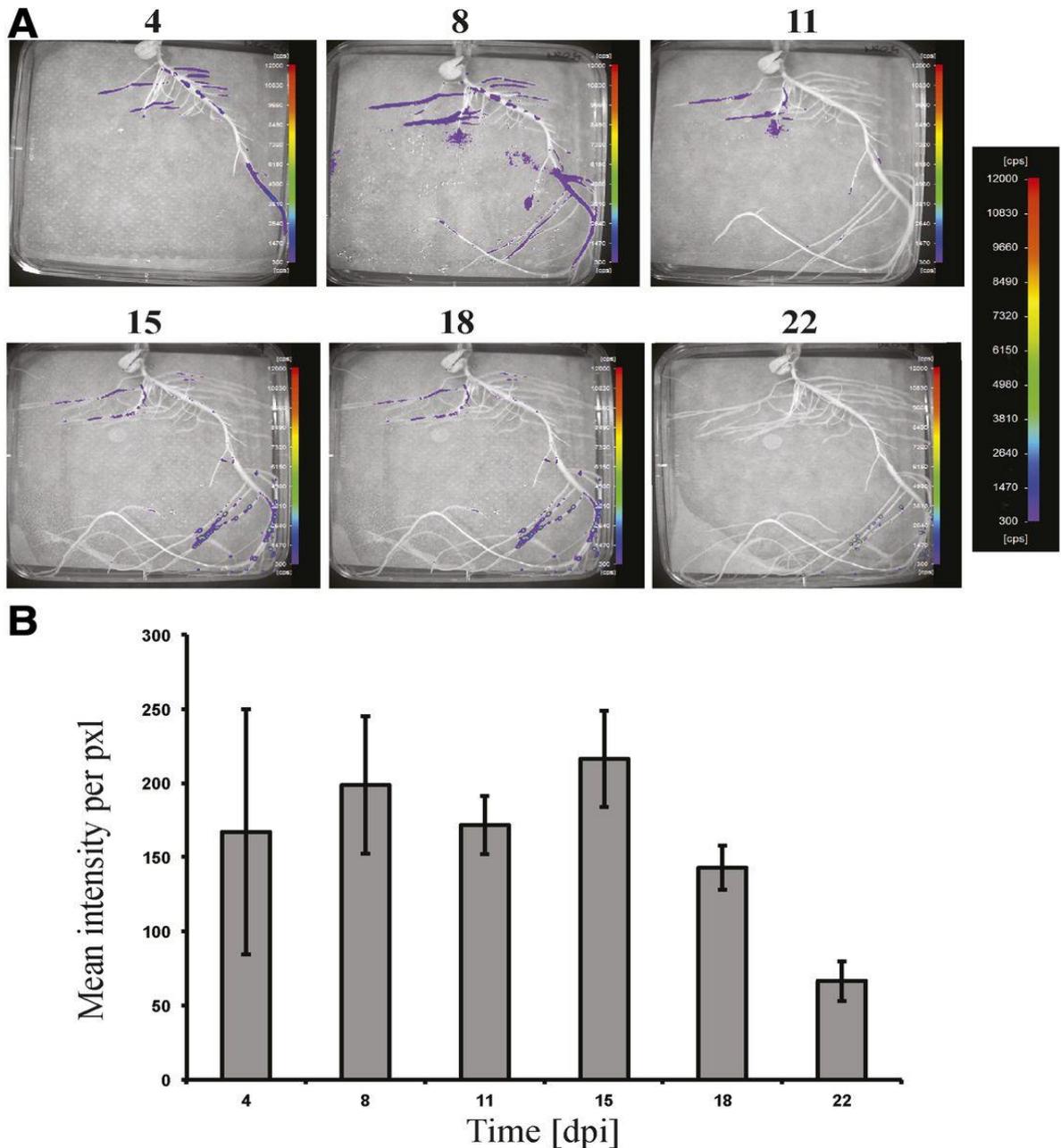
The identification of root exudation phenotypes responsible for different microbiome-mediated functions that can be connected to loci in the plant genome is of great interest to modern plant breeding efforts. Initial approaches towards identifying a genetic basis for microbial associations yielded quantitative trait loci (QTL) associated with the suppression of the seed pathogen *Pythium torulosum* by the biocontrol agent *Bacillus cereus* UW85 which could explain 38% of the phenotypic variation amongst the tomato lines assessed by Smith *et al.* (1999). More recently, genome-wide association studies (GWAS) of *A. thaliana* accessions have proved most fruitful in the identification of plant loci linked to the bacterial community structure on the leaves of plants grown in a field experiment when using the number of reads for individual taxa as quantitative phenotypes (Horton *et al.*, 2014) as well as the capacity to profit from specific root-associated microbes (Wintermans *et al.*, 2016). However, whilst successful in the discovery of a few candidate genes that correlate with the variation in composition of the plant microbiota in different plant genotypes, the mechanisms driving these changes that may be more easily exploited remain relatively overlooked. There is an argument to be made for the targeted investigation of root exudation by plant genotypes with contrasting microbial associations. Indeed, by mechanistically investigating the chemical interplay between plant roots and the rhizosphere microbiome it should be possible to pinpoint the genetic basis of specific exudation phenotypes that may be exploited to encourage beneficial microbial associations.

## 1.3 Measuring Root Exudation

Targeting root exudation as a means by which to understand the genetic basis of microbial selection in the rhizosphere holds great promise as has been described above. However, the quantification of root exudation presents a significant hurdle for our understanding of root microbiome recruitment. The identification of specific metabolites and determination of total root exudate composition are key to this approach but have both necessitated convoluted and time-consuming methodologies (Oburger and Jones, 2018). For example, the use of gas or liquid chromatography coupled with mass spectrometry that has become the standard for the identification and quantification of root-exuded compounds is a highly labour-intensive procedure, with different sample preparation methods required for different classes of metabolites. Thus, in order to collect sufficient quantities for multiple preparations, individual samples often comprise all the compounds released over a period of days, hindering the temporal resolution of such investigations (Chaparro *et al.*, 2013b). Moreover, the soluble exudates collected using the hydroponic systems employed in these investigations may not be totally representative of the full extent of rhizodeposition actually occurring (Oburger and Jones, 2018). The effects of utilising different rooting substrates on exudation (Mimmo *et al.*, 2011; Miller *et al.*, 2019) must be considered, whilst also acknowledging the dynamic sorption of exudates to certain substrate components (Jones and Edwards, 1998). Even the widespread use of sterile 'germ-free' plant growth systems, employed in order to minimise the microbial catabolism of exudates (Kuijken *et al.*, 2015) may actually impact exudation itself (Canarini *et al.*, 2019) - by altering concentration gradients generated by the natural source-sink dynamics of certain compounds (Meharg and Killham, 1995; Groleau-Renaud *et al.*, 2000) or removing the influence of certain exudation stimulating microbial products (Phillips *et al.*, 2004). As with any belowground trait, balancing optimal cultivation conditions that accurately reflect plant growth conditions in the field with the most desirable methodologies used to quantify said phenotypes is paramount, and inevitably involves compromise.

The utilisation of bacterial 'biosensors' that are routinely used in the examination of microbial perception of a variety of other biotic and abiotic environments presents an exciting opportunity for future studies of root exudation. Indeed, the ability of such biosensor strains to non-destructively perceive and report on the rhizosphere environment should allow the incorporation of spatial and temporal measurements of root exudation *in situ* (Gage et al., 2008). Furthermore, such reporter systems can be constructed to reflect not only whole-cell metabolic activity (Darwent *et al.*, 2003; Herron *et al.*, 2013) but also the activity of individual catabolic pathways (Jaeger *et al.*, 1999; Yeomans *et al.*, 1999), thus yielding information on total exudation or on a single component of exudation respectively. For this purpose, a suite of inducible bacterial bioreporters have recently been developed by Pini *et al.* (2017) using a system based on the bioluminescence (*lux*) genes of *Vibrio fischeri*. The *luxCDABE* gene cassette encodes all the enzymes needed for luminescence using only endogenous substrates (Close *et al.*, 2012) such that its activity may be precisely transcriptionally regulated. Indeed, Pini *et al.* (2017) employed the promoter regions found directly upstream of a selection of genes encoding compound-specific transport systems and metabolic enzymes that were identified in a comparative transcriptomic study of *Rhizobium leguminosarum* biovar *viciae* 3841 (Rlv3841) during rhizospheric growth (Ramachandran *et al.*, 2011). Naturally, the transcription of these genes is induced upon detection of the compound to which they are specific (Ramachandran *et al.*, 2011), such that luminescence of the resulting biosensor strains reflects the presence of these solutes in root exudates above a minimum concentration according to the extent of promoter expression (Pini *et al.*, 2017). Whilst the precise nature of the relationships between biosensor luminescence and substrate concentrations above these detection thresholds have not yet been characterised, measures of luminescence indicative of variable promoter expression effectively enable semi-quantitative comparisons of individual root exudation phenotypes amongst plants. Theoretically these biosensor strains may be used for the detection of specific root-exuded compounds from any plant species assuming its roots are adequately colonised by Rlv3841 as seen in figure 1a for pea (*Pisum sativum*) roots in the Pini *et al.* (2017) study. Thus, the

quantification of root exudation necessitates the use of gnotobiotic mesocosms that ensure no other organisms can compete with the biosensor strain, either for space on the root surfaces, or for the compound of interest itself (Kuijken *et al.*, 2015), which would lead to a misleading decrease in luminescence. However, assuming these conditions are rigorously adhered to, such bacterial biosensor reporter systems represent a powerful toolset with which to examine these hitherto elusive plant traits and how they may vary.



**Figure 1 - *In vivo* spatiotemporal mapping images of pea (*Pisum sativum*) root colonization with luminescently labelled *R. leguminosarum* biovar *viciae* 3841 (Rlv3841) where Lux expression is controlled by the constitutive neomycin phosphotransferase promoter pNeo (reproduced from Pini *et al.*, 2017) A - Images were acquired at 4, 8, 11, 15, 18, and 22 dpi respectively, [cps]= counts (numbers of photons emitted) per second represented by the colours on the 'heat-map' image overlay. B - Mean luminescence (pixels mm<sup>-2</sup>)  $\pm$ SEM.**

## 1.5 Wheat (*Triticum aestivum*)

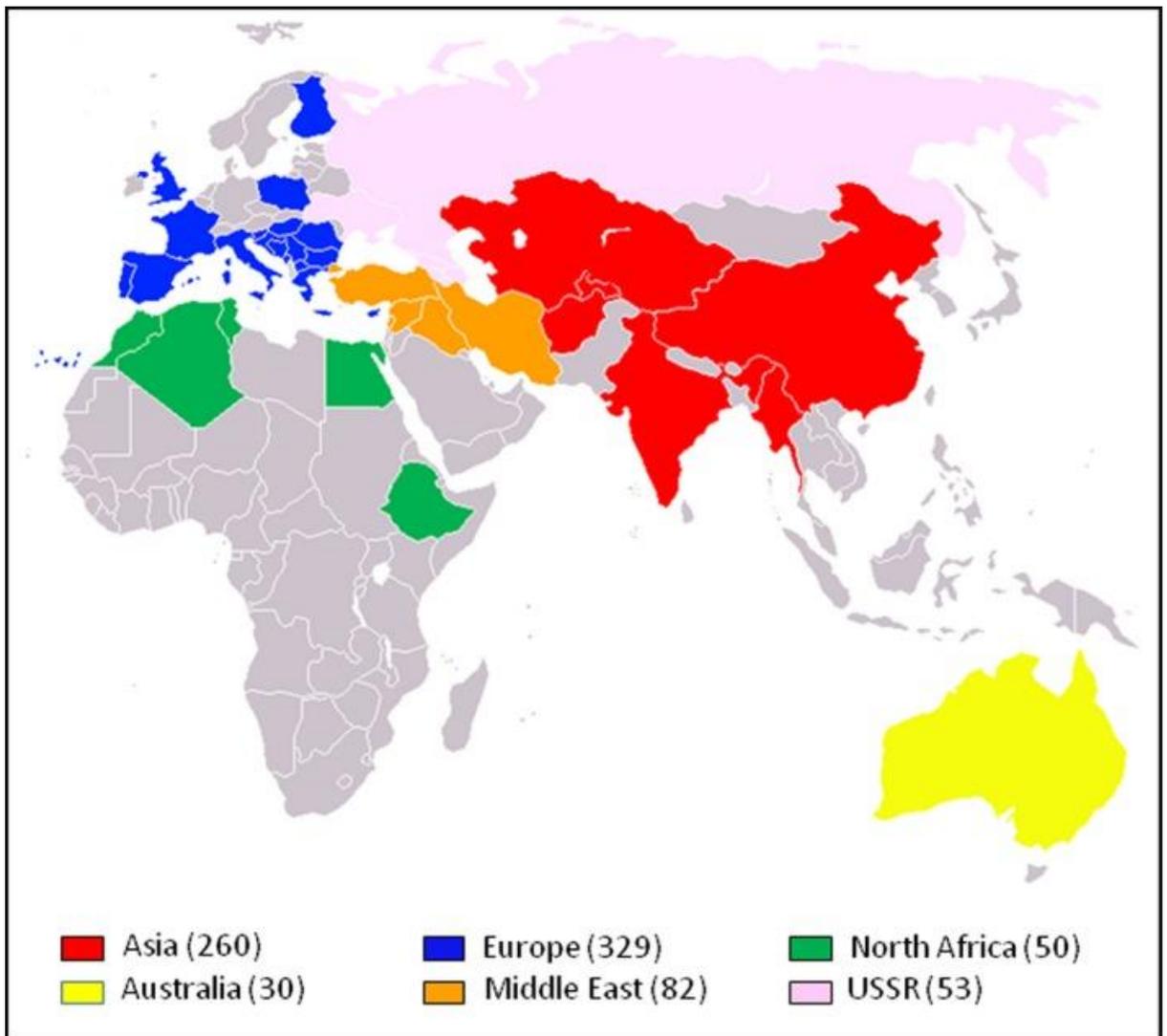
Hexaploid bread wheat (*Triticum aestivum*) is the world's most important crop for direct human consumption and represents ~30% of global cereal production across an unrivalled geographic range of cultivation (Gustafson *et al.*, 2009; Shewry, 2009). Cultivated wheat has an unmatched nutritional profile when compared to other cereals and constitutes roughly 20% of all human caloric consumption and 25% of protein intake ([www.fao.org/faostat](http://www.fao.org/faostat)). However, whilst the global demand for wheat is expected to increase by 60% by 2050 ([www.wheatinitiative.org](http://www.wheatinitiative.org)) due to population growth and shifting patterns of consumption (Lobell *et al.*, 2009), current yields appear to have plateaued (Grassini *et al.*, 2013). Concurrently, the unsustainable high-input agricultural practices that have largely enabled the increases in crop yields over the last 50 years are being held to account (Vitousek *et al.*, 1997; Pingali, 2012). Therefore, the real challenge is to further increase agricultural productivity in a way that also minimizes the harmful effects of chemical fertilizers and pesticides on the environment (Adesemoye and Kloepper, 2009; Godfray *et al.*, 2010; Foley *et al.*, 2011).

One particularly attractive approach to this two-fold problem would be to harness the potentially beneficial functions of microbes in the rhizosphere (Bakker *et al.*, 2012). Indeed, if the diversity of resident soil microorganisms is considered as a genetic reservoir of rhizosphere functions that may be incorporated into the extended genome of the host plant (Turner *et al.*, 2013a), there exists a plethora of potentially Plant-Growth-Promoting (PGP) microbes (Tkacz and Poole, 2015). The potential of such microbes to reduce fertiliser dependence in some crops has started to be realised already, with new agricultural practices incorporating PGP microbes achieving the same yields as intensive farming but with fewer agricultural inputs (Adesemoye *et al.*, 2009). For wheat itself, the benefits afforded by rhizosphere microorganisms are perhaps best characterised by the phenomenon of disease suppressive soils (Weller *et al.*, 2002). Indeed, wheat yields are known to benefit strongly from the protective effects of 2,4-DAPG-producing fluorescent *Pseudomonas* bacteria that

are enriched in soils suppressive to the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Kwak and Weller, 2013). Interestingly, the capacity of wheat plants to enhance resident populations of these 2,4-DAPG-producers appears to differ across cultivars (Mazzola *et al.*, 2004; Meyer *et al.*, 2010a, Kwak *et al.*, 2012) with cultivar-specific accumulations of these pseudomonads observed as early as the seedling stage (Okubara and Bonsall, 2008). Indeed, the spontaneous decline in take-all severity after the cultivation of certain wheat cultivars in agricultural field trials has been formally attributed to the take-all inoculum build-up (TAB) trait (McMillan *et al.*, 2018) whereby low TAB cultivars minimise the increase of take-all inoculum in the soil as measured by post-harvest infectivity bioassays (McMillan *et al.*, 2011). The influence of high and low TAB varieties on the rhizosphere microbiome was explored in a 2015 study by Mauchline *et al* which found *Pseudomonas spp.* populations to be affected more than any other bacterial group. By analysing the phenotypic and genetic diversity of *Pseudomonas spp.* populations in agricultural field trials, the authors revealed a considerable effect of wheat cultivar choice on rhizospheric selection pressures (Mauchline *et al.*, 2015). This research has suggested the existence of a genetic basis for such beneficial relationships between wheat and certain rhizosphere microbes that may well be exploited in the development of new varieties.

The polyploid wheat genome does however make genetic analyses more cumbersome than those of other major cereal crops and has hindered our genetic understanding of many important productivity traits (Bevan *et al.*, 2017). Although recently, a number of important genomic advancements and the proliferation of open access resources have streamlined the elucidation of traits using wheat genetics (Uauy, 2017; Borrill *et al.*, 2019). Unfortunately, modern wheat germplasm is still inherently vulnerable to the challenges faced by modern agriculture due to its highly reduced genetic diversity - a result of the intense selection pressures imposed by modern breeding and cropping practices (Haudry *et al.*, 2007; Winfield *et al.*, 2017). It is posited that during the domestication of wild ancestors and subsequently in breeding modern high yielding crop varieties, genes associated with beneficial microbial interactions may have been lost if such phenotypes also incurred a loss of yield or were

simply not selected for as strongly as they would be in nature (Wissuwa *et al.*, 2009; Perez-Jaramillo *et al.*, 2016; Hassani *et al.*, 2019). Moreover, plant breeding conducted under 'ideal' stress-free conditions largely prevents the adaptation of crops to the diverse environments encountered within their range of cultivation - including the local microbiota. This, along with the so called 'cost of domestication' whereby domesticated species exhibit increased accumulations of deleterious and non-advantageous mutations which genetically 'hitchhike' along with loci conferring the target traits (Lu *et al.*, 2016), has left us with crop plants that may well be less fit in terms of their associations with potentially beneficial microbes. Observations that would appear to support this notion are described regularly, mostly for intensively bred crop plants such as wheat itself. Amplicon sequencing of the ITS locus has revealed that fungal endophyte communities harboured by wild grass relatives of wheat for example, are much more taxonomically diverse than those associated with modern wheat, which was even found to lack some taxa known to benefit host plant growth (Ofek-Lalzar *et al.*, 2016). Such findings are consistent with earlier observations suggesting modern wheat cultivars harbour less diverse rhizobacterial communities than ancient landraces of domesticated wheat (Germida and Siciliano, 2001) with decreased incidences of mycorrhizal symbioses (Hetrick *et al.*, 1993). Interestingly, some recent studies have even linked the effects of domestication and breeding to root exudate composition, with an investigation of the closely related tetraploid wheats (*Triticum turgidum* spp.) revealing major effects on the secretion of certain rhizosphere metabolites (Iannucci *et al.*, 2017). Despite this, the importance of locally adapted bread wheat landrace populations that can be directly cross bred into modern varieties was only really realised as recently as the 1980s (Feldman and Sears, 1981).



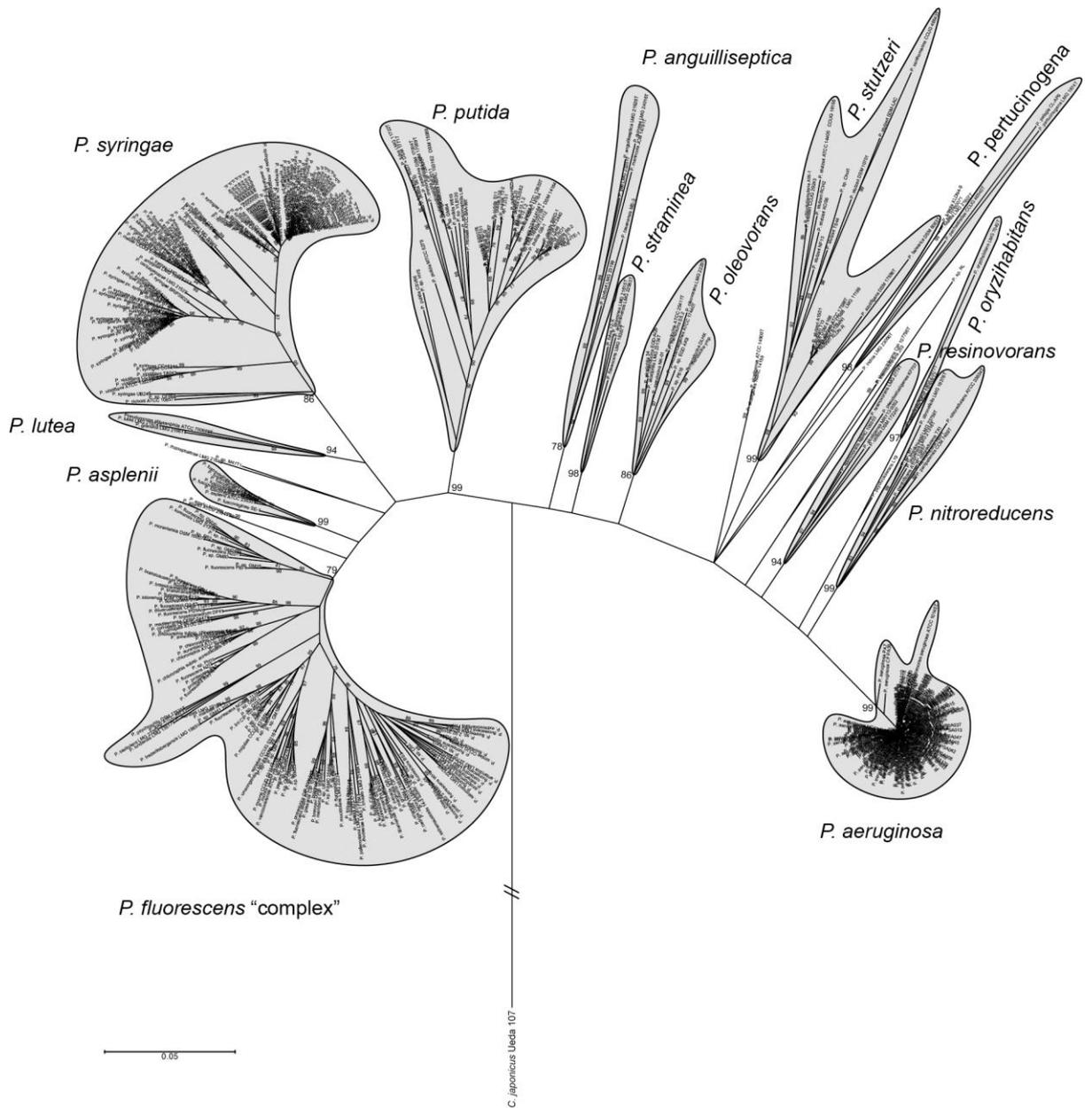
**Figure 2 - Geographic origins of the A.E. Watkins landrace collection c. 1930 (reproduced from Winfield *et al.*, 2017)** The numbers in brackets are the number of accessions from each region, totalling 804 accessions. The European accessions include 13 from the Canary Islands.

