Agricultural impacts on plant beneficial pseudomonads

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This thesis is dedicated to Elaine and Peter Ruscoe

Thank you for your unconditional love and support.

Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisors Prof. Penny Hirsch, Dr Tim Mauchline and Prof. Ian Dodd.

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Abstract

The soil microbiome is a dynamic and complex environment that offers numerous ecosystem services. Beneficial *Pseudomonas* spp. are agriculturally relevant bacteria with a plethora of plant growth promoting (PGP) traits, making them desirable targets for microbial inoculant development. Microbial inoculants have typically failed to produce reliable results, which can be attributed to the introduction of microbes into ecologically unsuitable environments. Its therefore important to better understand factors that can alter *Pseudomonas* spp. community structure and functioning. Crop domestication and land management have both played important roles in the development of agriculture over the last 10,000 years, however they have been associated with negative impacts on the soil microbiome. Here, the impacts of these agricultural components on soil pseudomonads was investigated.

The study of 17 domesticated and ancestral wheat genotypes cultivated in a grassland soil revealed no clear difference in pseudomonad community structure within rhizosphere or bulk soil. The Highfield experiment at Rothamsted Research tests the impact of land management and revealed various impacts to soil properties, wheat physiology and total microbial abundance across grassland, arable and bare fallow managed soils. However, pseudomonad abundance was not found to significantly differ in bulk soil and rhizosphere communities. Additional studies looking at the more closely associated root compartment of wheat grown in soils from distinct land uses, revealed differences in abundance and phylogeny of cultivated pseudomonads. A range of PGP genetic and functional potentials including siderophore production, anti-fungal activity and phosphate solubilisation differed in isolates according to land use. The presence of the 1-Aminocyclopropane-1-carboxylate (ACC) deaminase gene (*acdS*) was of particular interest, due to its potential to reduce levels of stress ethylene in plants by degrading its precursor ACC. Intriguingly, *acdS* gene abundance, phylogeny and functional activity appeared to differ in pseudomonads associated with the different land uses. The rhizosphere and root compartments of wheat had a higher *acdS* gene abundance, particularly in the bare fallow soil which is known to have degraded soil properties. This suggests factors associated with wheat grown in different land managements were driving the selection of ACC deaminase producing pseudomonads. *In vitro* attempts to promote wheat growth under salt stress by applying ACC deaminase-containing isolates was not successful. Overall this thesis evidences the functional potential of pseudomonads for use in microbial inoculants, whilst providing an insight into the complexity of soil-plant-microbe interactions

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List of Abbreviations and Acronyms

1-aminocyclopropane 1-carboxylate (ACC)

1-aminocyclopropane 1-carboxylate (ACCd)

1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP)

2-aminoethoxyvinyl glycine (AVG)

Abscisic acid (ABA)

Adenosine monophosphate (AMP)

Analysis of variance (ANOVA)

Basic Local Alignment Search TooL (BLAST)

Carbon (C)

cAMP receptor protein (CRP)

Cetrimide, Fucidin and Cephalosporin (CFC)

Chrome azurol S (CAS)

Coding sequences (CDS)

Colony Forming Units (CFU)

cis-zeatin (cZ)

Cytokinin (CK)

D-cysteine desulfhydrase (DCyD)

Deoxyribonucleic acid (DNA)

Di-acylphloroglucinol (DAPG)

Dimethylallyl pyrophosphate (DMAPP),

Dworkin and foster (DF)

Ethylene (ET)

Ethylenediaminetetraacetic acid (EDTA)

Fumarate-nitrate reduction regulator (FNR)

Genetically engineered (GE)

Gibberellic acid (GA)

Hasegawa, Kishino and Yano 1985 (HKY85)

Hexadecyltrimethylammonium bromide(HDTMA)

Hours (hrs)

Hydrogen cyanide (HCN)

Inole-3-acetic acid (IAA)

Indole-3-acetamide (IAM)

Indole -3-pyruvic (IPA)

Indole-3-acetonitrile (IAN)

Induced systemic resistance (ISR)

In vivo expression technology (IVET)

Isolation chip (ichip)

Isopentenyladenine (iP),

Isopentenyl transferase (IPT)