The A.E. Watkins landrace collection, comprising a geographically diverse set of accessions (Figure 2) is thus of particular note for having been collected in the 1930s before the advent of modern breeding practices (Wingen *et al.*, 2014). These accessions, purified by single-seed descent, possess extensive novel genetic diversity absent from modern germplasm (Winfield *et al.*, 2017) and have been used to generate a suite of fully genotyped genetic resources for trait discovery (Wingen *et al.*, 2017). Such exotic germplasm that has not been modified through modern breeding techniques represents an important source of genetic variation not present in modern elite varieties. For disease resistance breeding in particular, historical varieties of modern crops and even their wild relatives from outside the primary gene pool have already proved to be highly valuable, most recently in the targeted *R* gene

identification and cloning approaches of Arora *et al* (2019) in wheat. Moreover, variation from within the Watkins collection itself has in fact already yielded the identification of novel loci for both rust and eyespot resistance (Toor *et al.*, 2013; Burt *et al.*, 2014). These findings seem to point towards the existence of readily identifiable genetic variation responsible for relationships with the diverse rhizosphere microbial communities that are predicted to be associated with these largely unadulterated accessions. Together with the aforementioned evidence of the effects of wheat breeding on exudate composition, the present literature highlights the enormous potential to improve our understanding of the genetic control of interactions with beneficial rhizosphere microorganisms through targeted investigations of contemporary and historical accessions.

#### 1.4 *Pseudomonas spp.*

Pseudomonads are generalist Gram-negative gamma-proteobacteria found throughout almost all terrestrial habitats in a range of environmental niches (Loper *et al.*, 2012). Living saprophytically, *Pseudomonas spp.* commonly reside both on the surfaces of, and within the tissues of many higher organisms (epi- and endo-phytically respectively) including almost all plant species (Silby *et al.*, 2011). However, as well as the numerous commensal pseudomonads that live off nutrients secreted from (e.g. root exudates) or those that are present within (e.g. apoplast constituents) plants with no fitness effects on the host, some members of the genus engage in more parasitic or mutualistic relationships (Preston, 2004). The plethora of *P. syringae* pathovars (Figure 3) for example, cause disease symptoms in a number of economically important crop plants via the secretion of a variety of hypersensitive response and pathogenicity (Hrp) effector proteins directly into host cells (Ichinose *et al.*, 2013). On the other hand, many other plant-associated pseudomonads - most notably those in the *P. fluorescens* 'complex' (Figure 3) - promote the growth of their host by antagonising pathogenic microorganisms, synthesizing growth-stimulating hormones and promoting increased disease resistance (Haas and Defago, 2005).



**Figure 3 - Phylogeny of the *Pseudomonas* genus inferred by multi-locus sequence analysis reflecting the diverse lifestyles of the groupings (reproduced from Garrido-Sanz *et al.*, 2016)**

Phylogenetic tree of 451 *Pseudomonas* strains along with 107 type strains based on the concatenated partial sequences of the 16S rDNA, *gyrB*, *rpoD* and *rpoB*, ML method, Tamura-Nei. Only bootstrap values above 75% (from 1,000 replicates) are shown. *Cellvibrio japonicum* Ueda 107 was used as outgroup.

The remarkable diversity and widespread distribution of the genus are often attributed to the metabolic plasticity demonstrated by its members (Silby *et al.*, 2011). This plasticity is exemplified perfectly by the elegant transcriptional regulation of primary carbon metabolism by the RpiR family transcription factors HexR and RccR. These regulators rapidly respond to carbon source availability by sensing a single key intermediate; 2-Keto-3-deoxy-6-phosphogluconate (KDPG) (Daddaoua *et al.*, 2009; Campilongo *et al.*, 2017). This capacity to rapidly adapt to, and then utilise a range of carbon sources makes *Pseudomonas* highly successful in the exudate-rich rhizosphere compartment, with *Pseudomonas spp.* representing a major constituent of most plants' rhizosphere microbiomes (Mauchline and Malone, 2017). Indeed, rhizospheric pseudomonads are fierce competitors, able to outcompete other organisms through their characteristically rapid growth and ability to quickly swarm and form exopolysaccharide-based biofilms on root surfaces (Chin-A-Woeng *et al.*, 1997; Lugtenberg *et al.*, 2001). This rhizosphere competence allows *Pseudomonas spp.* to non-specifically colonise plant roots and as such they have been studied extensively, with bacteria from this genus often employed as model root colonisers (Lugtenberg *et al.*, 2001).

Aside from the impressive physical colonisation abilities of the pseudomonads, the intriguing capacity of members of the *P. fluorescens* complex to effectively suppress soilborne plant pathogens is of particular interest with some members touted as PGP rhizobacteria (Haas and Defago, 2005; Santoyo *et al.*, 2012). These remarkable abilities are primarily the result of the action of a chemically diverse array of bioactive secondary metabolites and secreted proteins, the production of which characterises the high taxonomic diversity of the pseudomonads (Loper *et al.*, 2012; Seaton and Silby, 2014). Accordingly, these molecules may antagonise pathogens through various means including siderophore-mediated competition for iron (Kloepper *et al.*, 1980), the production of relevant exoenzymes such as AHL lactonases that disrupt pathogenic quorum sensing (Jafra *et al.*, 2006), as well as classical antibiosis via the production of dedicated antimicrobial compounds. These

compounds include pyoluteorin, pyrrolnitrin and phloroglucinols like 2,4-DAPG (Haas and Defago, 2005) alongside more broad-spectrum toxins like hydrogen cyanide (Blumer and Haas, 2000). The enormous importance of these compounds for the control of plant diseases has spurred investigations of the genotypic diversity, population dynamics and ecological functions of pseudomonads in the rhizosphere (Lemanceau *et al.*, 1995; Blumer and Haas, 2000; Bergsma-Vlami *et al.*, 2005; Landa *et al.*, 2006) such that we might uncover the true potential of introduced inoculants as well as naturally occurring populations of producing strains. This chemical diversity is reflected by the enormous pan-genome of the *P. fluorescens* complex, totalling approximately 30,000 coding sequences, within which as few as 1,334 are conserved among all members (Garrido-Sanz *et al.*, 2016). The significance of this diversity for plant health was strikingly demonstrated by Haney *et al* (2015) via the 16S ribosomal RNA sequencing of the rhizobacterial communities associated with various *A. thaliana* accessions grown in natural soils. The authors found that between-accession differences in the rhizosphere microbiome were in fact largely restricted to strains belonging to a subset of the Pseudomonadaceae, with divergent consequences for plant health and productivity depending on the precise biotic and abiotic stresses faced (Haney *et al.*, 2015). Furthermore, for *Pseudomonas spp.* introduced into naturally complex tomato rhizosphere microbial communities, Hu *et al* (2016) found that the level of strain diversity in the inoculum determined the efficiency of pathogen suppression and resulting disease incidence - proposing that the increased variety of bioactive metabolites produced by diverse consortia could act synergistically to suppress the pathogen. Rhizosphere *Pseudomonas spp.* populations continue to be studied as a model for their highly influential role in plant health. With an increasing understanding of the factors governing their recruitment by crop plants, we may start to exploit these pseudomonads and other PGP microbes as part of the sustainable intensification of agriculture.

## 1.6 Conclusions and Research Goals

By targeting wheat root exudation as one of the molecular mechanisms underpinning the diversity of the rhizosphere microbiota it should be possible to identify and then determine the precise effects of specific exudation phenotypes. The use of bacterial biosensors in gnotobiotic mesocosms allows for a reliable phenotyping method that could be used to investigate the genetic basis for the exudation of a range of compounds. Furthermore, given the huge genetic variability of resident soil-dwelling pseudomonads, examining the phenotypic diversity of *Pseudomonas spp.* populations recruited by different wheat accessions from the same starting soil substrate represents an attractive system in which to characterise the role of root exudation in the recruitment or activation of specific functional characteristics of the rhizosphere microbiome. Indeed, if we can better understand the precise nature of the genetic determinants that contribute to efficient colonisation of the wheat rhizosphere by PGP rhizobacteria then there is huge potential for the production of improved, low-input wheat varieties that are desperately needed for the sustainable intensification of agriculture.

## Chapter 2

# MATERIALS AND METHODS

## 2.1 Plant Material

This study employed a diversity panel of wheat (*Triticum aestivum*) accessions (Table 1) acquired from the Germplasm Resources Unit at the John Innes Centre (Norwich, UK).

**Table 1 - Wheat (*Triticum aestivum*) accessions used in this study**

<b>Name</b>	<b>Origin</b>	<b>Sowing season</b>
Hereward	UK elite cultivar	Winter
Cadenza	UK elite cultivar	Spring
Avalon	UK elite cultivar	Winter
Chinese Spring (line 42)	Chinese landrace	Spring
Paragon	UK elite cultivar	Spring
Watkins 044*	Moroccan landrace	Spring
Watkins 079* ('Dolatkhani [white]')	Indian landrace	Spring
Watkins 246* ('Soor Ghanum')	Indian landrace	Spring
Watkins 349* ('Golema Franga')	Bulgarian landrace	Spring
Watkins 360* ('Veliko Hoce')	Yugoslavian landrace	Spring
Watkins 440*	Chinese Landrace	Spring
Watkins 624* ('Svalene')	Bulgarian landrace	Winter
Watkins 651*	Chinese landrace	Winter
Watkins 698*	Chinese landrace	Spring
Watkins 722*	Chinese landrace	Spring
Watkins 784* ('Oberdan')	Italian landrace	Spring
Watkins 811*	Tunisian landrace	Spring

\*A.E. Watkins landrace collection 'core set' accession numbers

Seeds of each accession were surface sterilised with 70% ethanol for 1 minute, 25% sodium hypochlorite solution (bleach) for 3 minutes and several washes with sterile distilled water in order to minimise the vertical transmission of microorganisms. Disinfected seeds were germinated on water-agar (1.5%) for 48 hours in darkness at room temperature before use.

## 2.2 Bacterial Strains and Growth Conditions

The strains used in this work are listed in Table 2 below.

**Table 2 - Bacterial strains used in this study**

Organism	Strain	Description	Reference
<i>R. leguminosarum</i>	LMB483	Phenylalanine biosensor; Rlv3841 [pLMB577]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB590	Xylose biosensor; Rlv3841 [pLMB684]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB592	myo-Inositol biosensor; Rlv3841 [pLMB686]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB593	Sucrose biosensor; Rlv3841 [pLMB687]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB608	Tartrate biosensor; Rlv3841 [pLMB708]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB610	Formate biosensor; Rlv3841 [pLMB710]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB614	C4-dicarboxylate biosensor; Rlv3841 [pLMB714]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB617	Mannitol biosensor; Rlv3841 [pLMB705]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB619	Erythritol biosensor; Rlv3841 [pLMB717]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB638	Malonate biosensor; Rlv3841 [pLMB737]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB639	GABA biosensor; Rlv3841 [pLMB738]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB667	Fructose biosensor; Rlv3841 [pLMB757]	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB542	Rlv3841 carrying <i>lux</i> genes with no promoter	Pini <i>et al.</i> (2017)
<i>R. leguminosarum</i>	LMB743	Rlv3841 carrying <i>lux</i> genes under pNeo promotion	Pini <i>et al.</i> (2017)
<i>P. fluorescens</i>	SBW25- <i>lacZ</i>	SBW25 carrying a neutral, chromosomal ' <i>lacZ</i> marker in a defective prophage locus	Zhang and Rainey (2007)
<i>P. fluorescens</i>	SBW25- <i>lacZ</i> $\Delta rccR$	SBW25- <i>lacZ</i> with the <i>rccR</i> gene ( <i>PFLU_6073</i> ) deleted	Pacheco-Moreno (personal gift)
<i>P. fluorescens</i>	SBW25 $\Delta hexR$	Wildtype SBW25 with the <i>hexR</i> gene ( <i>PFLU_4840</i> ) deleted	Campilongo <i>et al.</i> (2017)

Unless stated otherwise, *Pseudomonas spp.* were grown at 28 °C using LB media (Bertani, 1951), solidified with 1.5% agar where appropriate. Initially, rhizosphere-dwelling pseudomonads were isolated using Pseudomonas Selective Agar, consisting of Pseudomonas Agar Base (Oxoid, CM0559) with Pseudomonas C-F-C Supplement (10µg ml<sup>-1</sup> Cetrimide, 10µg ml<sup>-1</sup> Fucidin, and 50µg ml<sup>-1</sup> Cephalosporin) (Oxoid, SR0103E). For root colonisation assays, strains were grown in M9 minimal media with 0.4% pyruvate as

the sole carbon source for the final 32 hours before inoculation to ensure that the only available nutrients in the experiment were plant derived. The final co-cultured root colonisation samples were plated onto blue-white selection plates (LB with 100 µg ml<sup>-1</sup> Carbenicillin, 50 µg ml<sup>-1</sup> X-Gal, and 0.1 mM IPTG). For the luminescent biosensor assays, all growth conditions follow Pini *et al* (2017). Briefly, all *Rhizobium leguminosarum* bv *viciae* 3841 (Rlv3841) *lux* biosensor strains were grown on tryptone yeast (TY) agar (Beringer, 1974) or universal minimal salts (UMS) at 28°C. UMS is derived from acid minimal salts (AMS; Poole *et al.*, 1994) with the following changes: EDTA-Na<sub>2</sub> (1 µM), CoCl<sub>2</sub>O·6H<sub>2</sub>O (4.2 µM), FeSO<sub>4</sub>O·7H<sub>2</sub>O (0.04 mM), and CaCl<sub>2</sub>O·2H<sub>2</sub>O (0.51 mM). Antibiotics were added when necessary at the following concentrations: streptomycin (500 µg ml<sup>-1</sup>), tetracycline (2 µg ml<sup>-1</sup> in UMS, 5 µg ml<sup>-1</sup> in TY).

## 2.3 Isolation of Rhizosphere *Pseudomonas* spp. Populations

For each cultivar, 5 successfully germinated seedlings were transferred to pots containing JIC Cereal Compost Mix (40% Medium Grade Peat, 40% Sterilised Soil, and 20% Horticultural Grit; with 1.3kg/m<sup>3</sup> PG Mix™ 14-16-18 + 1 MgO Trace Elements (TE) (0.03% B, 0.09% Fe, 0.16% Mn, 0.15% Cu, 0.20% Mo, and 0.04% Zn) Base Fertiliser, 1kg/m<sup>3</sup> Osmocote® Exact Mini 16-8-11 + 2 MgO + TE (0.02% B, 0.45% Fe, 0.06% Mn, 0.050% Cu, 0.020% Mo, and 0.015% Zn) Base Fertiliser, H2Gro® (Wetting Agent), 3kg/m<sup>3</sup> Maglime (dolomitic limestone), and 300g/m<sup>3</sup> Exemptor® (Insecticide); final pH 6.0 - 6.5). Plants were grown for 4 weeks at 25 °C in a Controlled Environment Room with a 16-hour light cycle and irrigated twice weekly with sterile tap water alongside several pots left unplanted as bulk soil control treatments. The rhizosphere compartments of 3 wheat plants from each cultivar were individually sampled as follows: Ethanol flamed scissors were used to remove the aerial parts of each plant and bulk soil was shaken from the root systems before transferring to individual sterile 50 ml falcon tubes. Each rhizosphere sample tube was filled up to 15 ml with sterile phosphate buffered saline (PBS) and vortexed for 10 minutes at 4 °C. Concurrently, three 50 ml falcon tubes were each filled with ~30 ml of loose sub-surface bulk

soil from three of the unplanted pots, filled to 45 ml with sterile PBS, and similarly vortexed. Dilution series of  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  were created for each of the samples using sterile PBS and plated onto Pseudomonas Selective Agar. After 48 hours of incubation at room temperature in darkness, 20 representative *Pseudomonas spp.* colonies were randomly sampled from the most appropriate dilutions and re-streaked onto L media agar plates to confirm single colony isolation. All isolates were catalogued in a 96-well plate-based system from which overnight cultures were transferred by microplate replicator onto assay plates.

## 2.4 Phylogenetic Analysis of Soil-Dwelling Pseudomonads

A collection of *Pseudomonas* strains similarly isolated from the rhizosphere compartment of full-term glasshouse-grown wheat plants as well as bulk soils was established as part of a previous project (Chloe Ormrod, unpublished data). Overnight cultures of a selection of 66 of these isolates - originally associated with the roots of the wheat cultivars Hereward and Cadenza or unplanted bulk soils - were used as templates for *gyrB* PCR by Chloe Ormrod with the UP-1 and UP-2r primers described by Yamamoto and Harayama (1995) and sequenced through Eurofins Genomics following Mauchline *et al* (2015). All phylogenetic analyses were performed in Geneious 10.2.3. All 66 *gyrB* sequences from each of the isolates as well as several *Pseudomonas* (*P. fluorescens* SBW25 and F113, *P. protegens* Pf-5, *P. putida* KT2440, and *P. syringae* DC3000) reference sequences were aligned within a 664 base pair (bp) fragment using the MUSCLE sequence alignment programme. The final phylogeny was constructed by neighbour-joining using the default settings of the 'Geneious tree builder' with an *Agrobacterium fabrum* C58 *gyrB* sequence provided as an outgroup.

## 2.5 Phenotypic Characterisation of *Pseudomonas spp.* Isolates

Up to 60 strains per isolation from the rhizosphere compartment of each wheat genotype were analysed in terms of 4 rhizobacterial traits related to success in the rhizosphere. Overnight cultures of the isolates were created in 96-well plates with gentle agitation at 28 °C and subsequently transferred to large 25 x 25 cm square assay plates using a microplate

replicator. The production of fluorescent compounds such as pyoverdine siderophores was determined by exposing colonies to UV light after 48 hours of growth at 28 °C on KB agar such that producing colonies would fluoresce. To assess protease activity, Kings medium B (KB) agar supplemented with 1% milk powder was used as the growth medium such that degradation halos would appear around those colonies producing proteases after 48 hours at 28 °C. To determine differences in polysaccharide production, the extent of red pigmentation of colonies was assessed after 48 hours of growth at 28 °C on KB agar with 0.005% Congo Red dye. Finally, cyanogenic bacteria were detected following a modified Castric and Castric (1983) methodology, whereby 96-well plates containing liquid cultures were used instead of colonies grown on solid media. Briefly, filter paper soaked in Feigl-Anger solution suspended above the 96-well plates changes colour after 48 hours of incubation at 28 °C if the corresponding isolates produce HCN. To obtain an objective basis for the comparison of isolates, colonies were visually examined and allocated ordinal values between 0 and 2 depending on the extent to which the phenotype was observed in the test where 0 = not present, 1 = weakly present, 2 = strongly present. Representative examples of isolates scored as such for each of the phenotypes outlined above are included in the appendix (Figure S1). The distribution of trait scores exhibited by different collections of isolates were compared using Chi-square tests and Bonferroni corrections to adjust *P* values where multiple comparisons were made.

## 2.6 Bacterial Carbon Source Availability Assessments

Successfully germinated seedlings were individually transplanted into 50 ml Falcon tubes containing sterilised pre-wetted small-grain vermiculite at a depth of 1.5 cm with 10 ml rooting solution (1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 100 µM KCl, 800 µM MgSO<sub>4</sub>, 10 µM FeEDTA, 35 µM H<sub>3</sub>BO<sub>3</sub>, 9 µM MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.8 µM ZnCl<sub>2</sub>, 0.5 µM Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.3 µM CuSO<sub>4</sub>·5H<sub>2</sub>O, 6 mM KNO<sub>3</sub>, 18.4 mM KH<sub>2</sub>PO<sub>4</sub>, and 20 mM Na<sub>2</sub>HPO<sub>4</sub>). Seedlings were allowed to grow for 7 days in a controlled environment room at 25 °C with a 16 h light cycle before inoculation of roots with 1 ml aliquots of a PBS-based mixed-culture inoculum prepared with equal

concentrations ( $1 \times 10^3$  CFU ml<sup>-1</sup>) of the two *P. fluorescens* SBW25 primary carbon metabolism mutants  $\Delta rccR$  and  $\Delta hexR$  (Campilongo *et al.*, 2017). Here the *hexR* deletion had been made in a wildtype SBW25 background whereas the *rccR* deletion was made in the neutrally marked SBW25-*lacZ* strain (Zhang and Rainey, 2007) such that the gene product ( $\beta$ -galactosidase) of the *lacZ* reporter catalyses the hydrolysis of X-Gal, producing a blue colour. After an additional 7 days of plant growth in the same controlled environment, all aerial shoots were removed before adding 20 ml sterile PBS and vortexing for 5 min at 4 °C to resuspend bacteria. Dilution series of  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  were created from 100  $\mu$ l of surface liquid extracted immediately post-vortex using additional sterile PBS and plated onto LB blue-white selection plates containing X-Gal such that resultant colonies of the SBW25-*lacZ*  $\Delta rccR$  strain would appear blue whereas those of the  $\Delta hexR$  strain would remain white. Following 48 h incubation at 28 °C the number of blue vs white colonies on the plates of the most appropriate dilutions were enumerated. Data are presented as the ratio of  $\Delta rccR$  to  $\Delta hexR$  CFU ( $\Delta rccR$  CFU /  $\Delta hexR$  CFU) recovered divided by the ratio of  $\Delta rccR$  to  $\Delta hexR$  CFU ( $\Delta rccR$  CFU /  $\Delta hexR$  CFU) in the initial inoculum - also determined by blue-white selection as above.

## 2.7 Bacterial Luminescence-based Root Exudate Biosensors

Luminescent biosensor strains were grown from glycerol stocks on tryptone yeast (TY) agar to confirm single strain isolation before streaking onto UMA (UMS with 16 g L<sup>-1</sup> agar) slopes with 10 mM NH<sub>4</sub>Cl and 30 mM pyruvate in universal tubes and incubation at 28°C for three days. Successfully germinated seedlings were transferred to individual 10 x 10 cm square petri dishes containing Fahraeus agar (Somasegaran and Hoben, 1994) and 'sandwiched' between two sterile 10 x 10 cm square filter paper sheets. Bacterial cultures were resuspended and washed three times in UMS without additions, before inoculating  $3 \times 10^7$  CFU (300  $\mu$ l inoculum at OD<sub>600</sub> = 0.1) directly onto seedling roots between the filter paper layers. All plates were placed upright on their sides and covered with aluminium foil to minimise exposure of roots to light. A 1 cm<sup>2</sup> hole was cut into the plastic along the top edge

of every petri dish with a sterile hot scalpel such that the shoots would emerge through them during their subsequent growth in a controlled environment room at 25°C with a 16-hour light cycle. Plates were photographed using a NightOWL cooled-CCD camera system (Berthold Technologies) at 2, 4, 7, and 14 days post inoculation (dpi). CCD images of light output under 0% illumination (ie. luminescence) were captured using a 60 s exposure, low camera gain, and slow readout. After acquisition and export using the IndiGO imaging software (Berthold Technologies) all images were analysed using the Fiji distribution package of the ImageJ image processing software (Schindelin *et al.*, 2012). Each image consisted of an array of 1024 by 1024 pixels, each with a pixel value (0-65535) determined by the luminous intensity of the subject matter it recorded. Data are presented as relative luminescence units, calculated as the 'integrated density' of luminescence by multiplying the total number of pixels (ie. area) detecting luminescence (defined as any pixel value over 30 in order to exclude background 'noise') by the mean value of those pixels (ie. intensity), giving an indirect but semi-quantitative measurement of the exudation of specific compounds *in vivo*. In cases where the results of independent experimental batches were to be compared, data are presented relative to a common 'reference' treatment (Paragon + LMB614 in 4.2.1) to account for non-biological batch-batch variation.

### 2.7.1 Statistical Analyses

Distributions of univariate data were initially inspected for normality and adherence to a Gaussian distribution tested using the D'Agostino-Pearson normality test. For data normally distributed, student's t-test and one-way Analysis of Variance (ANOVA) were implemented, the results of which are presented having met the assumption that the data have equal variances (standard deviations) as tested by the Brown-Forsythe test. Where the mean luminescence values of more than 2 treatments were to be compared, appropriate multiple comparisons methods were applied following significant one-way ANOVA hypothesis testing - Šídák's tests when comparing selected pairs of means, or Dunnett's tests when comparing every mean to one reference mean.

## 2.8 Quantitative Trait Loci Mapping

The F<sub>4</sub> Paragon x Watkins accession 440 mapping population was acquired as per Chapter 2.1 from the Germplasm Resources Unit at the John Innes Centre (Norwich, UK). The C4-dicarboxylate exudation trait was evaluated for 3-5 biological replicates of 63 Kompetitive Allele Specific PCR (KASP™) genotyped recombinant inbred lines (RILs) using the luminescence of the C4-dicarboxylate specific biosensor LMB614 7dpi as per Chapter 2.6 above. Paragon was included as a common reference line in every experimental batch of 8 to 14 genotypes such that non-biological batch-batch variation could be minimised using the relationship between the mean Paragon value of each batch and the mean Paragon value across all batches to transform the mean luminescence values of the rest of the genotypes from each batch.

A single marker regression analysis was initially undertaken by James Simmonds (Uauy group, JIC) using data on KASP™ SNP markers generated by Wingen *et al* (2017) ([http://wisplandracepillar.jic.ac.uk/results\\_resources.htm](http://wisplandracepillar.jic.ac.uk/results_resources.htm)) as independent variables and mean luminescence values of RILs as dependent variables. QTL effects were subsequently estimated as regression coefficients at marker positions with significant ( $P < 0.05$ ) marker-trait associations as determined by Wald tests. Simple interval mapping was subsequently performed using a small step size of 2 cM to resolve candidate QTL above a low arbitrary detection threshold of  $-\text{Log}_{10}[P] = 2$  for Wald test probability values. Finally, a multiple linear regression-based multi-QTL model of phenotypic variation was fitted using backward selection of all candidate QTL from simple interval mapping - retaining only those loci whose removal caused significant ( $P < 0.05$ ) deteriorations to the fit of the model. Genstat 20<sup>th</sup> edition was used for all QTL detection and estimation of QTL effects.

## Chapter 3

# CHARACTERISING CULTIVAR-SPECIFIC WHEAT RHIZOSPHERE ENVIRONMENTS

## 3.1 Introduction

The response of diverse soil bacterial communities to the presence of plant roots is an inevitably complex phenomenon and as such it has been hard to determine the influence of plant genetics *in situ*. Plant genotype is however believed play at least some role in governing the precise physical and chemical properties of the rhizosphere (Kamilova *et al.*, 2006; Ladygina and Hedlund, 2010) such that at the level of inter-species comparison at least, host plants are observed to select for distinct microbial communities (Lemanceau *et al.*, 1995; Garbeva *et al.*, 2004; Berg and Smalla, 2009; Hartmann *et al.*, 2009; Turner *et al.*, 2013b). The extent to which the host plants influence over its microbiome differs amongst contemporary accessions of important crop plants is however not so well defined and thus the potential for exploitation is more or less unknown. Early observations made by Mauchline *et al* (2015) are the starting point for this work. In their study, the authors phylogenetically analysed the *gyrB* sequences of *Pseudomonas* field isolates, revealing groupings that reflected the wheat cultivar that had previously been grown in the field from which they were sampled. The two wheat cultivars (Hereward and Cadenza) shown to exert these distinct selective pressures on *Pseudomonas spp.* genomic diversity (Mauchline *et al.*, 2015) have subsequently been used by our lab as starting points for further characterisation of the role of plant genetics in the rhizosphere. Indeed, populations of rhizospheric pseudomonads associated with full-term glasshouse-grown Hereward and Cadenza plants isolated in 2017 (Chloe Ormrod, unpublished data) were used to develop high-throughput bacterial phenotyping protocols. These methodologies revealed differences in the abundance of certain rhizobacterial traits at the population level according to wheat genotype. Subsequent findings with *Pseudomonas spp.* populations associated with a diversity panel of barley (*Hordeum vulgare*) accessions isolated after just 3 weeks of growth (Alba Pacheco-Moreno, unpublished data) suggest that such plant genotype specific differences in rhizosphere selection may also be detected at this stage in wheat.

It stands to reason that investigation of even the very earliest stages of rhizobacterial community assembly may very well be pertinent for the distinction of plant genotype specific rhizosphere traits. The importance of the initial stages of microbiome assembly for the structure and function of microbial communities throughout the rest of development is well defined (Pagaling *et al.*, 2014) and has recently been linked to contrasting plant health outcomes (Wei *et al.*, 2019). Indeed, Edwards *et al* (2015) were able to experimentally determine that axenic rice seedlings transplanted into natural soils acquire a steady-state microbiome within the first 14 days of soil contact that closely resembles those of well-established plants grown in the same soils. The mechanisms controlling the initial recruitment of different microbial genotypes *in situ* remain poorly understood. However, by targeting quantifiable traits such as root exudation in the early stages of plant development we may find target traits for screening efforts to identify loci responsible for differential rhizobacterial recruitment. Indeed, any mechanisms encouraging rhizosphere colonisation by PGP microbes may be exploited in the development of new crop varieties using plant genetics in order to improve agricultural sustainability.

The identification of any cultivar-specific differences in root exudation affecting the success of specific microbial populations in the early rhizosphere environment is an exciting prospect. The quantity, identity and diversity of exudates could all conceivably contribute to the differential assembly of rhizosphere microbial communities. This is particularly apparent for exudates that act as carbon substrates that may be more or less readily metabolised by different microbes, as investigated by defined soil amendments in seminal laboratory experiments by Orwin *et al* (2006). With this in mind, our group has developed an *in vivo* carbon source availability assay using two *Pseudomonas* strains that are compromised in their use of either more 'complex' or 'simple' carbon substrates respectively. The transcriptional regulation of primary carbon metabolism by the RpiR family transcription factors RccR and HexR is disrupted in these strains such that the *rccR* mutant grows best on 'simple' carbon sources (pyruvate, acetate, succinate, etc) whereas the *hexR* mutant strain is only able to metabolise more 'complex' carbon substrates (glucose, glycerol, etc). When co-

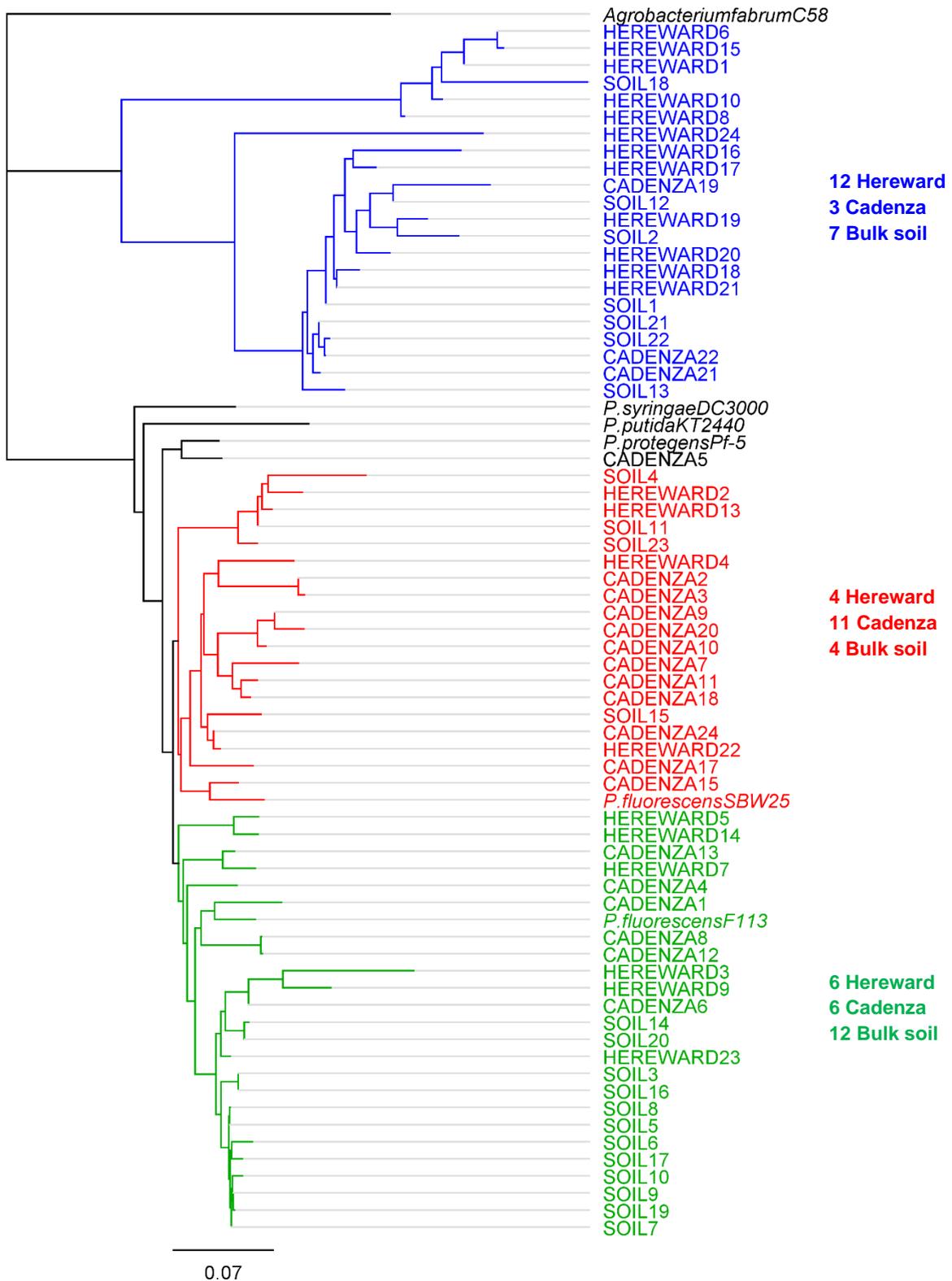
inoculated onto wheat seedlings in gnotobiotic mesocosms and subsequently recovered, the ratio of the two strains can then be determined by blue-white selection. This should give an indication of any bias in the proportion of simple and complex carbon sources in root exudates - potentially favouring specific root-associated microbial populations. Subsequent investigations have also suggested that certain bacterial taxa respond preferentially to the addition of specific low molecular weight carbon compounds despite variable community-level responses (Eilers *et al.*, 2010). Through root exudation, large quantities of these carbon compounds are released into soils mainly in the form of simple sugars, amino acids, and organic acids (Krafczyk *et al.*, 1984; Bais *et al.*, 2006; Chaparro *et al.*, 2013b; Zhalnina *et al.*, 2018). A selection of the *R. leguminosarum* Rlv3841-lux fusion biosensors specific to sugars, polyols, amino acids, or organic acids from the lab of Phil Poole at the University of Oxford should be able to determine the relative importance of the identity of these readily metabolizable substrates in the wheat rhizosphere.

The first aim of this chapter was to align and build a phylogenetic tree using *gyrB* sequences previously amplified from pseudomonads isolated from full-term Hereward and Cadenza plants as well as unplanted soils (Chloe Ormrod, unpublished data). Given the phenotypic differences already observed, we hypothesise that this analysis should reveal a degree of grouping according to the genetic identity of the plant. Furthermore, rhizosphere pseudomonads isolated from the roots of wheat plants grown for 4 weeks in a controlled environment were phenotypically screened to see if similar cultivar-specific trends are observable in early development. Finally, thorough characterisations of the influence of root exudation on the rhizosphere environment were undertaken in gnotobiotic conditions utilising *Pseudomonas* strains impaired in primary carbon metabolism (Campilongo *et al.*, 2017) as well as a suite of the *Rhizobium* biosensors (Pini *et al.*, 2017). Ultimately this should yield information on the nature of root exudation from two contemporary wheat cultivars and will provide a platform for further investigations into the influence of plant genetics on the rhizosphere microbiome.

## 3.2 Results

### 3.2.1 Phylogenetic Analysis of Wheat Root Associated Pseudomonads

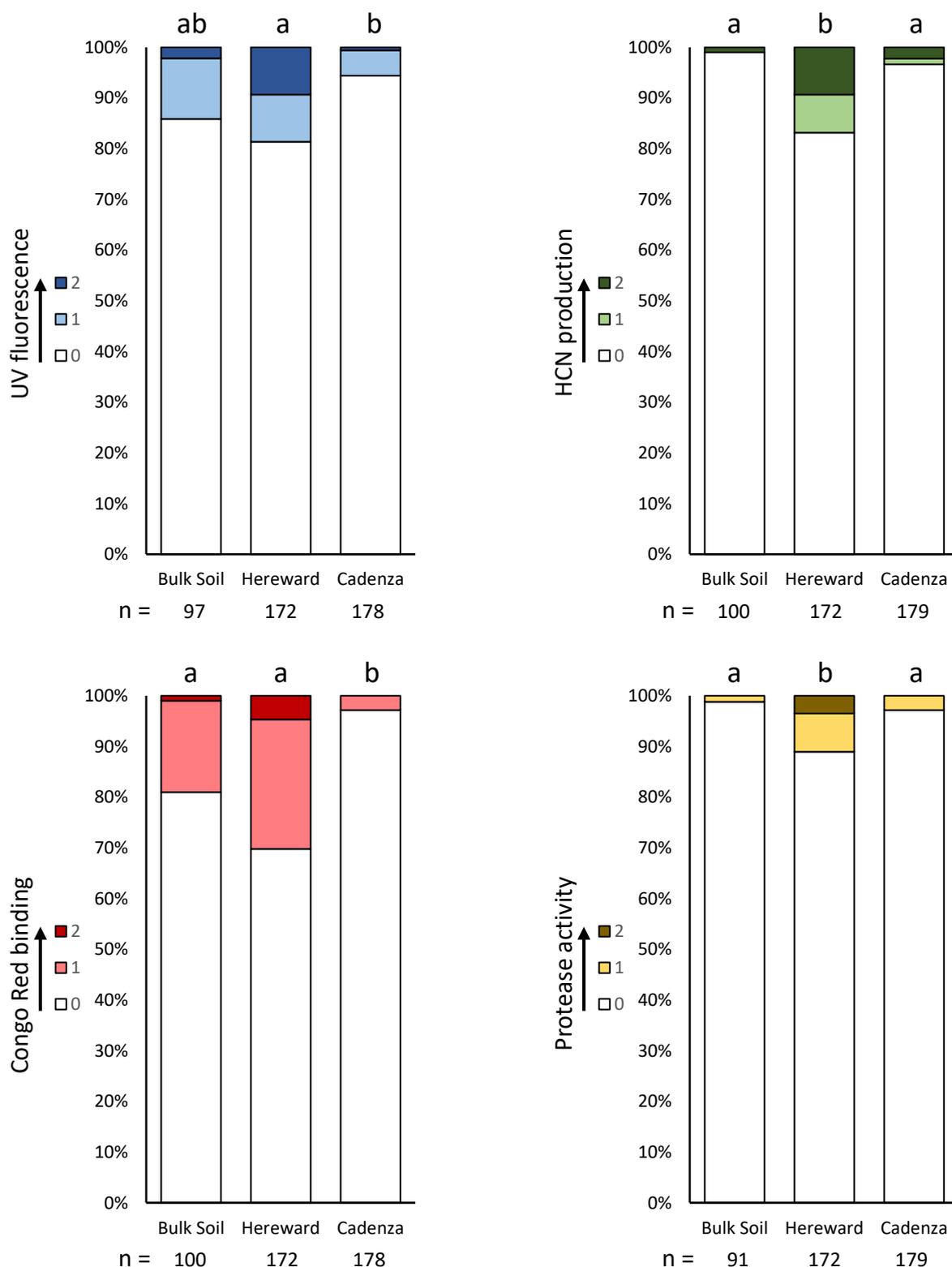
The phylogenetic relationship between a total of 66 *Pseudomonas spp.* isolates previously collected from full-term glasshouse-grown cv. Hereward and cv. Cadenza wheat plants as well as unplanted soils was inferred from sequenced fragments of the *gyrB* gene. The analysis from which the phylogenetic tree in figure 4 was built utilised *gyrB* as opposed to 16S rRNA sequences in order to enable a finer resolution of intraspecies phylogeny (Yamamoto and Harayama, 1995). The strains examined here span much of the diversity expected in the *Pseudomonas* genera, with some such as those in the blue grouping in figure 4 even clustering towards the *Agrobacterium fabrum* outgroup indicating the highly disparate nature of the *gyrB* sequences in this analysis - not all of which may have originated from pseudomonads *sensu stricto*. Some evidence of clustering between *gyrB* sequences from the Hereward, Cadenza, and bulk soil samples was observed here. The three broad groupings into which almost all isolates fell (coloured red, blue, and green in figure 4) were each characterised by the predominance of isolates associated with one of these treatments (Figure 4). Within each of these groupings, there were at least twice as many sequences from strains associated with the dominant treatment than the other two combined, with clear subgroupings dominated almost entirely by strains from these treatments in evidence.



**Figure 4 - Phylogenetic tree of *Pseudomonas* spp. genotypes based on *gyrB* sequence homology**  
 Evolutionary relationship of *Pseudomonas* isolates according to the sequence homology of fragments of their *gyrB* gene aligned within a 664 bp consensus sequence region. A total of 66 sequences from strains isolated in a glasshouse experiment detailed in Materials and Methods 2.4 are included here alongside 5 reference sequences from the model pseudomonads *P. fluorescens* SBW25 and F113, *P. protegens* Pf-5, *P. putida* KT2440, and *P. syringae* DC3000, as well as an *Agrobacterium fabrum* outgroup. Taxon labels indicate whether strains were collected from the rhizosphere compartment of the wheat (*Triticum aestivum*) cultivars Hereward or Cadenza, or unplanted bulk soils, the number of which that fell into the 3 coloured groupings here are shown to the right of the tree. Scale shows the number of base substitutions per site.