Jasmonic acid (JA)

Knockout (KO)

Leucine responsive regulatory protein (LRP)

Luria-Bertani (LB)

Maximum likelihood (ML)

Methyl Acceptor Proteins (MCP)

Multi-locus sequence analysis (MLSA)

Multiple Sequence Comparison by Log-Expectation (MUSCLE)

Nematode associated molecular patterns (NAMPS)

National Center for Biotechnology (NCBI)

Nitrogen (N)

Oil Seed Rape (OSR)

Optimal Density (OD)

Pathogen associated molecular patterns (PAMPS)

Polymerase chain reaction (PCR)

Phosphate buffered saline (PBS)

Phosphorous (P)

Plant growth promoting (PGP)

Pseudomonas Selective Agar (PSA)

Plant growth promoting rhizobacteria (PGPR)

Potassium (K)

Potato Dextrose Agar (PDA)

Rapid Annotation using Subsystem Technology (RAST)

Revolutions per minute (rpm)

Ribonucleic acid (RNA)

S'adenosyl-L-methionine (SAM)

Salicylic acid (SA)

Seconds (s)

Secretion system (SS)

Soil Organic Carbon (SOC)

Soil Organic Matter (SOM)

Single polynucleotide (SNP)

Sustainable development goals (SGDs)

Transfer ribonucleic acid (tRNA)

Transitional model (TIM 012032)

Trans-zeatin (tZ),

Tris-borate-EDTA (TBE)

Tryptamine (TAM)

Tryptone Soya Broth (TSB)

Ultraviolet (UV)

United Nations (UN)

Whole genome sequencing (WGS)

Zeatin riboside (ZR)

1.0 General introduction

1.1 The global food system and its stability

1.1.1 The global food system

The global food system is highly complex and interconnected, with various pressures and interactions that can affect food security. Broadly, five main factors constitute the food system, these are the environment, technology, industry, governance and consumers (Lindgren 2018). The stability of the food system relies on a balancing of these factors, which can each drive impacts at various points along the food chain. Its therefore important when addressing issues of food security, that the wider context is considered with a systems perspective. This comes with its difficulties since food systems have constantly been evolving since the birth of agriculture.

Agriculture began over 10,000 years ago when human civilisation transitioned away from a hunter-gather lifestyle, to living in permanent settlements which centred around farming (Doebley, Gaut, and Smith 2006) . The targeted cultivation of crops selected by humans, consequently gave rise to domesticated descendants which differ in their genetic and phenotypic makeup. The crude selection of desirable aboveground traits has since developed into more precise genetic selections and hybridizations. Thanks to the technological advancements equipping the crop scientist of today, agricultural outputs have been dramatically increased through deliberate genetic breeding (Pingali 2012). Other components of importance have focused on agronomic management strategies to enhance yields, through a combination of increased irrigation, fertilizer and pesticide application. Food productivity is one of the main aspects of achieving food security, but the processing, transport, storage, consumption and disposal of food are also crucial factors when viewing the modern food system.

The development of agriculture is one of the greatest successes for our civilisation, with a

focus on productivity, a yield-centric approach has provided for billions. Despite the clear advantages for human sustenance, the food system can bear negative social, political, economic and environmental implications. Conversely, there are many factors that can have negative impacts to agriculture, a threat that is predicted to become worse, for example with climate change. This brings into question the stability of our current food system and whether it can sufficiently provide for future generations (Lindgren, 2018).

1.1.2 Risks relating to food security

Producing enough food for the world's population is arguably one of the top concerns for food security. With an annual 1.1% population increase yielding an extra 83 million people each year, the global population is expected to reach over 9 billion by 2050 (United Nations 2017). As highlighted in figure 1.1, an increase in population in most continents will mainly be attributed to an aging demographic, with improvements in healthcare and nutrition significantly extending human lifespans. This has obvious implications to food productivity levels, with studies indicating that the world would need to increase crop production by 26%-68% from 2014 baseline levels (Hunter et al. 2017). Such predictions also acknowledge increasing sustainability issues, a demand for crops utilized as animal feed in the meat and dairy industry, along with the production of bio-crops for alternative energy sources and pharmaceutical use (Hunter et al. 2017; Lam 2011).