### 3.2.2 Characterising Rhizospheric *Pseudomonas spp.* Populations

Capturing any differences in the selective pressures imposed on rhizosphere organisms by the growth of wheat plants was deemed to be most likely at the level of rhizobacterial communities. Thus, collections of pseudomonads (minimum 172 strains) isolated from three independent batches comprising three 4-week-old controlled-environment grown wheat plants per cultivar were screened for four physiological traits relevant to success in the rhizosphere as outlined in Materials and Methods 2.5. The proportions of isolates exhibiting each of these phenotypes were always higher amongst the Hereward-associated pseudomonads examined here (Figure 5). Accordingly, the distributions of scores for each of these phenotypes were found to differ significantly between collections of pseudomonads isolated from Hereward and Cadenza roots - as denoted by the different letters in figure 5. Additional comparisons were made with a collection of 100 strains isolated from bulk soils in pots left unplanted in two of the three experimental batches from which the rhizosphere pseudomonads were collected. Where statistically significant differences did exist between the distributions of scores recorded for these bulk soil isolates and the rhizosphere collections - as they did for three out of the four traits assessed here - they were only seen for one of the two cultivars at a time (Figure 5). The distributions of scores for HCN production and Protease activity amongst bulk soil pseudomonads were significantly different from Hereward-associated isolates but not from the collection of strains associated with Cadenza which exhibited similarly low representations of these traits. However, for Congo Red binding the distribution of scores for the Cadenza-associated isolates were significantly different to that of the bulk soils (Figure 5) - suggesting that Congo Red-binding strains were selected against in the Cadenza rhizosphere. Furthermore, whilst the distributions of UV fluorescence trait scores differed significantly between Hereward and Cadenza, neither were significantly different to that of the bulk soil isolates (Figure 5).



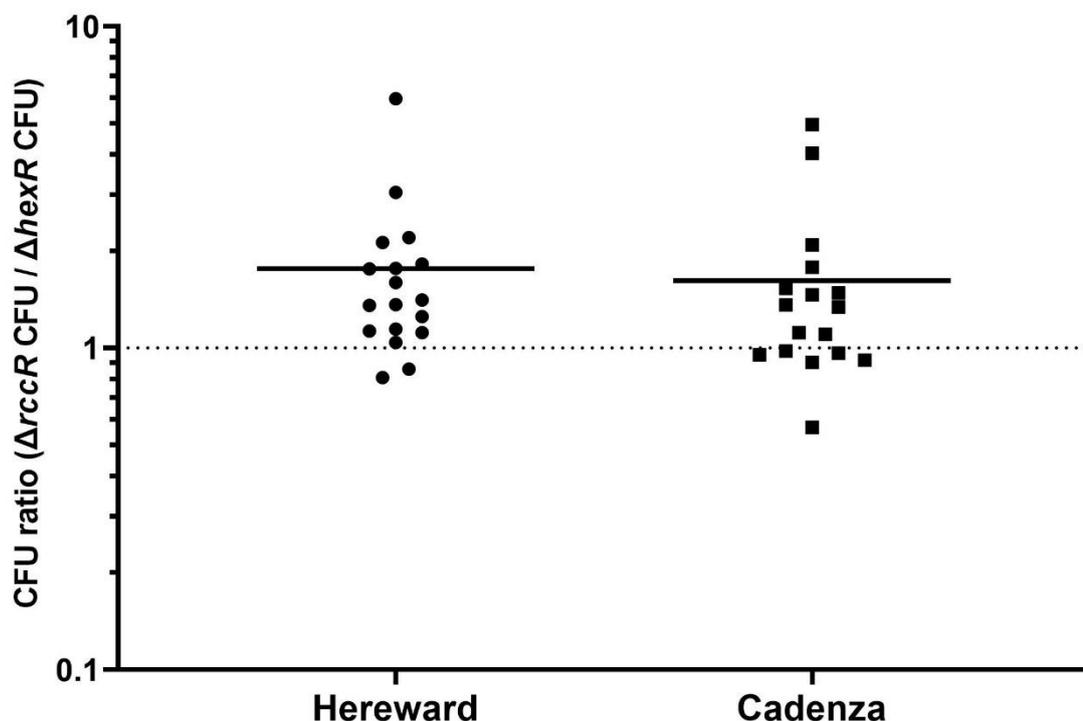
**Figure 5 - The effect of wheat genotype on rhizospheric *Pseudomonas spp.* populations**

Plots showing the percentage of isolates associated with the roots of the wheat (*Triticum aestivum*) cultivars Hereward and Cadenza - as well as unplanted bulk soils - that exhibit particular phenotypes *in vitro* where 0 = not present, 1 = weak presence, 2 = strong presence. The rhizosphere *Pseudomonas spp.* isolates here are pooled from 3 independent isolations (following Methods 2.3) of a maximum of 60 strains per treatment, each sampled from 3 individual pots containing one plant each, whilst the bulk soil isolates were collected from just 2 of these experiments. The results of statistical comparisons (Chi-squared tests) between the distributions of trait scores recorded for each collection of isolates are denoted by a compact letter display whereby treatments not annotated with the same letter differ significantly (Adjusted  $P < 0.05$ ).

### 3.2.3 Dissecting the Role of Wheat Root Exudation

#### 3.2.3.1 Bacterial Carbon Source Availability

The aim of this work was to determine whether a nutritional bias exists in the composition of root exudates for either of the wheat cultivars presently investigated. Figure 6 shows the similarity of the results of the *Pseudomonas* mutant strain-based assay from Hereward and Cadenza, with no observable differences in the mean ratio of mutant CFU recovered from the two cultivars. This suggests that a similar proportion of 'simple' to 'complex' carbon exudates were released from the roots of both cultivars here and would thus refute the existence of such a broad-scale nutritional bias across all of the many carbon-containing primary metabolites exuded. The mean ratio of  $\Delta rccR$  to  $\Delta hexR$  CFU recovered did however increase significantly from that of the initial inoculum (dotted line) applied to Hereward ( $t_{17} = 2.747$ ,  $P = 0.0138$ ) and Cadenza ( $t_{16} = 2.217$ ,  $P = 0.0415$ ) roots, indicating that the utilisation of 'simple' carbon sources was more important for competitive rhizosphere colonisation here.



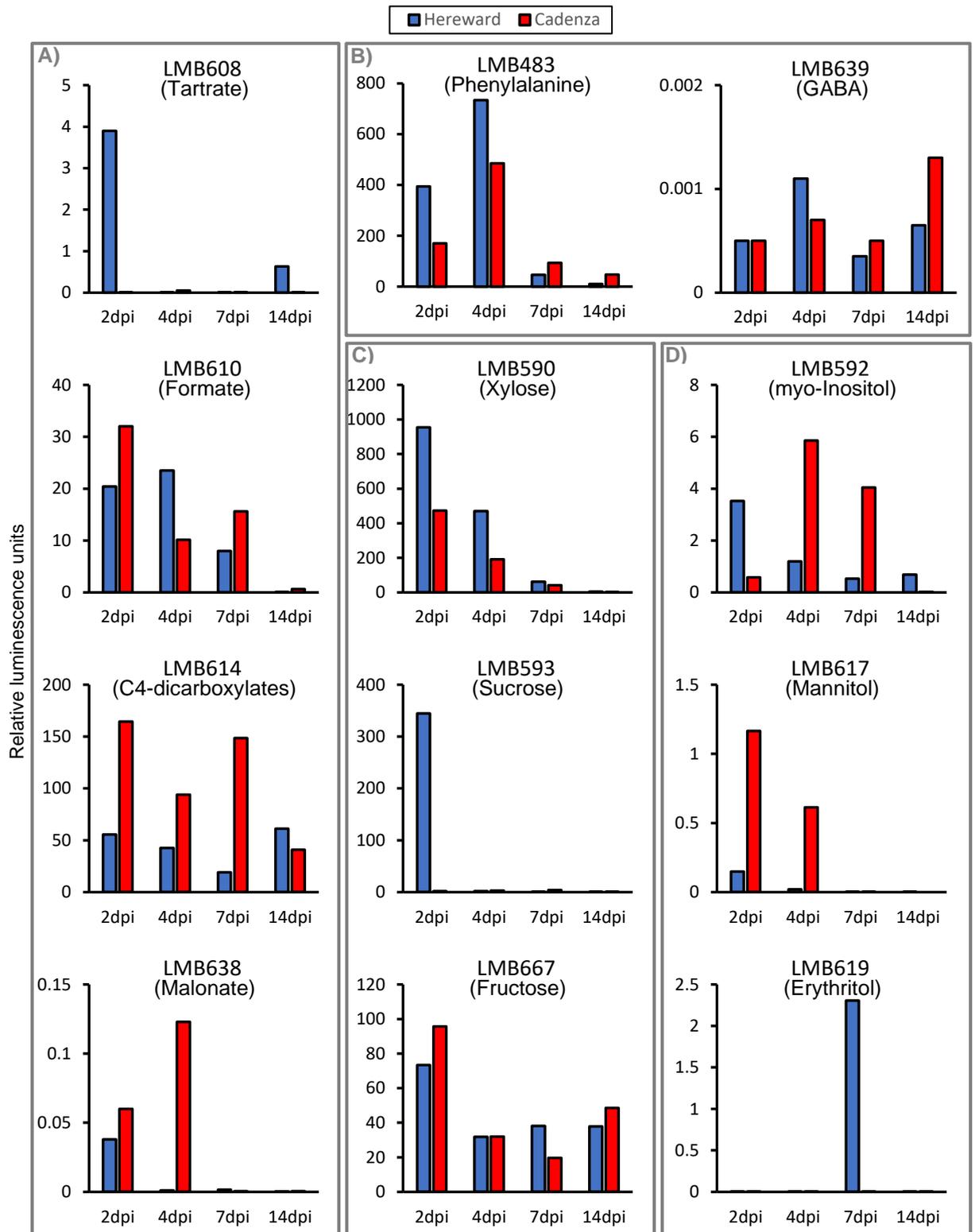
**Figure 6 - Necessity of 'simple' and 'complex' carbon metabolism in rhizobacterial competence**  
Competitive wheat rhizosphere colonisation efficiency of two contrasting *Pseudomonas* spp. primary carbon metabolism mutants as inferred from the ratio of CFU recovered 7 days after their co-inoculation onto 7-day old axenic wheat (*Triticum aestivum*) seedlings. Each data point represents the ratio of  $\Delta rccR$  CFU to  $\Delta hexR$  CFU recovered from individual plants divided by the same ratio for the inoculum initially applied to them in one of 2 independent experiments comparing the Hereward (n=18) and Cadenza (n=17) cultivars. All data are presented on a log10 scale and mean values are indicated by horizontal bars.

### 3.2.3.2 Bacterial Luminescence-based Root Exudate Profiling

To gain a preliminary understanding of the performance of the luminescent biosensor bacteria on wheat roots, all strains were inoculated separately onto the roots of two seedlings each of the Hereward and Cadenza wheat cultivars previously shown to select for distinct *Pseudomonas* populations (Mauchline *et al.*, 2015). These root systems were assessed *in vivo* at 2, 4, 7 and 14 dpi in order to understand the general patterns of exudation in these wheat cultivars (Figure 7). The assumption of thorough root colonisation required for accurate quantification of exudation was supported by the detection of luminescent activity across all root surfaces (Figure S2 in appendix) when inoculated with the constitutive *lux* fusion biosensor (LMB743). This also indicates that the total amount of carbon in the root exudates of both cultivars was sufficient for bacterial growth even under the significant metabolic burden of constant luminescence - 20 ATP molecules per photon of light produced (Close *et al.*, 2012). However, several of the compound specific biosensors (LMB592, LMB608, LMB617, LMB619, LMB638, and LMB639) including all those specific to polyols (sugar alcohols) exhibited negligible levels of luminescence (below 10 rlu) at all timepoints that are probably attributable to background noise (Figure 7). The lack of detectable *lux* activity in this system does not however rule out the possibility that these metabolites were present in the root exudates, indeed it may be that their concentrations are below the threshold of detection for some of the reporter constructs. However, for the purposes of developing biosensor-based assays for wheat root exudation, these strains were not investigated further.

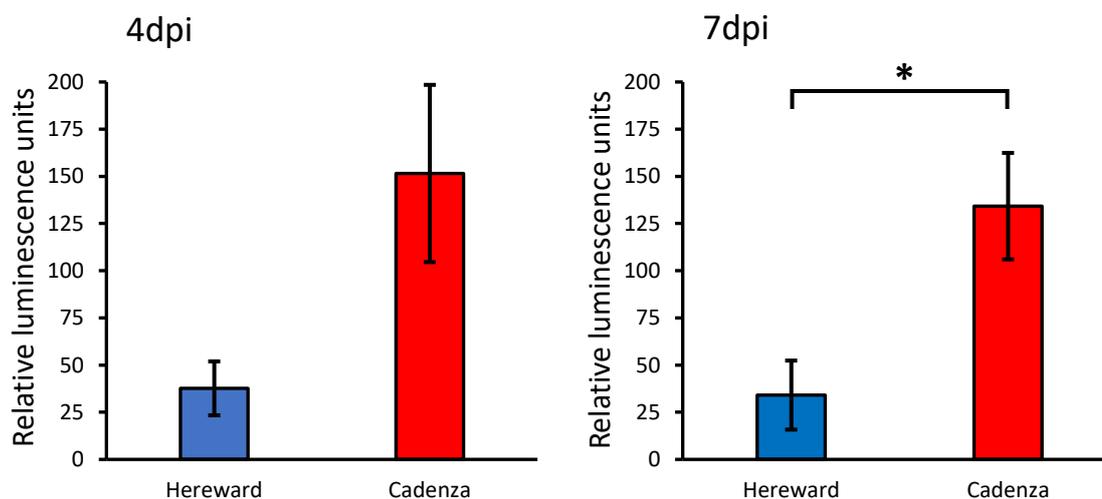
The luminescence of the remaining biosensors tended to decline over time in this experiment, likely reflecting the accumulated effects of growth in this stressful gnotobiotic system - be that through the exhaustion of energy stores in the seed and the lack of exogenous nutrients in the growth substrate or because of the less than favourable growth conditions for the bacteria. This was however to be expected and such a decline was indeed also observed by Pini *et al* (2017) when first testing these biosensors (Figure 1b). This

reduction in luminescence was more apparent for some strains (LMB483 and LMB590) than others, which may conceivably reflect some developmentally pre-programmed change in exudation strategy. The levels of luminescence emitted by the strains specific to phenylalanine (LMB483), xylose (LMB590), fructose (LMB667), and C4-dicarboxylates (LMB614) did however continue to be detected until the end of this experiment. This together with the observation that the luminescence of all of these strains was detected when inoculated onto the roots of both of the cultivars in this experiment suggests that these compounds may well form part of a common wheat exudation profile that is likely shared between all wheat cultivars. Several differences did however exist in the extent of exudation detected by multiple biosensors at various timepoints, however few of these trends were consistent across multiple timepoints. The only distinctions between the levels of luminescence detected from the two cultivars that applied to 3 or more of the timepoints in this experiment were afforded by LMB614 and LMB590, which revealed greater levels of xylose and C4-dicarboxylate exudation by Hereward and Cadenza respectively (Figure 7). The more changeable nature of the exudation detected by some other biosensors does not imply that these are spurious trends for which a genetic basis does not exist, just that they might be less easily screened for using these methodologies - and so were not investigated further here.



**Figure 7 - In vivo bioluminescence of a suite of *R. leguminosarum lux* fusion biosensors over time**  
Mean luminescence values recorded from twelve Rlv3841 biosensors growing on the roots of two plants each of the wheat (*Triticum aestivum*) cultivars Hereward (blue bars) and Cadenza (red bars) 2, 4, 7, and 14 dpi. Relative luminescence units represent the integrated density (no. luminescent pixels x mean pixel value) of the luminescent area in each CCD image. The groupings A-D reflect the chemical classification of the compounds detected by these strains, where group A respond to the organic acids Tartrate (LMB608), Formate (LMB610), Malonate (LMB638) and a group of C4-dicarboxylates (LMB614); group B respond to the amino acids Phenylalanine (LMB483), and GABA (LMB639); group C respond to the sugars Xylose (LMB590), Sucrose (LMB593), and Fructose (LMB667); and group D respond to the polyols (sugar alcohols) myo-Inositol (LMB592), Mannitol (LMB617), and Erythritol (LMB619).

These initial experiments seemed to suggest that the exudation profiles of two cultivars (Hereward and Cadenza) known to exert distinct selective pressures on the rhizosphere may be more similar than might have been imagined. However, it may be that there are more pronounced differences in the exudation of metabolites for which we do not have specific biosensors. Moreover, as only two individual plants were inoculated with each biosensor per cultivar, our conclusions require further investigation with larger sample sizes to reduce sampling variation and gain statistical power. This being said, the most striking difference in luminescence values between the two cultivars observed here (LMB614 at 7dpi) provided the most interesting results upon further investigation. Indeed, inoculating 5 plants of each cultivar with the biosensor specific to the C4-dicarboxylates malate, succinate, and aspartate (LMB614) revealed a clear trend for higher levels of exudation by Cadenza at both 4 and 7 dpi (Figure 8). The difference in luminescence was however only statistically significant 7dpi ( $t_8 = 2.977$ ,  $P = 0.0177$ ) reflecting the reduced within-cultivar (plant-to-plant) variability by this time point, perhaps indicative of a convergence of biological seed-to-seed variation or the more consistent levels of root colonisation by the biosensors across replicates by this stage.



**Figure 8 - *In vivo* bioluminescence of the C4-dicarboxylate biosensor on wheat roots 4 and 7 dpi**  
Mean luminescence values recorded from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of five plants each of the wheat (*Triticum aestivum*) cultivars Hereward (blue bars) and Cadenza (red bars) 4 and 7 dpi  $\pm$  SEM. Here, relative luminescence units represent the integrated density (no. luminescent pixels x mean pixel value) of the luminescent area in each CCD image. Appropriate statistical analyses (Student's t test) revealed the significance of differences between cultivar means where \* =  $p \leq 0.05$ .

### 3.3 Discussion

The multifaceted approach to rhizosphere interrogation outlined above has yielded diverse data drawing together several key indications of wheat cultivar-specific dependencies. Some evidence of phylogenetic clustering has been observed in soil-dwelling pseudomonads according to the genetic identity of the wheat plant with which they associated (Figure 4). These groupings were less easily distinguished from those isolates collected from unplanted soils but nonetheless represent a host-genotype dependent stratification of rhizospheric *Pseudomonas spp.* populations. Indeed, this background 'noise' of bulk soil isolates was to be expected given the breadth of microbial diversity sequestered in the soil from which subsets are selected for by the growing wheat plant. Whilst inferences cannot be drawn about the nature of the selection of rhizosphere pseudomonads from background bulk soil communities for each of the wheat genotypes investigated here, we are able to show some degree of phylogenetic clustering according to wheat genotype suggesting an adaptation of soil *Pseudomonas spp.* to the precise physical and chemical influences of the host plant during its development. Previous works have identified phylogenetic clustering of taxa that proliferate in rhizosphere soils (Shi *et al.*, 2015) and propose a conserved evolutionary basis for the response of complex soil communities to plant roots, however host-genotype dependencies are rarely observed. These data would however seem to corroborate the observations of wheat cultivar specific selection at the level of individual bacterial genomes made by Mauchline *et al* (2015) in field isolates associated with a second wheat crop cultivated after the Hereward and Cadenza cultivars. Indeed, by similarly constraining the complexity of the phylogenetic analysis to just the pseudomonads - a successful rhizobacterial taxa ubiquitous in the rhizosphere - this work also presents an indication of such a relationship in glasshouse-grown plants. However, the reproducibility of these initial signs of host genotype-specific stratification in the rhizosphere microbiome should now be investigated rigorously and replicated in other microbial lineages.

Nonetheless, these results could still be indicative of a wider trend in which closely related host plants exert more similar selective pressures on rhizosphere bacteria than those more distantly related. Indeed, Bouffaud *et al* (2014) were able to demonstrate a relationship between the phylogenetic distance between Poaceae genotypes and the taxonomic relatedness of their associated rhizobacterial communities using 16S rRNA microarray data. This study was however built on previous results that found rhizobacterial community composition to be dependent on the diversification history of the maize accessions investigated, but not on the actual genetic distances between host plants (Bouffaud *et al.*, 2012). This perhaps indicates the taxonomic resolution at which it has been possible to identify microbiome-wide correlations with host genetic relatedness. Fitzpatrick *et al* (2018) were able to correlate plant evolutionary relatedness with microbial community diversity and composition across their diversity panel of 30 plant species (divergent for up to 140 million years). However, no such relationships could be reported for a similar number of accessions from a single crop species (Peiffer *et al.*, 2013). Utilising high resolution single nucleotide polymorphism (SNP) data, neither species diversity estimates nor the abundances of the most commonly isolated microbes from 27 highly diverse maize inbreds correlated with their relatedness, despite finding a subset of 'heritable' bacteria whose abundance associated significantly with individual accessions (Peiffer *et al.*, 2013). Moreover, even for the highly divergent species investigated by Fitzpatrick *et al* (2018), covariance between host kinship and the diversity and composition of root microbial communities only held for the endosphere compartment, despite a similarly strong host plant species effect on the rhizosphere microbiota. Conclusions from the present work only can however only be drawn from a limited comparison of two wheat genotypes, and so correlations with host-relatedness cannot be assessed. However, the identification of a host genotype dependency in the assembly of populations of an important representative of the rhizosphere microbiota between contemporary wheat cultivars still indicates the potential of wheat genetics for microbiome modulation.

Given the apparent phylogenetic conservation of rhizobacterial adaptations to plant genotype it should follow that microbial traits with more or less relevance to success in the rhizosphere environments of genetically distinct host plants should be more or less abundant accordingly. The prevalence of the production of fluorescent siderophores, HCN, exopolysaccharides and proteases as evaluated by high-throughput *in vitro* bioassays differed significantly between collections of pseudomonads isolated from the two cultivars investigated here (Figure 5). However, whilst always more abundant in collections of isolates associated with Hereward, these results were not always reflective of enrichments of isolates exhibiting these traits from bulk soils. Of these increases, the secretion of HCN and proteases capable of visibly degrading milk powder did appear to have been selected for in the Hereward rhizosphere based on their similarly significant levels of enrichment relative to those observed in a collection of bulk soil isolates (Figure 5). The proportions of Congo Red-binding isolates however were quite similar amongst Hereward-associated isolates and those from bulk soils, and it was actually the distribution of trait scores exhibited by isolates from the Cadenza rhizosphere which differed significantly from those of bulk soils (Figure 5) - indicating a selection against isolates producing Congo Red-binding exopolysaccharides here. It is worth noting however that these findings could also be indicative of a selection for or against functions which co-occur with the traits measured here, or indeed for a community of pseudomonads that just so happens to be more or less diverse in terms of these traits. Nonetheless, these findings still seem to suggest that differences in the selective pressures exerted on rhizosphere microbes by Hereward and Cadenza do exist as early as 4 weeks after the transplantation of seedlings into complex soil substrates.