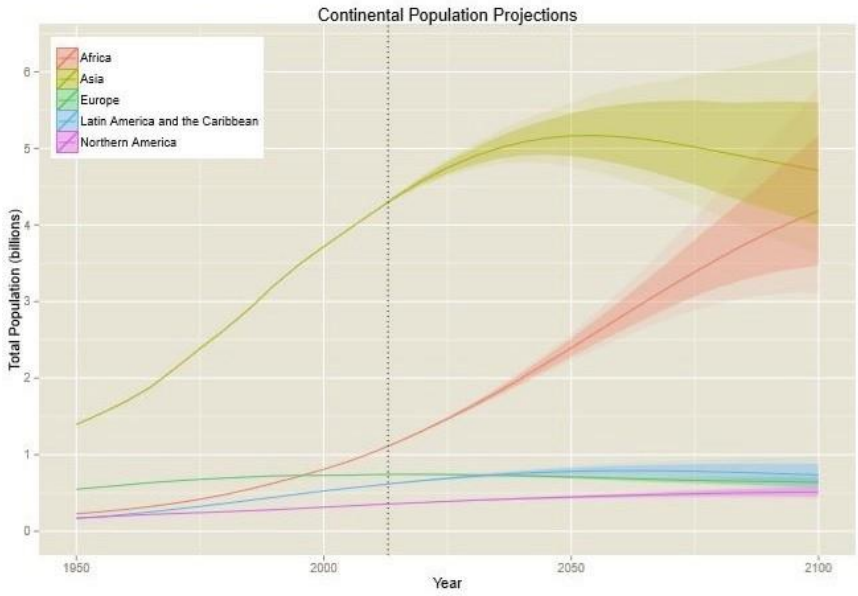


Figure 1.1: UN 2012 population projections by continent: The United Nations population projections from 2012 highlights a growing population, with the highest increases in birth rates occurring in African and Asian continents (Gerland et al. 2014).

Decreases in land allocated for food production is further compounded by soil erosion, which can occur from climatic events, poor agricultural practices and deforestation. A healthy soil is vital for agriculture and greatly benefits crop production through various ecosystem services. Soil degradation however can result in reduced soil fertility, increases in compaction, salinization and acidification which negatively impacts productivity. Additionally, soil erosion affects water security, energy security (bio-crop production), biodiversity protection and can contribute to climate change through destruction of carbon sinks (McBratney, Field, and Koch 2014). Further agricultural contributions to climate change occur through meat and dairy production, which have been shown to produce higher emissions of greenhouse gases compared to crop production (IPCC 2018).

Reducing impacts and adapting to climate change will be vital for agriculture, particularly with predicted increases in the occurrence and duration of drought and flooding events. Future

temperature and precipitation trends have been associated with reduced crop production and yields, with wheat and maize likely to be the most negatively affected (Lobell, Schlenker, and Costa-Roberts 2011). Future crops will therefore need to be resilient to varying climatic stressors. Resilience in ecosystems is commonly associated with higher levels of biodiversity, however our current food system has replaced many native crops, for relatively few staple crops. Additionally, the process of crop domestication has caused a genetic bottlenecking in modern crop varieties, reducing genetic variability when compared to ancestral genotypes (Doebley, Gaut, and Smith 2006). Promoting biodiversity in agriculture is essential, since it preserves genetic variability which can aid in resilience towards various environmental stressors. Additional impacts surround pesticide use, which can affect the diversity of wild plants and animal species, particularly birds and invertebrates. Agricultural intensification has therefore been described as the biggest contributor to biodiversity loss globally (IPCC 2018). An additional concern with the widespread and excessive use of pesticides, is the development of resistance in target pests and pathogens, which creates complications for pest control.