The possibility that the assembly of microbial communities associated with domesticated crops is controlled at least in part by the contribution of a few major alleles (Peiffer *et al.*, 2013) means the lack of one of these major genes may conceivably have affected the recruitment of microbes with the traits measured here. Several key root traits have already been implicated in rhizosphere microbiome assembly (Robertson-Albertyn *et al.*, 2017), further highlighting the relevance of investigations into the genetic basis of phenotypes that

influence the rhizosphere environment. However, compared with traits that determine physical aspects of the rhizosphere microhabitat such as those of the barley root hair mutants of Robertson-Albertyn *et al* (2017) which explained 18% of the variation between rhizosphere samples, genes that control specific chemical properties of the rhizosphere may hold greater promise for more targeted microbiome manipulations. Indeed, the plethora of pre-existing, yet largely unknown root exudation phenotypes are arguably the most logical targets for investigations into functional alterations of the rhizosphere microbiota using plant genetics. Inevitably a much more thorough understanding of the relationship between any such traits and their potential effects on the microbiota is required before any implementation of these principles is possible. However, the identification of genetic loci that may potentially be used as such would represent an important proof of concept.

Whilst no differences in the relative levels of root colonisation of the carbon metabolism mutant strains could be detected between Hereward and Cadenza (Figure 6) this was not totally unexpected. The overall proportion of 'simple' to 'complex' carbon compounds in wheat root exudates is likely to remain relatively constant simply as a result of physical constraints on the flux of these metabolites from root cells regardless of any differences in the identities of the individual root exudates. What might be expected however is for the relative importance of specific compounds to differ both within the 'simple' and 'complex' carbon compound classes measured here but also for other classes of common root exudates. Consistently, both 'simple' and 'complex' carbon substrates were detected in the root exudates of both wheat cultivars in the *R. leguminosarum* biosensor assays (Figure 7) - respectively the C4-dicarboxylates (malate, succinate and aspartate), and the sugars xylose and fructose - as well as an amino acid (phenylalanine). Thus, the compounds detected in wheat exudates here are actually represent all three major classes of root-exuded metabolites - organic acids (OAs), sugars, and amino acids - often collected and analysed as pooled groups of compounds (Chaparro *et al.*, 2013b). Whereas conclusions cannot be drawn on the relative importance of these specific compounds within the main classes of

metabolites they represent, the potential uses of these compound-specific biosensors for the development of tractable root exudation phenotyping assays are clear.

Of the main classes of primary metabolites exuded, it was a group of organic acids (OAs) that were picked out here as a potential source of variability between wheat cultivars (Figure 8). These ubiquitous cellular metabolites are implicated in several key processes within soils, many afforded by the carboxyl groups that define their chemistry (Jones, 1998; Meyer *et al.*, 2010b). Organic acid exudation represents a significant selective carbon sink, with certain compounds increasingly secreted from the roots in order to mediate certain environmental stresses - for example those best able to complex toxic metal cations (Ma *et al.*, 2001). Indeed, whilst the exudation of OAs and other primary metabolites is an active, rather than a passive process, their efflux tends to follow a strong concentration gradient. As a result of their central role in primary metabolism, the synthesis and cellular concentrations of these compounds are strictly regulated, and as such, concentration gradients are principally determined by the fate of these readily metabolised substrates in the rhizosphere. While some evidence exists for reuptake by plant roots and sorption to mineral phases (Jones and Darrah, 1993; Jones and Edwards, 1998), it has long been understood that the facilitated-diffusion driven exudation of many primary metabolites is profoundly affected by microorganisms that act as a strong sink of plant photosynthates (Groleau-Renaud *et al.*, 2000; Kuijken *et al.*, 2015; Canarini *et al.*, 2019). In terms of their utilisation by microbes however, not all primary metabolites are created equal. A general trend towards the microbial incorporation of carboxylic acids over amino acids and sugars is observed (Jones and Edwards, 1998; Gunina *et al.*, 2017) which may correspond to the ease with which these compounds may enter into the respective metabolic pathways of the microbiota. In fact, the physio-chemical properties of low molecular weight compounds may even be predictive of uptake by soil microbes and subsequent mineralisation, with higher carbon-oxidation state substrates exhibiting reduced soil half-life for example (Gunina *et al.*, 2017). Thus, rhizobacterial adaptation to readily metabolizable exudates such as the di- and tri-carboxylic acid intermediates of the TCA cycle, with their high carbon-oxidation states, is inevitable.

Moreover, when compared to the sugar components of the model root exudate solutions applied to soil microcosms by Shi *et al* (2011), OAs not only caused greater increases in bacterial community richness, but also induced significantly larger shifts of the dominant soil taxa - a key characteristic of rhizosphere microbiome assembly.

Indeed, amongst the generally increased capacity for carbohydrate metabolism observed in plant-associated bacteria (Levy *et al.*, 2018), an abundance of organic acid transporters has been noted in strains that proliferate in the rhizosphere (Zhalnina *et al.* 2018). The expression of genes related to the utilisation of C4-dicarboxylates in particular was highlighted by Ofek-Lalzar *et al* (2014) as a specific quality of microbial communities colonising the surfaces of wheat roots, hinting at the importance of these compounds as substrates for growth within this closely plant-associated niche. As well as this intriguing evidence for the active utilisation of these OAs in this specific microhabitat, the authors also observed an enrichment of C4-dicarboxylate sensing and transporting *dct* genes - whose promoter controls luminescence in LMB614 here - within the wider wheat root community metagenome (Ofek-Lalzar *et al.*, 2014). This serves to highlight the additional importance of exuded OAs in the assembly of root associated microbial communities through their central role in bacterial chemotaxis - the crucial first step in root colonisation. Indeed, the predominance of genes related to these OAs in rhizosphere and rhizoplane communities is likely an artefact of their necessity for successful recruitment into this plant-defined niche.

Several of the common OA components of root exudates have been implicated in the success of rhizobacterial colonisation. Whereas biofilm formation is reportedly induced by fumaric acid (Yuan *et al.*, 2015), citric acid and in particular malic acid appear to have well conserved roles in chemotaxis towards roots (de Weert *et al.*, 2002; Yuan *et al.*, 2015). Consistently, *in vivo* colonisation of root tip surfaces was impaired in the *P. fluorescens* organic acid sensing mutants of Oku *et al* (2014) for which the chemotactic response to dicarboxylic acids was abolished. Moreover, an abundance of receptors specific to a diversity of OAs are to be found in root-colonizing isolates, often with overlapping specificities for

certain compounds (Parales *et al.*, 2013). This apparent functional redundancy may simply represent an insurance against loss of function, or indeed the need to detect certain compounds at different concentrations as is seen for the two *P. aeruginosa* receptors that sense high and low phosphate concentrations (Wu *et al.*, 2000). This could even be indicative of a requirement to distinguish between specific cocktails of OAs exuded by different host plants. The functional importance of specific receptors is in fact known to vary from strain to strain, likely due to different expression patterns, and presumably altering their response to root exudates (Parales *et al.*, 2013). As such, it is conceivable that these differences represent rhizobacterial adaptations to particular host plants based on the chemical profile of their root exudates.

The influential role of root-exuded OAs in the assembly of rhizobacterial communities around the roots is clear to see, as is their potential for manipulating the rhizosphere microbiota. Indeed, whilst the quantities of metabolites exuded from roots may be primarily governed by source-sink dynamics, the expression and regulation of certain efflux carriers that facilitate the diffusion of specific compounds can majorly influence the chemical profile of root exudates. The central role of aluminium-activated malate transporters (ALMTs) and multi-drug and toxin extrusion (MATE) transporters in OA mediated mineral nutrition and aluminium toxicity resistance is a well-established one (Delhaize *et al.*, 2007; Ryan *et al.*, 2009). For malate in particular, which the C4-dicarboxylate biosensor LMB614 used here is most responsive to (Pini *et al.*, 2017), the contribution of a membrane localised transporter gene *TaALMT1* (Sasaki *et al.*, 2004) to the variation in OA-mediated aluminium tolerance between wheat cultivars has long been known (Delhaize *et al.*, 1993). Detailed promoter analyses carried out as a consequence of the relevance of Al tolerance for wheat breeding have subsequently revealed the existence of eight classes of promoter sequence variations that determine malate efflux directly by altering *TaALMT1* transcript levels as a function of the number and order of upstream tandem sequence repeats (Sasaki *et al.*, 2006; Raman *et al.*, 2008). Interestingly, the expression of the corresponding efflux transporter gene in *Arabidopsis* (*AtALMT1*) has even been implicated in rhizobacterial colonisation. Foliar

pathogen-induced upregulation of *AtALMT1* increases root exudation of malate, enabling selective recruitment of the beneficial rhizobacterium *Bacillus subtilis* FB17 in a dose-dependent manner (Rudrappa *et al.*, 2008). Subsequent experiments with *AtALMT1* overexpression lines also demonstrate the recruitment of this biocontrol strain (Kobayashi *et al.*, 2013), highlighting the potential of such manipulations. More relevantly, field trials incorporating near-isogenic wheat lines carrying either Al-tolerant or -sensitive *TaALMT1* promoter sequence variants have even indicated that differences in the abundances of certain rhizobacterial taxa could be malate exudation dependent (Mahoney *et al.*, 2017). Consideration should however be given to the pleiotropic roles of malate exudation as well as the nuances of its existing regulatory networks before implementing any such modifications for exploitation of the microbiota. Here, evidence of differential malate exudation by two wheat cultivars known to exert contrasting selective pressures on the rhizosphere has been presented. In concert with the observed differences in the abundance of relevant rhizobacterial phenotypes in collections of isolates associated with either cultivar and phylogenetic clustering as such, this would seem to suggest that root exudates are indeed shaping the wheat rhizosphere microbiome.

## Chapter 4

# RESOLVING THE GENETIC BASIS FOR DIFFERENTIAL WHEAT ROOT EXUDATION

## 4.1 Introduction

Whilst the basis for the exudation of certain compounds from plant roots is understood to be principally determined by a few well characterised genes, the genetic regulation of most exudation remains unknown. The identification of novel variation in root exudation traits, as was observed for the levels of C4-dicarboxylates detected in exudates from two contemporary wheat cultivars above (Figure 8) does however present the potential for further characterisation and exploitation using plant genetics. This finding in particular opens up the possibility that the contrasting selective pressures imposed on the rhizosphere microbiota by Hereward and Cadenza (Mauchline *et al.*, 2015) are mediated at least in part by C4-dicarboxylate exudation. Indeed, if patterns of microbial trait abundances similar to those observed for Hereward and Cadenza associated *Pseudomonas spp.* isolates above (Figure 5) prove to be consistent amongst other accessions with similarly contrasting C4-dicarboxylate exudation phenotypes, the potential for targeted microbiome manipulations as such will be further substantiated.

From what is known about malate efflux mediated Al tolerance in wheat and the major contribution of *TaALMT1*, some level of variation for C4-dicarboxylate exudation as detected by the luminescent biosensors employed here should be expected within modern wheat germplasm (Raman *et al.*, 2008). Moreover, additional and potentially novel variation for exudation traits may well be present within collections of historic accessions less affected by the losses of genetic diversity during domestication and modern breeding (Haudry *et al.*, 2007; Iannucci *et al.*, 2017). Wheat landraces have indeed yielded novel genetic variation for the exudation of OAs in previous studies. For *TaALMT1* itself, several new promoter alleles that altered expression of the transporter and subsequent malate exudation were identified among the landraces surveyed by Raman *et al.* (2008). The diverse wheat germplasm of the A.E. Watkins landrace collection may thus serve as a rich source of variation for exudation phenotypes. Indeed, further elucidation of the role of C4-dicarboxylates in the assembly of

the rhizosphere microbiome would benefit hugely from investigations of any such accessions with more pronounced exudation phenotypes.

Moreover, to facilitate the discovery of novel alleles from the collection a suite of genetic resources have been established under the powerful nested association mapping (NAM) design first implemented in maize (Yu *et al.*, 2008) whereby several diverse accessions are each crossed with a common reference line - here the UK elite wheat cultivar Paragon (Wingen *et al.*, 2017). This approach affords statistically powerful high-resolution genetic dissections of diverse phenotypes that have even been used to resolve complex quantitative traits such as flowering time and leaf architecture in maize (Buckler *et al.*, 2009; Tian *et al.*, 2011). The resultant recombinant inbred lines (RILs) themselves constitute numerous biparental mapping populations of Paragon crossed with a particular Watkins line, thus also serving as permanent resources for conventional linkage-analysis based QTL mapping. Indeed, NAM populations have attracted substantial research interest, not least from studies of the rhizosphere microbiome (Peiffer *et al.*, 2013; Walters *et al.*, 2018). The large-scale replicated field studies of the NAM parental lines by Walters *et al.* (2018) which revealed the existence of 'heritable' microbes were themselves conducted in part to justify further genetic dissections using the maize NAM population to map the plant genes responsible.

Investigation of loci responsible for variation in the C4-dicarboxylate exudation phenotype studied here using luminescent biosensor assays represents an important assessment of the wider utility of these methodologies for expanded studies of the genetic architecture of other root exudation traits in the Watkins collection. Indeed, given enough variance within the collection there exists the possibility of being able to identify loci responsible for the exudation of any individual compound or group of compounds that a biosensor can be constructed to detect.

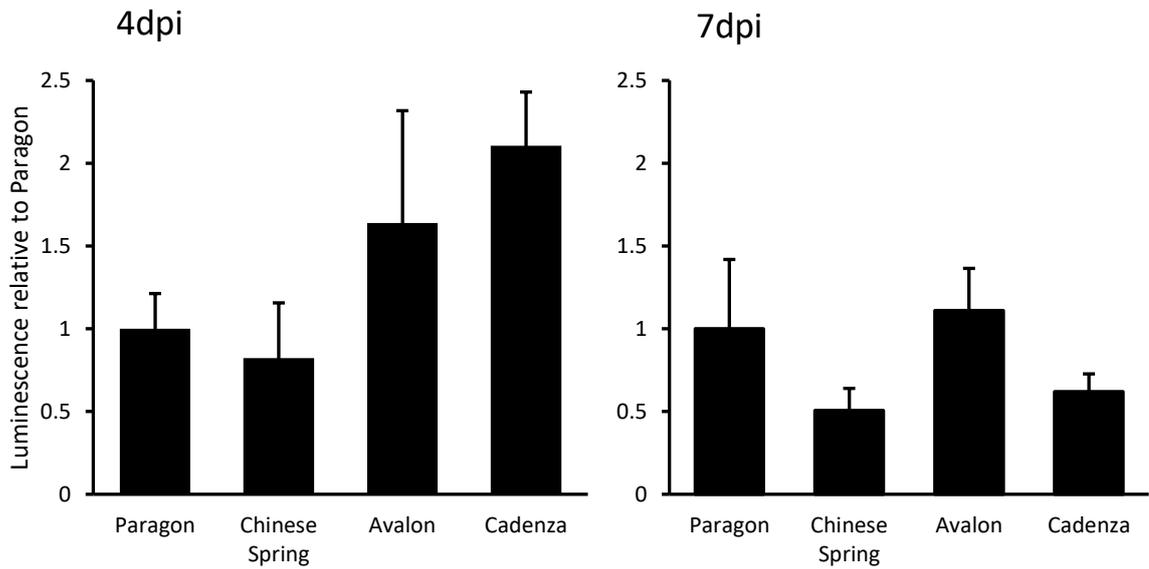
In this chapter the extent of variation for the C4-dicarboxylate exudation phenotype amongst a selection of wheat landraces and modern elite cultivars will be determined using bacterial luminescence-based biosensor assays. These wheat accessions, each a founding line of a

biparental mapping population, could thus be used to dissect the basis of any variation observed for the C4-dicarboxylate exudation trait. The recruitment of microbes with particular traits by accessions with contrasting C4-dicarboxylate exudation phenotypes will then be assessed as in 3.2.2 to ascertain the consistency of the effects of C4-dicarboxylate exudation on microbial community assembly in the rhizosphere. Finally, the utility of the biosensor assays employed here for mapping QTL will be assessed by screening a biparental mapping population for the variable C4-dicarboxylate exudation phenotype. This proof-of-concept experiment should begin to reveal the loci conferring this root exudation phenotype which could inform future plant breeding efforts for altered microbial associations.

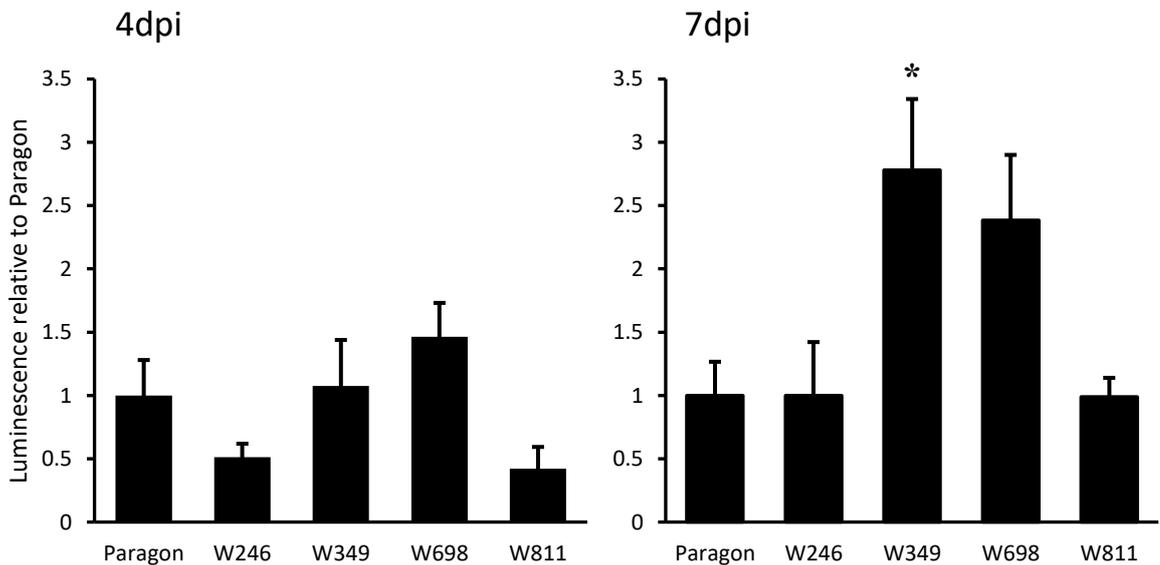
## 4.2 Results

### 4.2.1 C4-dicarboxylate root exudation in the primary wheat gene pool

The biosensor strain LMB614 which responds specifically to the presence of the C4-dicarboxylates malate, succinate, and aspartate was inoculated onto seedlings and incubated as detailed in Materials and Methods 2.7 before *in situ* quantification of luminescence at 4 and 7 dpi. Initially, C4-dicarboxylate exudation was assessed as such for a limited selection of contemporary accessions (Figure 9) and historically cultivated germplasm from the Watkins collection (Figure 10) all of which are associated with pre-existing genotyped mapping populations for trait discovery. Genotype-specific variation in the levels of luminescence indicative of C4-dicarboxylate exudation 7 dpi was much more extensive amongst the landrace accessions ( $F_{4, 10} = 4.540$ ,  $P = 0.0239$ ) than modern cultivars ( $F_{3, 12} = 1.263$ ,  $P = 0.3308$ ), with a similar but less substantial difference in evidence 4 dpi. Attention should however be paid to the semi-quantitative nature of the luminescence output, whose relationship with exudation has not been defined by calibrating against concentrations of inducer molecules or direct measurements of exudation rate, preventing firm conclusions being drawn from this line of investigation. In addition, it should be noted that the extent to which the *R. leguminosarum* biosensor was able to colonise the roots of these disparate accessions may also interfere with the interpretation of these data without additional controls.



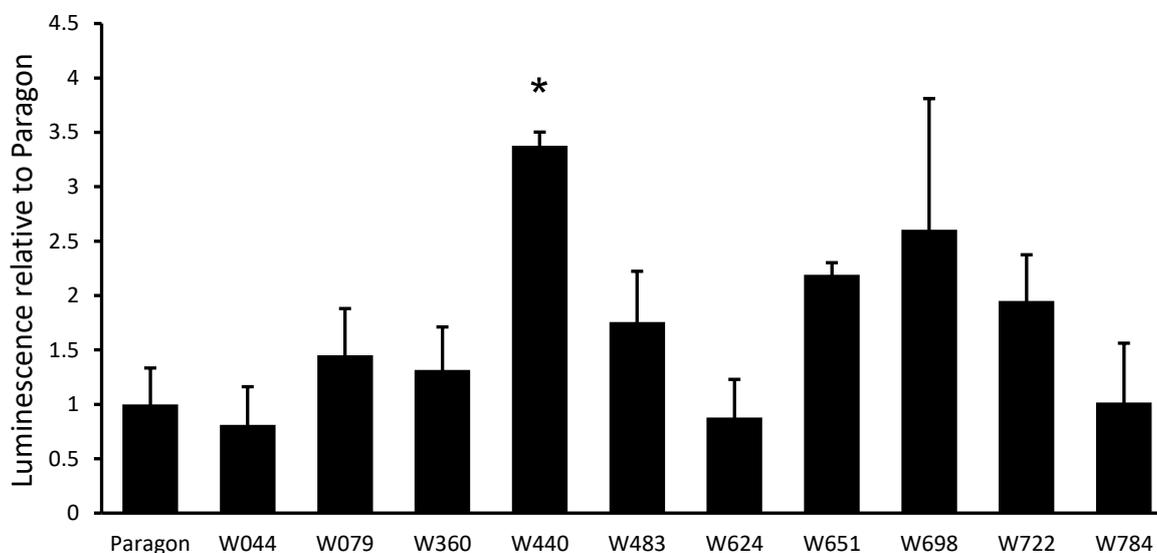
**Figure 9 - Variation amongst modern wheat cultivars for the C4-dicarboxylate exudation phenotype**  
 Mean luminescence emitted from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of four plants each of the modern wheat (*Triticum aestivum*) genotypes Paragon, Chinese Spring, Avalon and Cadenza, at 4 and 7 dpi, presented relative to that of Paragon  $\pm$ SEM. Šídák's multiple comparison tests were performed comparing Paragon with Chinese Spring and Avalon with Cadenza following one-way Analysis of Variance (ANOVA) hypothesis testing where \* =  $p \leq 0.05$ .



**Figure 10 - Variation amongst wheat landraces for the C4-dicarboxylate exudation phenotype**  
 Mean luminescence values recorded from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of three plants each of both the modern wheat (*Triticum aestivum*) cultivar Paragon and 4 historic landrace accessions (W246, W349, W698, and W811) at 4 and 7 dpi, presented relative to that of Paragon  $\pm$ SEM. Dunnett's multiple comparison tests comparing individual landrace accessions with Paragon were performed following one-way Analysis of Variance (ANOVA) hypothesis testing where \* =  $p \leq 0.05$ .

Nevertheless, none of the differences between the pairs of contemporary accessions from which biparental mapping populations have been established were found to be statistically significant at either timepoint (Figure 9), whereas one such significant difference did exist

between Paragon and a Watkins line (W349) 7 dpi (Figure 10). Moreover, upon repetition with additional replicates and only measuring luminescence once at 7 dpi (Figure S3 in appendix) the statistical significance of this difference only increased (Mean difference = 1.851, Adjusted  $P = <0.0001$ ), and so 7 dpi was selected as the sole measurement timepoint for all subsequent screening of exudation traits. As such, investigation of a wider selection of landrace accessions from the Watkins collection (Figure 11) further substantiated the high levels ( $F_{10, 22} = 2.511$ ,  $P = 0.0345$ ) of between-genotype variation for C4-dicarboxylate exudation. Based on the elevated levels of luminescence from LMB614 grown on the roots of W440 (Mean difference = 2.377, Adjusted  $P = 0.0244$ ) we selected the Paragon x W440 biparental mapping population for further investigation.



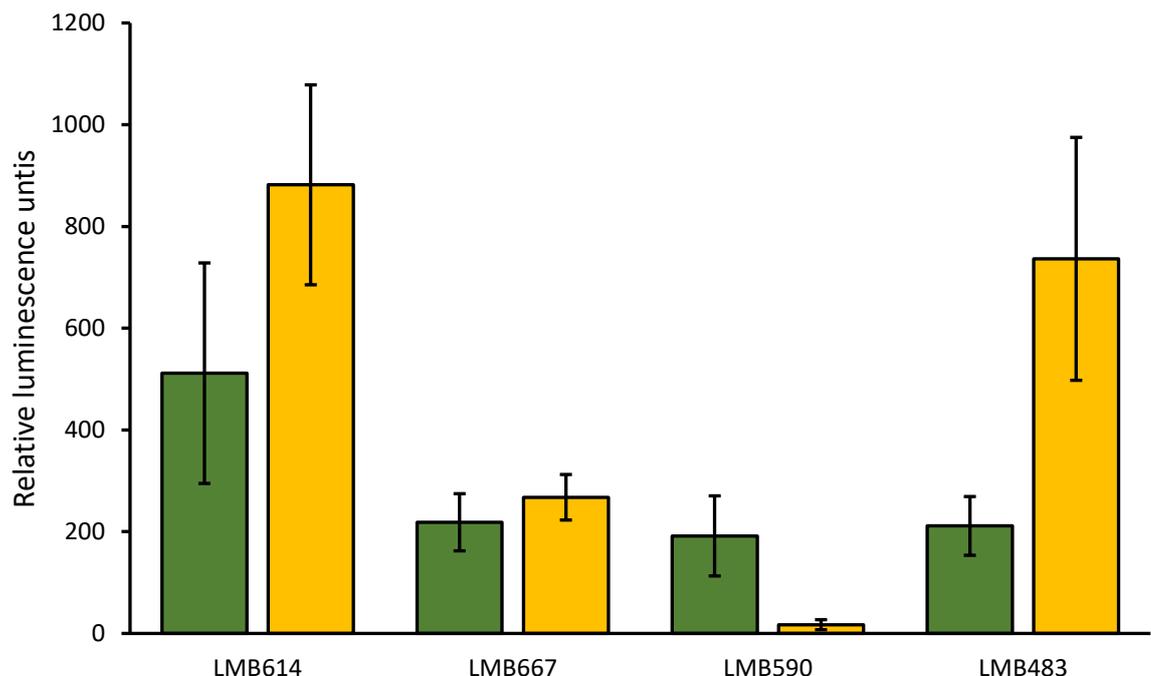
**Figure 11 - Variation amongst a wheat landrace panel for the C4-dicarboxylate exudation phenotype** Mean luminescence values recorded from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of three plants each of both the modern wheat (*Triticum aestivum*) cultivar Paragon and a panel of 10 historic landrace accessions (W044, W079, W360, W440, W483, W624, W651, W698, W722 and W784) at 7 dpi, presented relative to that of Paragon  $\pm$ SEM. Dunnett's multiple comparison tests comparing individual landrace accessions with Paragon were performed following one-way Analysis of Variance (ANOVA) hypothesis testing where \* =  $p \leq 0.05$ .

These results also seem to indicate that the level of C4-dicarboxylate exudation from Paragon roots sits on the lower bound of the range exhibited by the Watkins landrace panel (Figure 11). Moreover, when the luminescence of LMB614 on Hereward and Cadenza roots is also considered alongside W440 and Paragon (Figure S4 in appendix), the extent to which C4-dicarboxylate exudation is reduced in these modern cultivars is further accentuated.

## 4.2.2 Characterising the rhizosphere environment of the Paragon and W440 cultivars

### 4.2.2.1 Bacterial Luminescence-based Root Exudate Profiling

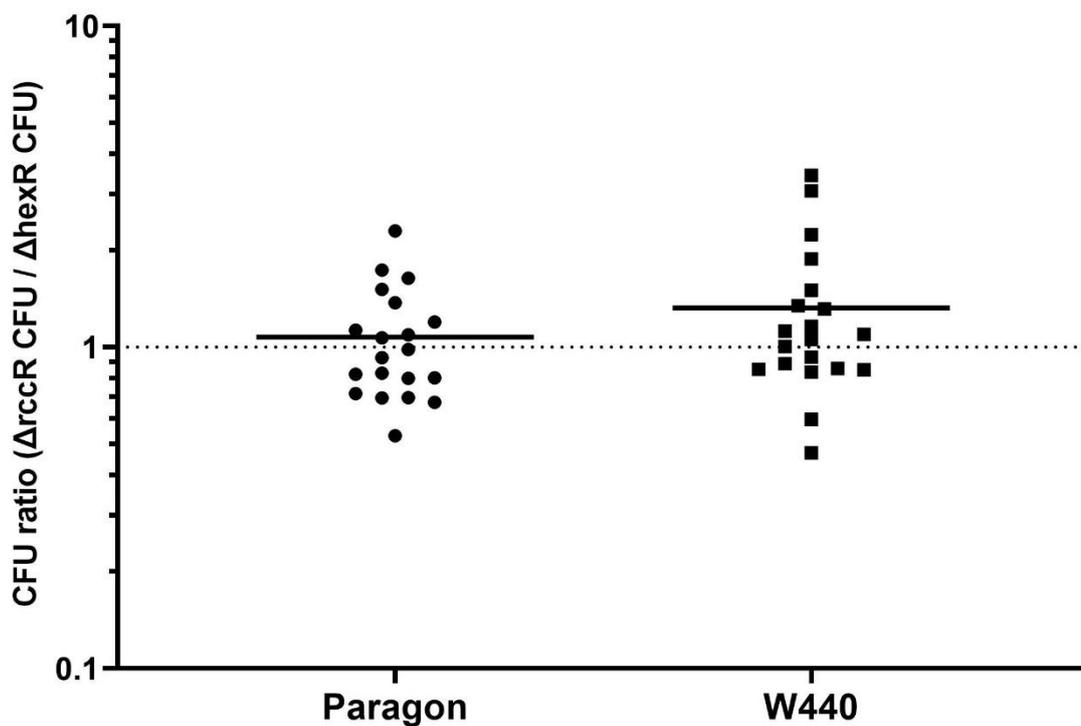
Assessments of the wider exudation profiles of Paragon and W440 were carried out using the biosensor strains that exhibited luminescence in 3.2.4, specifically those responding to C4-dicarboxylates, fructose, xylose, and phenylalanine (Figure 12). Whilst none of the differences between wheat accessions for individual biosensors were statistically significant ( $P < 0.05$ ) - including that of the C4-dicarboxylate biosensor in this instance - several indications of genotype-dependent effects were in evidence here. Aside from the difference in the levels of luminescence from the C4-dicarboxylate biosensor between Paragon and W440, the luminescence of the xylose (LMB590) and phenylalanine (LMB483) biosensors were notably higher and lower respectively for W440 in comparison to the relatively consistent levels of luminescence detected from the other 3 biosensors for Paragon.



**Figure 12 - *In vivo* bioluminescence of four *R. leguminosarum* Rlv3841 *lux* fusion biosensors 7 dpi** Mean luminescence values recorded from biosensors specific to C4-dicarboxylates (LMB614), fructose (LMB667), xylose (LMB590), and phenylalanine (LMB483) growing on the roots of five plants each of the wheat (*Triticum aestivum*) cultivar Paragon (green bars) and the Watkins landrace accession W440 (yellow bars) 7 dpi  $\pm$ SEM. Here, relative luminescence units represent the integrated density (no. luminescent pixels x mean pixel value) of the luminescent area in each CCD image.

#### 4.2.2.2 Bacterial Carbon Source Availability

The competitive rhizosphere colonisation abilities of the two *P. fluorescens* primary carbon metabolism mutants, impaired in their use of either 'simple' and 'complex' carbon sources were used as in 3.2.3.1 to reveal any bias in types of carbon sources present in the exudates of Paragon and W440. The mean ratio of mutant CFU recovered from the rhizosphere compartment of Paragon and W400 one week after co-inoculation onto axenically grown seedling roots differed little between wheat genotypes and was very similar to the initial inoculum ratio (dotted line) indicating that the utilisation of 'simple' and 'complex' carbon exudates were of equal importance to rhizosphere competence here (Figure 13).

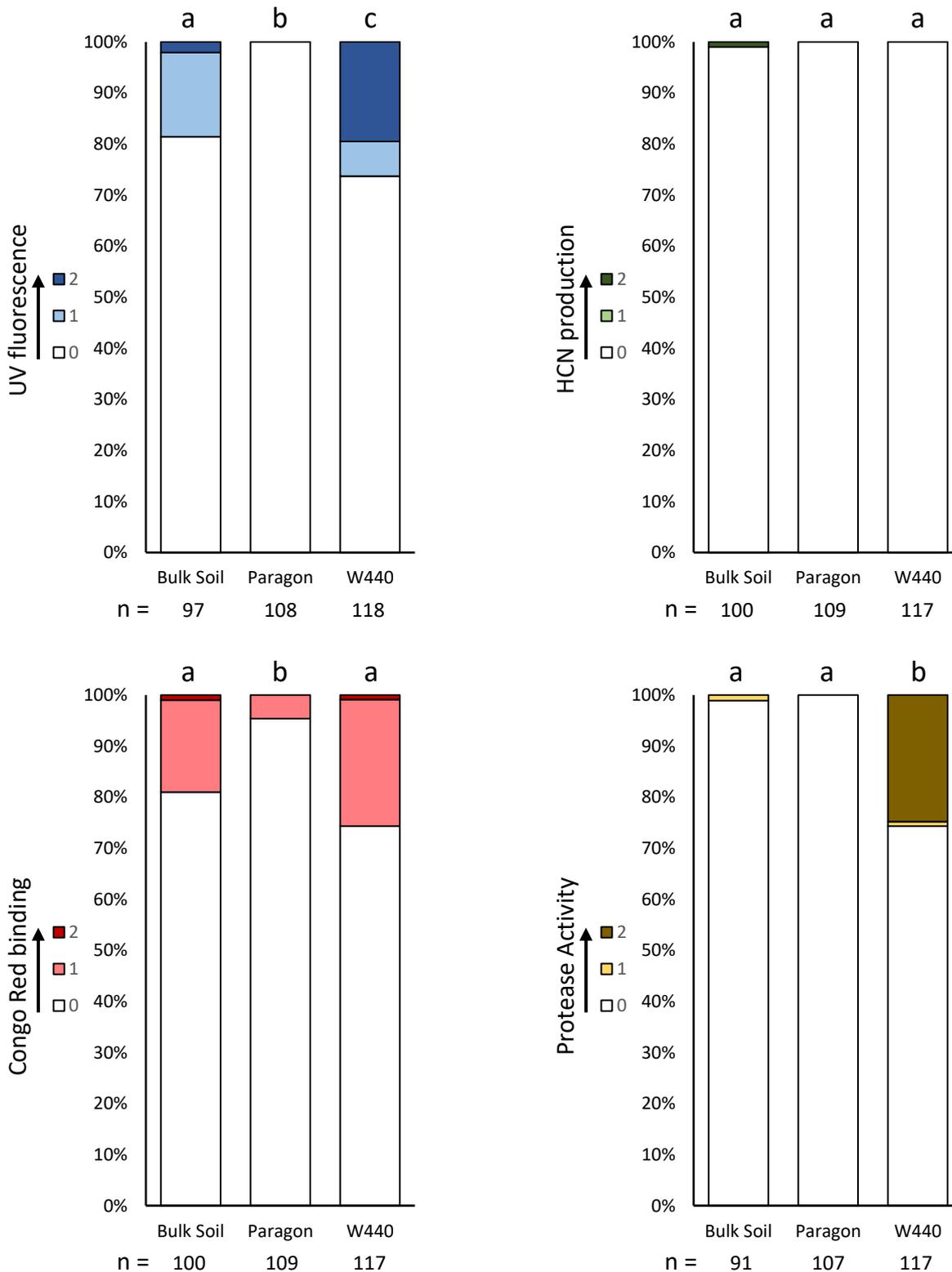


**Figure 13 - Necessity of 'simple' and 'complex' carbon metabolism in rhizobacterial competence**

Competitive wheat rhizosphere colonisation efficiency of two contrasting *Pseudomonas* spp. primary carbon metabolism mutants as inferred from the ratio of CFU recovered 7 days after their co-inoculation onto 7-day old axenic wheat (*Triticum aestivum*) seedlings. Each data point represents the ratio of  $\Delta rccR$  CFU to  $\Delta hexR$  CFU recovered from individual plants divided by the same ratio for the inoculum initially applied to them in one of 2 independent experiments comparing the Paragon (n=18) cultivar with the W440 (n=17) landrace accession. All data are presented on a log<sub>10</sub> scale with mean values indicated by horizontal bars.

#### 4.2.2.3 Rhizosphere *Pseudomonas* spp. Populations

Collections of root-associated pseudomonads (minimum 107 strains) isolated from two independent experimental batches (each comprising 3 plants/cv.) of 4-week old controlled-environment grown wheat cultivars were screened for 4 relevant rhizobacterial phenotypes as in 3.2.2 above. The distributions of trait scores presented in figure 14, obtained following Materials and Methods 2.5, were found to differ significantly between strains isolated from Paragon and W440 roots. Indeed, UV fluorescence, Congo Red binding, and Protease activity were all more prevalent amongst rhizosphere isolates in the W440-associated collection. The trait scores exhibited by a collection of 100 strains isolated from bulk soils in pots left unplanted in the same two experimental batches are also presented in figure 14. The notable absence of HCN production amongst all the strains examined here - bar one bulk soil isolate (Figure 14) precluded meaningful comparisons between treatments for this phenotype. However, the trait scores recorded for the other three phenotypes investigated here followed the same patterns of enrichment and diminishment relative to bulk soils as observed for the Hereward and Cadenza associated isolates in figure 5. In this case, isolates producing proteases capable of visible milk powder degradation were enriched in W440-associated strain collections, whilst Congo Red-binding isolates were selected against by Paragon. Additionally, the increased and decreased proportions of UV-fluorescent isolates associated with W440 and Paragon respectively were different enough here to have resulted in the detection of statistically significant differences in the distributions of their trait scores relative that of the bulk soils (Figure 5).

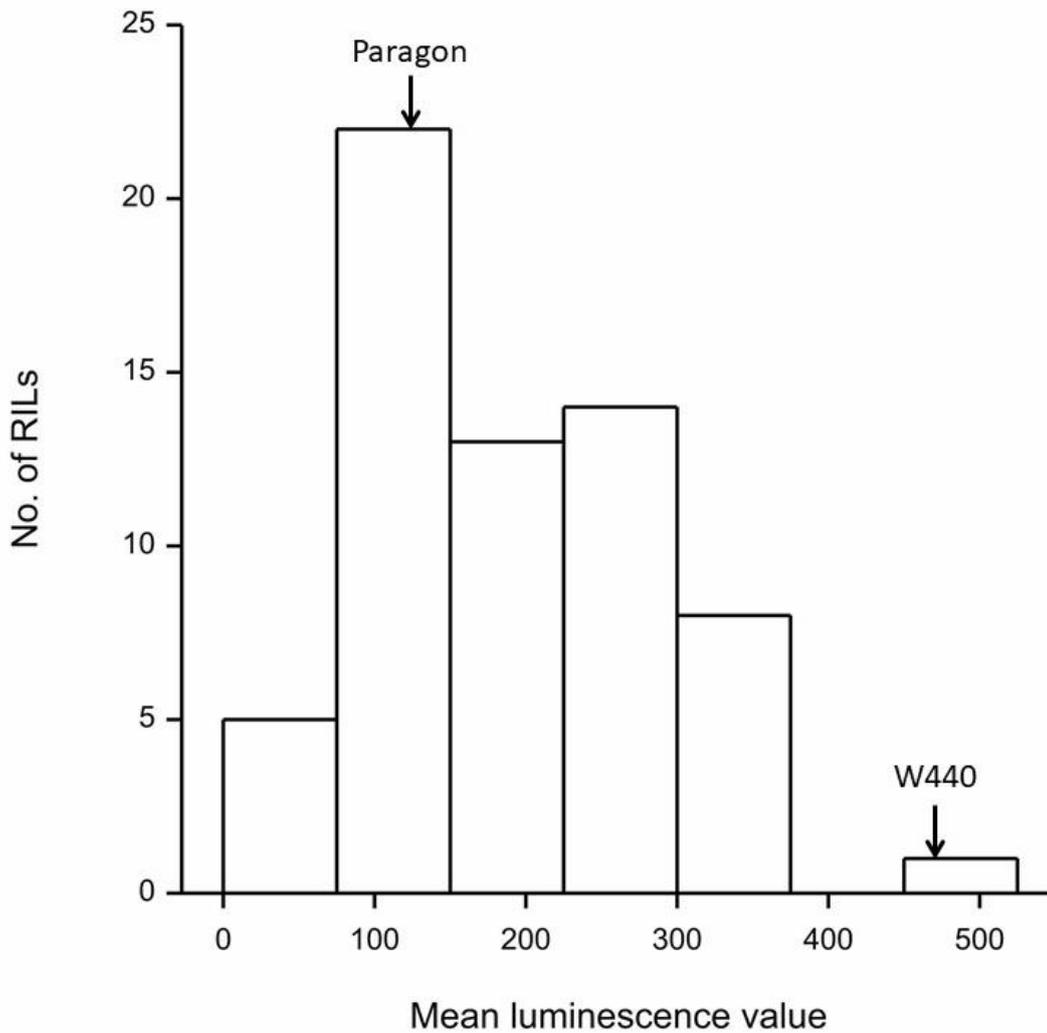


**Figure 14 - The effect of wheat genotype on rhizospheric *Pseudomonas spp.* populations**

Plots showing the percentage of isolates associated with the roots of the wheat (*Triticum aestivum*) cultivars Paragon and Watkins landrace 440 (W440) - as well as unplanted bulk soils - that exhibit particular phenotypes *in vitro* where 0 = not present, 1 = weak presence, 2 = strong presence. The *Pseudomonas spp.* isolates here are pooled from 2 independent isolations (following Methods 2.3) of a maximum of 60 strains per treatment, each sampled from 3 individual pots containing one plant each where applicable. The results of statistical comparisons (Chi-squared tests) between the distributions of trait scores recorded for each collection (of isolates) are denoted by a compact letter display whereby treatments not annotated with the same letter differ significantly (Adjusted  $P < 0.05$ ).

### 4.2.3 Mapping Quantitative Trait Loci

The extent of C4-dicarboxylate exudation from the roots of 63 F<sub>4</sub> RILs of the Paragon x W440 mapping population as well as both parental genotypes was assessed as detailed above using the luminescence of the rhizosphere-colonising biosensor strain LMB614 7dpi as an indirect but quantitative measurement. The frequency distribution of mean luminescence values was near-continuous amongst RILs with the major peak near to the luminescence values observed for Paragon and a positively skewed tail towards the higher mean of the parental Watkins accession (Figure 15).



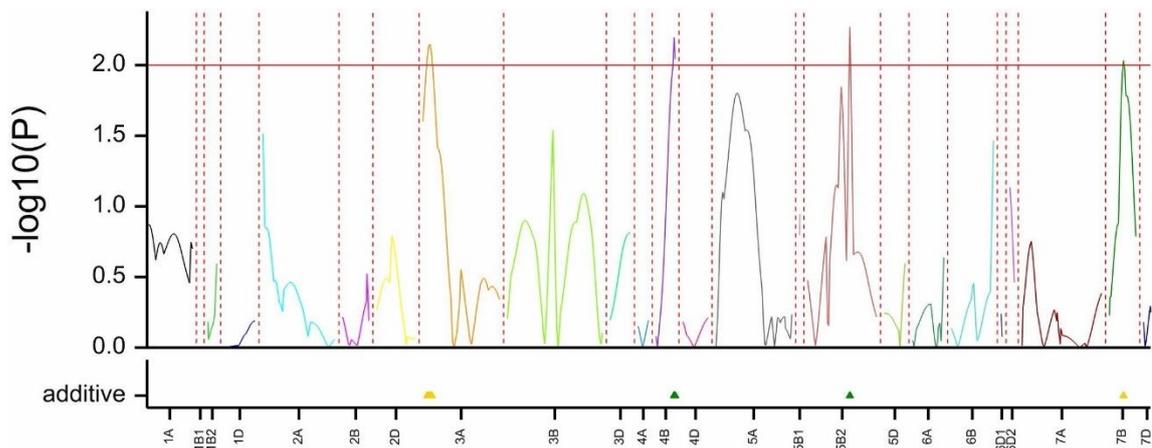
**Figure 15 - Frequency distribution of C4-dicarboxylate exudation in the Paragon x W440 population**  
Histogram of the mean luminescence values for 63 F<sub>4</sub> RILs from the Paragon x Watkins accession 440 bi-parental mapping population 7 dpi with the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614). Mean values for the parental genotypes are indicated by arrows.

A primitive single marker regression analysis initially conducted by James Simmonds (Uauy group, JIC) using genotyping data for total of 175 KASP™ SNP markers generated by Wingen *et al* (2017) revealed several minor marker-trait associations, but no major explanatory loci indicative of any individual W440-derived alleles being responsible for a large proportion of the phenotypic variance. In fact, out of the 14 significant ( $P < 0.05$ ) marker-trait associations presented in Table 3, the positive effects of the four most significant markers on C4-dicarboxylate exudation were conferred by alleles derived from Paragon.

**Table 3 - Single marker regression analysis for C4-dicarboxylate exudation from wheat roots**

Marker	Linkage group	Chromosome	P (Wald)	Allele for positive effect	R <sup>2</sup>
BS00049213	16	5B	0.007	Paragon	0.1127
BS00106143	12	4B	0.011	Paragon	0.1003
BS00022466	12	4B	0.012	Paragon	0.0980
BS00044388	16	5B	0.017	Paragon	0.0895
BS00049372	23	7B	0.020	W440	0.0861
BS00074359	23	7B	0.021	W440	0.0838
BS00022780	16	5B	0.024	Paragon	0.0808
BS00048491	8	3A	0.029	W440	0.0760
BS00089954	9	3B	0.032	W440	0.0702
BS00105208	14	5A	0.033	W440	0.0723
BS00001108	5	2A	0.035	W440	0.0712
BS00061384	19	6B	0.038	Paragon	0.0686
BS00042181	19	6B	0.038	Paragon	0.0686
BS00025739	8	3A	0.043	W440	0.0655

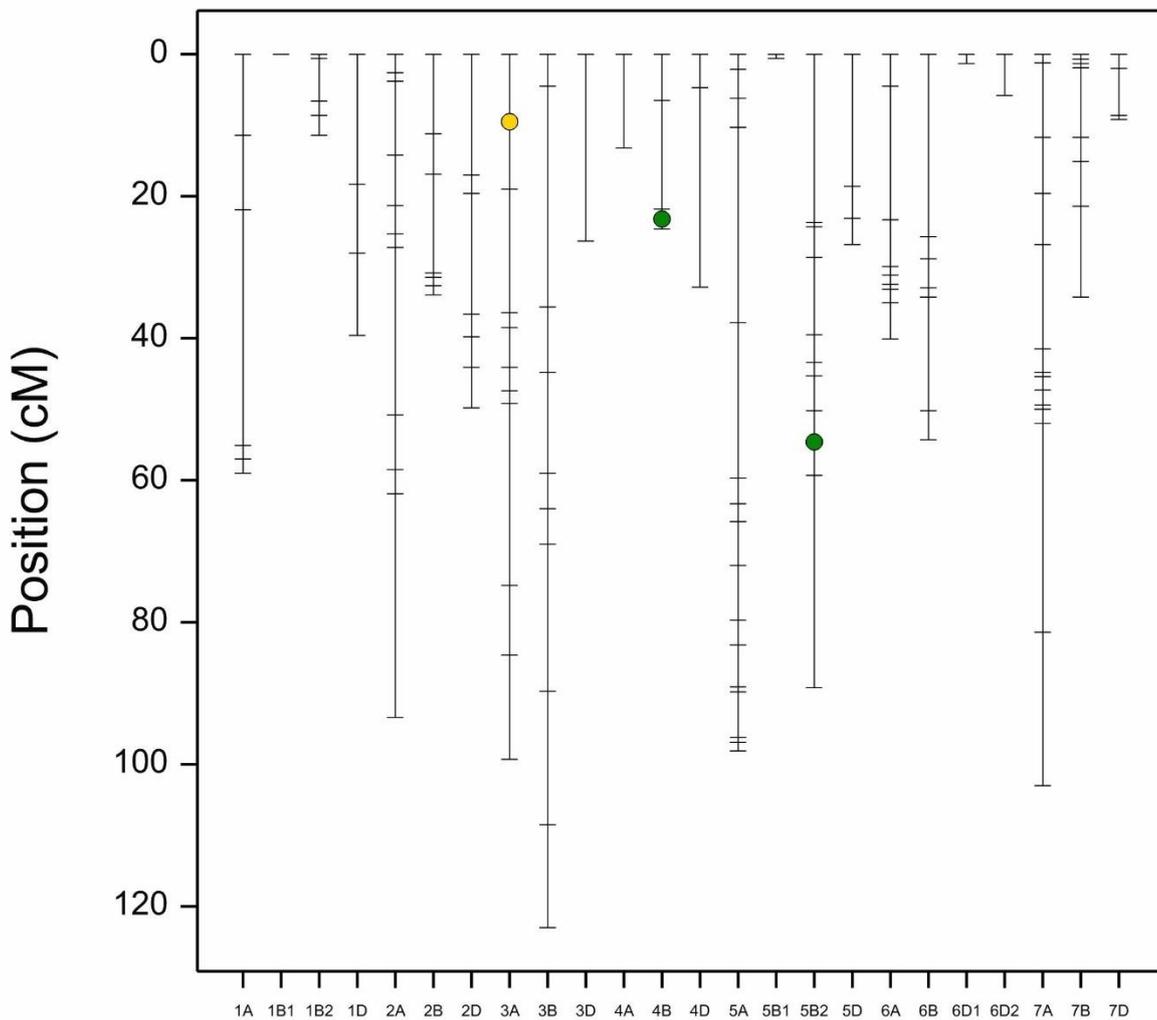
The two parents actually contributed an equal number of significant positive marker alleles (Table 3), although several marker-trait associations with the same allele for positive effect were detected by pairs of closely linked, adjacent markers on the same chromosome and may actually be indicative of a single QTL. Thus, a simple interval mapping approach incorporating additional genetic predictors ('pseudo-markers') at intervals of a maximum distance of 2 cM along the genetic map as well as at marker positions (641 loci in total) was employed to resolve the locations of any QTL. A total of 8 loci (2 markers and 6 pseudo-markers) exceeded the low arbitrary detection threshold, describing 4 regions from linkage groups on chromosomes 3A, 4B, 5B, and 7B (Figure 16) - two with alleles derived from Paragon (green triangles) and two from W440 (yellow triangles).



**Figure 16 - Profile plot of loci effecting variation for C4-dicarboxylate exudation from wheat roots**  
 Profile plot of  $P$ -values from Wald tests of QTL effects on the  $-\log_{10}$  scale obtained following simple interval mapping across 24 linkage groups of the Paragon x W440 mapping population labelled according to their chromosomal location, each depicted by a different coloured line. The locations of loci exceeding the threshold value ( $-\text{Log}_{10}[P] = 2$ ) are shown in the lower panel as triangles coloured according to the source of the allele (Paragon = green; W440 = yellow) for increased C4-dicarboxylate exudation from roots as quantified by bacterial luminescence-based biosensor assays.

Three of these candidate loci were fitted in a final multi-QTL model - each having sufficiently significant ( $p < 0.05$ ) effects on phenotypic variation as determined by backward selection.

Whilst two of these QTL carried Paragon-derived positive effect alleles (located on chromosomes 4B and 5B), the loci with the largest additive effect was conferred by a W440 allele on chromosome 3A which could explain 14.47% of the variance for the C4-dicarboxylate exudation trait amongst the RILs screened here. Locations of the peak markers (or pseudo-markers) for these QTL are displayed on a genetic map in figure 17 below.



**Figure 17 - Locations of three minor QTL conferring C<sub>4</sub>-dicarboxylate exudation from wheat roots**  
 Genetic map showing the locations of the peak markers of three minor QTL as circles coloured according to the source of the positive allele (Paragon = green; W440 = yellow) across 24 linkage groups of the Paragon x W440 mapping population labelled according to their chromosomal location.

### 4.3 Discussion

The extent to which variation in the novel C<sub>4</sub>-dicarboxylate root exudation trait described above for two modern elite cultivars (Figure 8) is also evidenced in the wider wheat gene pool was investigated here. Amongst the limited selection of modern and historical wheat germplasm initially characterised here (Figures 9 and 10), expectations of greater between-accession variation amongst the historical landrace accessions were strongly supported. How well results obtained under axenic conditions in non-soil substrates reflect the exudation dynamics of plants grown in various natural soils remains to be seen, especially for OAs implicated in plant responses to multiple edaphic factors (Jones, 1998). Nonetheless, these

baseline differences may well reflect the relevance of C4-dicarboxylate exudation in the specific environments to which each of these landraces are highly adapted - be that for Al tolerance, mineral solubilisation, or the recruitment of particular sets of microbes. Modern elite cultivars on the other hand, largely selected under idealised field conditions without regards for non-yield traits, are likely to exhibit much less variation for these rhizosphere processes. The observation that few landrace accessions induced lower levels of bacterial luminescence indicative of promoter upregulation by C4-dicarboxylate detection than the modern elite cultivar Paragon certainly attests to this fact, as do the further reduced levels in Hereward and Cadenza in Figure S4 (in appendix). If anything, such reductions are likely to have been inadvertently selected for in modern breeding programmes targeting high yields if the reduced levels of photosynthates leaving the roots as part of root exudation were instead diverted towards further plant growth or grain filling for example. It is not hard to imagine that certain mechanisms of root exudation may have been lost from the elite wheat gene pool in this way - particularly those resulting in the efflux of substantial quantities of primary cellular metabolites such as the C4-dicarboxylates. The novel variation observed for the C4-dicarboxylate exudation trait amongst the landrace accessions may well have been facilitated by such pathways, having escaped the selective pressures imposed on modern wheat germplasm since their collection in the 1930s.

However, in comparison to the highly C4-dicarboxylate exuding Watkins accession 440, the exudation of the other compounds as detected by the three additional luminescent biosensors strains employed in 4.2.2.1 was not reduced in Paragon. Indeed, the sugars fructose and xylose were detected in the W440 rhizosphere at low levels more characteristic of exudation from Paragon roots (Figure 12). Moreover, the negligible levels of luminescence emitted by the xylose biosensor (LMB590) on W440 roots were in fact much lower than the relatively consistent levels detected from this and two of the other biosensors (LMB667 and LMB483) shown in figure 12 after 7 days of growth on Paragon roots. These findings would appear to conflict with the predictions of reduced levels of exudation in modern cultivars as outlined above. However, they may in fact suggest that the exudation of certain compounds

have been selected upon less strongly by wheat breeding programmes, perhaps indicative of the smaller nutrient cost associated with their exudation or the increased relevance of these particular kinds of exudation under modern field conditions. Whilst conclusions drawn from comparisons of the exudation of the individual compounds investigated here cannot reasonably be applied to the compound classes to which they belong, it is certainly interesting to note that the compounds detected at higher levels in the landrace accession were organic and amino acids - primary metabolites also known to have greater effects on soil bacterial community composition compared to sugars (Shi *et al.*, 2011; Gu *et al.*, 2020).

Despite these differences, the importance of the active metabolism of such 'simple' carbon metabolites compared with more 'complex' ones during rhizosphere colonisation was indistinguishable for *Pseudomonas* mutant strains inoculated onto the roots of W440 and Paragon (Figure 13). The ratios of mutant CFU recovered in these experiments did however contrast with those recorded in 3.2.3.1 where the *rccR* mutant better able to utilise simple carbon sources was more successful in the rhizosphere. However, without replicating these experiments and applying the same inoculum to all 4 accessions simultaneously, conclusions cannot be drawn on the differences observed between these two pairs of wheat accessions. Once again, the increased exudation of certain compounds of interest may have been accompanied by corresponding reductions in the amount of other exudates released from roots, potentially obscuring any differences in the ratio of 'simple' to 'complex' carbon sources in the rhizosphere that might otherwise have been detected between Paragon and W440. Indeed, the ratio of these carbon metabolites exuded from wheat roots may be regulated as such, potentially reflecting some as yet unknown constraints on the efflux of these photosynthates. Alternatively, these results could serve as an indication that the role of the variable levels of exudation observed for individual compounds is not a purely metabolic one - at least in terms of rhizosphere microbiome assembly. Indeed, the role of particular OAs as chemo-attractants for certain sets of rhizosphere microbes as previously described may be independent of their utilisation as readily metabolised simple carbon sources.

It was hoped that investigation of the prevalence of a selection of relevant rhizobacterial traits amongst pseudomonads isolated from the roots of these disparate wheat accessions would further reveal the nuances of their influence on their rhizosphere microbiome. Apart from HCN production, which was notable in its absence from all but one of the strains isolated here, statistically significant differences were observed between the distributions of trait scores for all three of the other phenotypes recorded for Paragon- and W440-associated pseudomonads in 4.2.2.3. Intriguingly, the patterns of enrichments and diminishments of isolates displaying these traits relative to the levels recorded in bulk soils were near-identical to those observed for Hereward and Cadenza in Figure 5 - with Paragon scores analogous to those of Cadenza, and W400 to those of Hereward. These patterns of microbial trait enrichments did not however correspond with the C4-dicarboxylate exudation phenotypes of these accessions. Indeed, the accessions exuding the higher (Cadenza and W440) or lower (Hereward and Paragon) levels of C4-dicarboxylates in each of the pairs of wheat genotypes did not share correspondingly similar patterns of microbial trait enrichments. These admittedly anecdotal findings refute any suggestion that these C4-dicarboxylates are directly involved in determining these microbial trait enrichments. However, given the plethora of other factors that may have contributed to the differences in microbial trait abundances observed here, including differences in the exudation of other metabolites detected in this study in 4.2.2.1, we cannot draw meaningful conclusions about the direct influence of specific exudates on the rhizosphere microbiota. Moreover, even in field studies employing Near Isogenic Lines (NILs) differing only at the *TaALMT1* locus, the observed effects on community structure and species variation of the bacterial rhizosphere microbiome were background dependent - being evident in only one of the pairs of NILs, but not the other (Mahoney *et al.*, 2017).

The differences in C4-dicarboxylate exudation detected here might still play some role in the assembly of distinct rhizosphere microbiota. The relative amounts of these compounds exuded into the rhizosphere may in fact play an important role as part of a more complex signal, recognised by root-colonising bacteria with specific sets of OA chemoreceptors that

may be involved in the discrimination between different host plants with specific root exudation profiles as discussed in 3.3 above. Such a scenario would explain why the relative level of any individual root exudate such as the C4-dicarboxylates studied here cannot easily be used to predict the response of complex microbial communities. Indeed, the potential complexity of natural root exudate cocktails has made distinctions of the causal links between plant genetic identity and rhizosphere microbial diversity all the more elusive (Badri *et al.*, 2013; Steinauer *et al.*, 2016). Such speculation on the role of the compounds presently investigated is however unfounded without evidence of a reproducible effect on some aspect of the rhizosphere microbiota.

Nonetheless, identifying the basis of the C4-dicarboxylate root exudation trait would be an important proof-of-concept for the implementation of bacterial luminescence-based biosensors for trait discovery. Variation amongst the Paragon x Watkins 440 F<sub>4</sub> mapping population for the C4-dicarboxylate exudation trait was assessed in a total of 63 RILs using the bacterial biosensor assay developed above. The quantitative nature of this exudation trait was supported by the broadly unimodal distribution of mean luminescence values between the two parental values, with only a few transgressive values observed at very low levels of luminescence below that observed for the Paragon parent. These observations of heritable plant genetic control of rhizodeposition are in accordance with those of Mwafulirwa *et al.* (2016), who reported substantial variation of total rhizosphere microbial activity between the offspring of a similarly diverse cross between an elite barley genotype and a wild accession. Together, these results highlight the untapped potential of exotic germplasm to further our understanding and exploitation of plant-microbiome interactions, especially where extensive genetic resources for trait discovery already exist as they do for the A.E. Watkins landrace collection.

However, the lack of any strong QTL explaining the increased levels of C4-dicarboxylate exudation inferred from the luminescence of LMB614 on the parental Watkins accession (W440) does not support the assumption that simple exudation traits are controlled by a few

major genes with large effects such as the principle determinant of malate exudation *TaALMT1* located on chromosome 4D. The results presented instead indicate that the C4-dicarboxylate exudation trait measured here is highly polygenic, with multiple loci of small effect (Table 3) controlling aspects of the exudation of C4-dicarboxylates and their regulation. Whilst a variety of loci such as those controlling the extent of root colonisation by the *R. leguminosarum* biosensor may well have misleadingly affected the luminescence output measured here without modulating the expression of the *lux* cassette promoter, a number of potential sources of variation directly affecting C4-dicarboxylate exudation are also thought to exist. Indeed, evidence from investigations of aluminium induced malate efflux has indicated that genes other than *TaALMT1* may be involved in malate efflux in some wheat accessions. Upon observation of several Al-sensitive Japanese genotypes that possessed promoter alleles that should have conferred malate-mediated aluminium tolerance given their correspondingly high levels of *TaALMT1* expression, Sasaki *et al* (2006) concluded that some additional genes may be involved in post-transcriptional regulation of *TaALMT1* function. Moreover, the group of Al-tolerant landrace accessions investigated by Raman *et al* (2008) that lacked the blocks of sequence repeats in the *TaALMT1* promoter region thought to confer Al-tolerance could even be suggestive of independent malate exudation pathways in the primary wheat gene pool. Additionally, several motifs commonly associated with abiotic stresses in the region upstream of *TaALMT1* (Sasaki *et al.*, 2006) indicate that malate exudation could be further modulated by additional phytohormones or environmental stimuli that may not be controlled for. Indeed, the nuances of existing exudation patterns may be finely tuned to the environmental stressors faced in specific environments where the C4-dicarboxylate exudation mediated response to Al toxicity or bacterial pathogenesis for example, may be more or less relevant. Careful consideration should therefore be given to the many important functions of C4-dicarboxylates in the rhizosphere before attempting to manipulate their exudation by exploiting loci from exotic germplasm whose regulation is not fully understood.

Alternatively, these weak QTL may in fact be artefacts of the high level of background biological variability between the individual replicate plants screened here. Such excessive levels of plant-to-plant variability may well have undermined the power of the analyses performed and lead to the detection of spurious marker-trait associations and the proposal of a misleading final QTL model (Figure 17). In fact, this would not be unprecedented for measurements of root exudates. Substantial changes in the levels of *Sorghum*-derived malate exudates detected in the sandy substrate of Miller *et al* (2019) were similarly obscured by the large variation between plant replicates, contributing to the negligible levels of statistical significance this finding had in their analyses. The relative importance of this source of variation has even been investigated using a nested experimental design to dissect the total variance of *Arabidopsis* root metabolite profiles into that attributable to natural accession-accession variation, the experimental batch, and the individual variability between plants (Mönchgesang *et al.*, 2016b). The authors found plant-to-plant variability contributed around 50% of the total variation for all metabolic profiles, outweighing that contributed by both other sources of variation in their similarly designed experimental set-up - using 4 plants per accession per batch. Moreover, this trend was especially evident when considering classes of primary metabolites, increasing the minimal detectable magnitude of any accession-specific effects to impractical levels. As such, the overall efficacy of our bacterial luminescence-based screening assays in resolving the genetic basis for variable root exudation traits is questionable. However, by treating this work as a pilot study, approaches such as linear hierarchical regression modelling may be used to estimate individual variances of each level of the experimental design, and some optimal combination of biological and technical replicates may be calculated as such to allow effective resolution of genotype-specific differences in exudation traits needed to detect meaningful QTL in future studies.

## Chapter 5

# DISCUSSION AND FUTURE PERSPECTIVES

Understanding and exploiting the determinants of favourable microbiome assembly in crop plants could directly address some of the major challenges faced by 21<sup>st</sup> century agriculture. The manifestation of various microbiome-mediated functions could increase yields not only under increased pressures from biotic and abiotic stressors, but also with a decreased reliance upon the environmentally harmful chemical inputs currently employed. Here, several aspects of the influence exerted on the rhizosphere microbiota by two pairs of wheat accessions have been characterised with the aim of dissecting the genetic basis for any variation observed. Initially, populations of rhizospheric *Pseudomonas spp.* selected from the diversity of soil pseudomonads inhabiting the starting soil substrate were examined in terms of both the phylogenetic relationship between strains associated with different wheat cultivars and the representation of rhizobacterial traits amongst them. Phylogenetic groupings inferred from *gyrB* sequence homology tended to be dominated by *Pseudomonas* isolates associated with just one of the two cultivars examined indicating the fine level of control these plants have over individual microbial genotypes. Indeed, given the diversity of putatively PGP functions that may be carried out by different strains in this genus even within a single field location (Stefanato *et al.*, 2019) the proliferation of certain groups of pseudomonads over others may well determine the extent of PGP afforded by the microbiota. Moreover, after just four weeks of growth in the same starting soil substrates, these same two cultivars also appeared to exert significantly different selective pressures on *Pseudomonas spp.* with particular functional traits - a trend also observed for the other pair of wheat accessions subsequently investigated. Indeed, amongst collections of *Pseudomonas* isolates associated with these two pairs of wheat accessions, certain plant genotypes elicited intriguingly similar patterns of trait enrichments relative to bulk soils. It may therefore be interesting to see whether the similar patterns of trait enrichments by Paragon and the Watkins landrace accession 440 are also reflected by similar phylogenetic groupings of isolates associated with full-term wheat plants. Indeed, doing so could provide an important determination of the relevance of such functional differences in structuring the rhizosphere microbiota more generally, which may well translate to other more agriculturally relevant soil-based systems.

The targeted investigations of root exudation conducted here could not however determine any differences amongst these pairs of accessions that may have resulted in the patterns of trait enrichments observed. Indeed, differences in the C4-dicarboxylate exudation trait identified as the sole source of significant variation between the accessions investigated here were not consistent with these trends. Whilst these results do not invalidate the potential roles of these compounds in rhizosphere microbiome assembly, they do restrict the utility of the novel C4-dicarboxylate exudation trait in efforts to manipulate the microbiome at present. The exudation of primary metabolites such as these OAs should not however be written off in these lines of research. Indeed, compounds like these may even represent the crucial factors eliciting PGP functions from microbial communities that may well have a high potential for PGP but would otherwise remain focused solely on their own growth (Yang *et al.*, 2019). However, in future investigations the exudates responsible for the initial structuring of the microbiota should be targeted as a priority as their effects may be validated more easily and exploited as such. Admittedly, the absence of any exudation traits with direct consequences for rhizosphere microbiome assembly in this study may well have been a factor of the arbitrary choice of compounds investigated based on the availability of biosensors. However, given the ease with these biosensors were used to conduct a preliminary screen of a wheat mapping population here, this study can hopefully be viewed as a successful proof-of-concept for this novel approach towards the identification of plant traits responsible for microbiome assembly. This should in turn justify the construction of additional biosensor strains with novel specificities more likely to detect differences in relevant exudation traits, potentially informed by other methodologies such as gas or liquid chromatography-based identification of the biggest differences between root exudate profiles or based on the findings of other studies such as those implicating particular classes of plant metabolites in microbiome modulation (Huang *et al.*, 2019). Indeed, a pipeline for the identification of plant traits modulating microbiome assembly can be imagined that starts with the informed construction of novel biosensors and ends with the dissection of the genetic basis for these traits using resources such as the Watkins NAM panel for future crop improvements.

In these and other lines of investigation into the rhizosphere microbiota and its exploitation for agricultural sustainability, studies of the initial assembly of these microbial communities should be further prioritised. Microbial communities assembled within the early stages of plant growth are highly predictive of future microbiome structure (Edwards *et al.*, 2015) to the extent that important microbiome-mediated functions such as pathogen suppression are largely determined by the composition of the microbiota of the soil in which the plant germinates (Wei *et al.*, 2019). Moreover, the 'alternative stable states' - or 'microbiome types' - exhibited by the resultant communities are by their nature highly resilient (Lozupone *et al.*, 2012) and often resistant to amendments - even with putatively PGP inoculants (Castro-Sowinski *et al.*, 2007). Fortunately the early stages of plant microbiome assembly most amenable to manipulation are however quite easily accessed in most crop species due to their annual lifecycles, enabling efforts to promote the integration of PGP microbes and the establishment of beneficial microbiome types at the start of every cropping cycle (Toju *et al.*, 2018). Indeed, an early example of this contemporary approach towards the incorporation of PGP microbes in crop production systems successfully targeted the microbiota transmitted within elite crop seed embryos by introducing microbes to the flowers of the parent plants (Mitter *et al.*, 2017), resulting in the modulation of various growth traits in field grown wheat progeny. Attempts to influence this initial assembly process seek to exploit the historical contingency of microbial community composition via the facilitation or obstruction of subsequent colonisation events by early arrivals - so called 'priority effects' (Fukami, 2015). Indeed, much of the plant protection afforded by PGP members of genera such as the pseudomonads studied here is based on these principles. Highly motile pseudomonads often have the advantage of early arrival and may therefore preclude the establishment of potentially pathogenic species via their occupation of niches that are particularly vulnerable to pathogenesis such as those at the root surface with access to the most abundant exudate components (Lugtenberg *et al.*, 2001). Resident pseudomonads may also modify the nature of other niches available within the rhizosphere for which they do not directly compete via the production of antibiotics of varying specificities (Haas and Defago, 2005) and the formation of

protective biofilms (Morris and Monier, 2003). Whilst these activities may shift the membership of these niches away from potentially pathogenic states, it is important to note that it is not just pathogens that will be inhibited, and that the colonisation of some other species, for example those able to tolerate antibiotic compounds, will in fact be facilitated.

Indeed, small changes to the order of species arrival such as the pre-colonisation of a single strain can be highly deterministic of future community composition, with pivotal roles in the subsequent development of distinct microbiome types (Werner and Kiers, 2014; Hiscox *et al.*, 2015; Sprockett *et al.*, 2018). Moreover, certain species appear to have more pronounced effects on the development of community structure analogous to the highly (inter-)connected 'hub taxa' inferred from microbial co-occurrence networks that are disproportionately important in shaping the microbiome community structure of plants (Agler *et al.*, 2016). Indeed, in addition to the aforementioned importance of the early establishment of putatively PGP microbes, 'hub taxa' that may not themselves promote plant growth, but play important roles in the predictable assembly of beneficial microbiome types are increasingly seen as the most important targets for robust PGP solutions (Toju *et al.*, 2018; Qiu *et al.*, 2019). Interestingly, within the consistently small fraction of total microbial community variance explained by *Arabidopsis* accession identity across environments, Brachi *et al* (2017) report a significant enrichment of 'hub taxa', with estimates of microbial 'heritability' generally decreasing with increased distance from these 'heritable hubs' in network models. These inferences hint at the exciting possibility that amongst locally adapted *Arabidopsis* ecotypes at least, variation at particular host loci mediates interactions with these 'hub taxa', effectively extending their influence over the microbiome. The pre-existence of mechanisms by which particular plant accessions preferentially associate with taxa able to steer plant microbiome assembly towards particular outcomes is certainly intriguing and may feasibly represent the genetically optimised outcomes of millions of years of co-evolution between plant and microbial partners to reciprocally increase fitness in their shared environments. It should be stressed, however, that microbial hub taxa are classified using network theory alone by considering all possible microbe-microbe interactions a given strain

has the potential to mediate. Establishing causality in such relationships and defining the true ecological significance of designated 'hub taxa' demands rigorous hypothesis-driven investigations of microbial community dynamics *in natura* (Faust and Raes, 2012; Vorholt *et al.*, 2017).

The last few years have however witnessed the increasingly widespread adoption of reductionist approaches to plant microbiome research that enable such hypotheses of microbiome assembly and functioning to be tested through targeted manipulations (Busby *et al.*, 2017). Defined 'synthetic communities' (SynComs) of microbes, constructed to recapture the structure and functions of the naturally acquired plant microbiota under controlled, axenic conditions represent the basis of these highly experimentally tractable systems (Vorholt *et al.*, 2017; Liu *et al.*, 2019). As such, quantitative assessments of various host and microbial parameters have started to unravel the complexities of plant microbiome assembly and the manifestation of certain functions. The importance of microbial 'keystone' species for example, without which the structure of microbial communities is lost, has been demonstrated in SynComs comprised of as few as seven bacterial strains in a maize root system (Niu *et al.*, 2017). Indeed, the historical contingency of plant microbiome assembly have been further verified by the drop-out and late introduction experiments of Carlström *et al.* (2019) whose comprehensive investigations revealed the relative abundances of all SynCom members were subject to priority effects in the assembly of an *Arabidopsis* foliar microbiome model. This same system was in fact employed by one of the seminal SynCom-based investigations of the plant microbiome which identified the significant potential of plant genetic factors harboured by *Arabidopsis* ecotypes - as well as individual host genes - in modulating the microbiota (Bodenhausen *et al.*, 2014). Such approaches have become an increasingly popular means of verifying the effects of host factors hypothesised to affect the plant microbiome (Castrillo *et al.*, 2017; Zhang *et al.*, 2019). Indeed, these systems could be utilised as such to determine the relevance of loci controlling exudation traits found to differ between accessions using the methodologies outlined here, such as the aforementioned

*TaALMT1* NILs of Mahoney *et al* (2017) or even transgenic lines with altered exudation phenotypes such as *TaALMT1* overexpressing wheat (Pereira *et al.*, 2010) for example.

Of the many important insights that these lines of research have garnered, the most intriguing may in fact be that of the interaction between these plant and microbial parameters revealed by a recent study of the foliar microbiome amongst locally adapted accessions of the model tree species *Populus trichocarpa* (Leopold and Busby, 2020). The authors found that the priority effects observed as a result of changes in the order of arrival of SynCom members were actually dependent upon the genotype of the host - suggesting some sort of genetic basis for the differences in microbiome assembly, potentially reflecting beneficial adaptations to the challenges faced in their local environments. These results further highlight the importance of studying locally adapted germplasm in order to better understand the control of plant microbiome assembly and potentially uncover novel microbiome-mediated functions of relevance to sustainable agriculture. Indeed, historically cultivated crop accessions adapted to diverse environments such as those in the A.E. Watkins wheat landrace collection employed here are therefore of enormous importance, and likely possess a diversity of microbiome assembly cues absent from modern elite varieties, as was recently inferred from the increased prominence of neutral processes in the assembly of the microbiota associated with modern wheat germplasm (Hassani *et al.*, 2020). Moreover, the NAM population associated with the collection should enable the genetic dissection of any number of complex rhizosphere traits and points towards its future utility in resolving the basis of microbiome-mediated functions relevant to sustainable crop improvements. However, it is important to note that some level of stochasticity will always exist in terms of species arrival order and as such neither host nor microbiome should be considered in isolation (Oyserman *et al.*, 2018). Indeed, any efforts to manipulate the rhizosphere microbiome will undoubtedly be more successful when plant and microbial partners are engineered in tandem (O'Connell *et al.*, 1996; Ryan *et al.*, 2009; Dessaux *et al.*, 2016), a principle noted right at the dawn of rhizosphere engineering, the relevance of which continues to this day.

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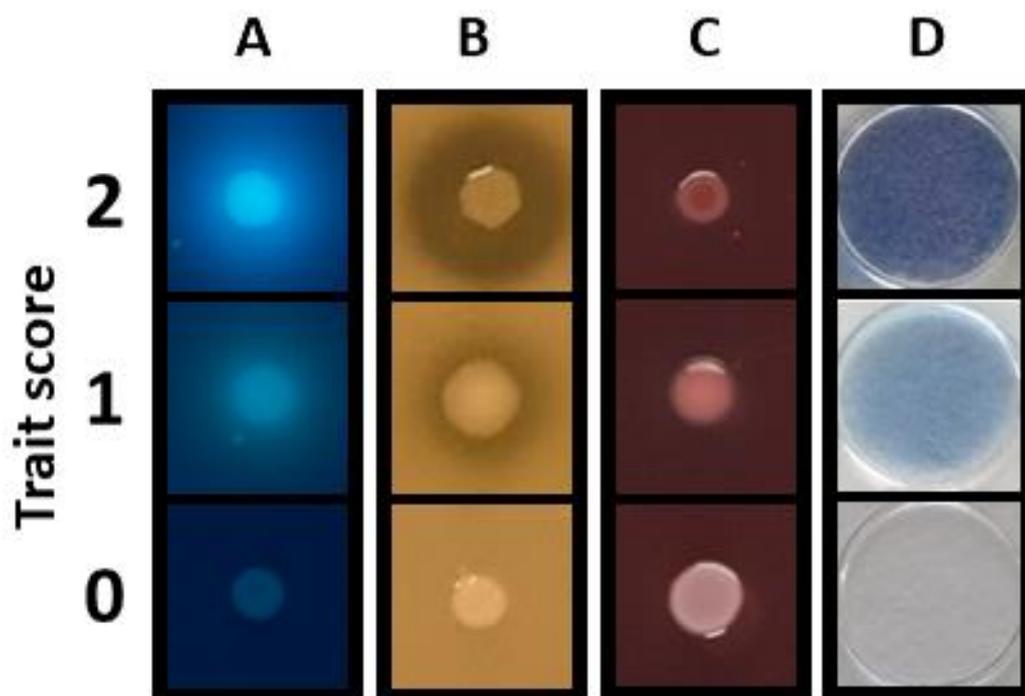
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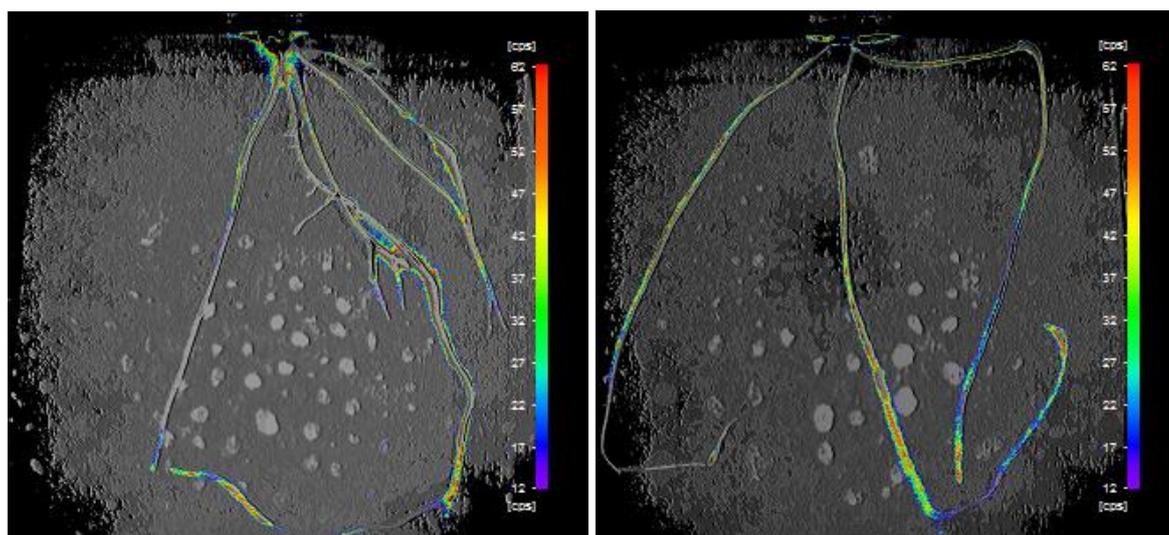
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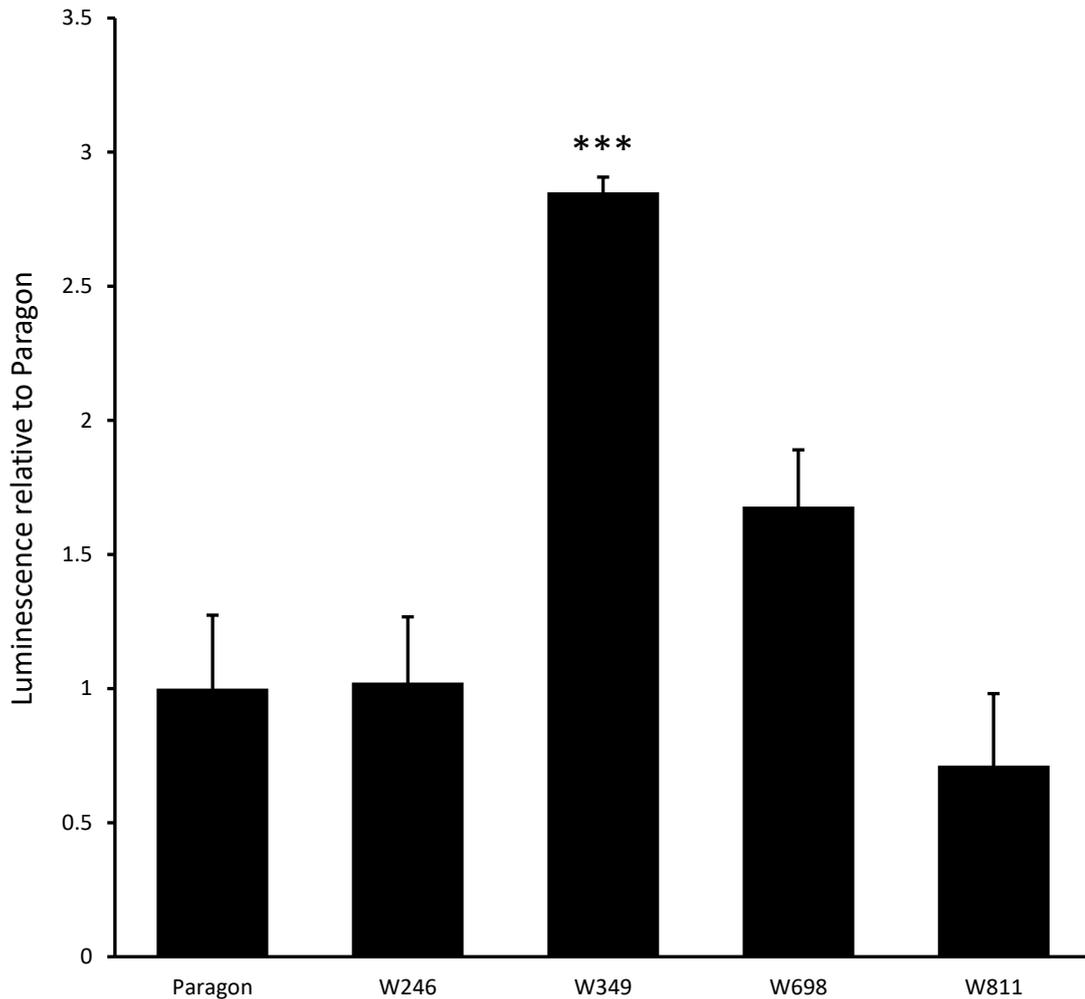
## Appendix



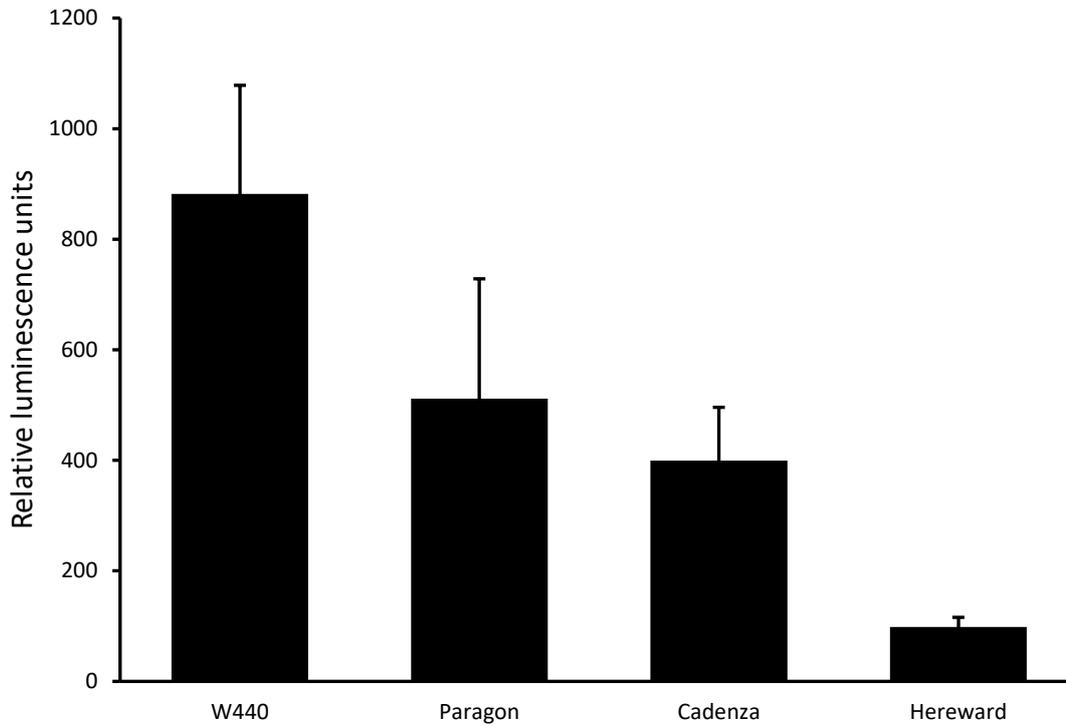
**Figure S1 - Variation for 4 rhizobacterial traits amongst *Pseudomonas spp.* *in vitro***  
Representative examples of *Pseudomonas* isolates scored 0, 1, or 2, based on the presence of four relevant rhizobacterial phenotypes as outlined in Materials and Methods 2.5 where A = fluorescent siderophore production; B = Protease secretion; C = exopolysaccharide production; D = HCN emission.



**Figure S2 - Spatial distribution of luminescent *R. leguminosarum* biosensor bacteria on wheat roots**  
*In vivo* bioluminescence of the constitutive *lux* fusion biosensor (LMB743) growing on the roots of the wheat (*Triticum aestivum*) cultivars Cadenza (left) and Hereward (right) 4dpi. Luminescent activity ([cps]= counts (numbers of photons emitted) per second) is represented by the colours on the 'heat-map' overlay.



**Figure S3 - Variation amongst four wheat landraces for the C4-dicarboxylate exudation phenotype**  
 Mean luminescence values recorded from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of five plants each of both the modern wheat (*Triticum aestivum*) cultivar Paragon and 4 historic landrace accessions (W246, W349, W698, and W811) 7 dpi, presented relative to that of Paragon  $\pm$ SEM. Dunnett's multiple comparison tests comparing individual landrace accessions with Paragon were performed following one-way Analysis of Variance (ANOVA) hypothesis testing where \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$ .



**Figure S4 - Variation amongst wheat accessions for the C4-dicarboxylate exudation phenotype**  
 Mean luminescence values recorded from the *R. leguminosarum* Rlv3841 *lux* fusion biosensor specific to C4-dicarboxylates (LMB614) growing on the roots of four plants each of the modern wheat (*Triticum aestivum*) cultivars Paragon, Hereward, and Cadenza and the historic landrace accession W440 7 dpi  $\pm$ SEM. Here, relative luminescence units represent the integrated density (no. luminescent pixels x mean pixel value) of the luminescent area in each CCD image.