Achieving sustainability whilst competing with increased supply demand is a challenging task. The use of fertilizers is of high importance in crop productivity, particularly in intensive systems that readily deplete soil nutrients without quick replacement. Nitrogen is the primary limiting factor in crop production, however, increased use of fertilizer is pushing the nitrogen cycle beyond its boundaries of sustainability (Stein and Klotz 2016). The production of nitrogen fertiliser via the Haber-Bosch process is very energy intensive and its excessive application to fields can contribute considerably to rising concentrations of the greenhouse gas nitrous oxide (Vitousek et al. 1997). Another concern is the sustainability of mining phosphate rock to produce phosphorous fertilizers. The predicted depletion of global phosphate reserves varies according to different studies, with total exhaustion thought to occur sometime within the next 50-600 years (Sharma et al. 2013; Walther and Kragler 2016). Additionally, both nitrogen

and phosphorous application can have detrimental effects to the environment, particularly when applied in excessive amounts. Leaching and runoff can cause contamination of ground and surface waters, affecting biodiversity as a result of eutrophication in freshwater ecosystems, in addition to reducing drinking water quality (Huang et al. 2017).

It's clear that there are many pressures applied to our global food supply but leading among the dominance of unsustainable agriculture are inadequate or inappropriate policies which include pricing, subsidy and tax policies that have encouraged the excessive, and often uneconomic use of inputs and the overexploitation of land (FAO 1995). Changes in policy could make a real contribution to food security, but a continued need to focus on encouraging scientific innovation, to progress technologically and for stakeholders to work collectively is also required. The achievements of modern agriculture have helped to increase yields, however with a better knowledge of the factors that govern our food system, technology now focuses on increasing efficiency and reducing environmental impacts. Food security, as defined by the United Nations (UN) refers to 'the condition in which all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life' (FAO 1996). Achieving global food security therefore requires a transdisciplinary approach. The UN sustainable development goals (SDG) for 2030, call for urgent action by member states in various areas to recognize the challenges of ending poverty and other deprivations, in addition to improving health and education, reducing inequality, and promoting economic growth, whilst tackling climate change and protecting biodiversity. There are 17 SDGs as seen in figure 1.2, with many of these goals requiring changes in the food system to better achieve sustainability.

Figure 1.2: UN sustainable development goals: A collection of 17 global goals set by the United Nations General Assembly in 2015 for the year 2030, many of which cover issues relating to the global food system (UN 2015).

1.1.3 Sustainable alternatives to conventional farming practices

Addressing issues of sustainability in agriculture is a challenging task, but researchers globally are exploring various options to tackle this issue. Widening the global use of native crop genotypes for cultivation, along with a focus on crop genetics to improve resilience and input efficiency is likely to play a central role of importance for food security (Vaughan, Balazs, and Heslop-Harrison 2007). Genetically engineered (GE) crops make use of biotechnology to introduce new traits that aren't naturally present in a crop genotype. This can include genes used to improve productivity and tolerance to a variety of abiotic and biotic stresses. GE crops have the potential to promote sustainable agriculture through reduction of pesticides, fossil fuels, CO_2 emissions in addition to conserving soil health (James 2011). For example, the mitigation of chemical applications such as broad-spectrum pesticides, has clear toxicological benefits to biodiversity and health. Despite this, GE crops are yet to receive widespread

acceptance with the technology remaining controversial. Reasons for this are complex with fears surrounding both the cultivation and consumption of crops leading to issues with human health, biodiversity loss and the social economic downfall of small hold farmers (Maghari and Ardekani 2011; Pixley et al. 2019).

Another promising area is agroecology, which utilises the natural ecology of a system to encourage ecosystem services that benefit crop production whilst minimising the negative socio-economic and environmental impacts of traditional agriculture (Wibbelmann 2013). For example, focusing on practices which encourage soil health, such as organic matter inputs and minimum tillage for sustainable productivity. Additional examples include integrated pest management, which focuses on understanding pests such as pathogenic microbes, insects, weeds and their interaction with crops. This allows for the deployment of techniques to manipulate pest-host relationships, whilst minimizing economic, environmental and health risks. The main approaches include chemical (e.g. pheromones), physical (e.g. inversion tillage, aeration), cultural (e.g. crop rotations) and lastly biological controls (e.g. natural enemies of the pest) (Trematerra 1997).

A growing area of agroecology is the use of bio-stimulants, which are a class of crop management products that contain substances and/or micro-organisms to stimulate natural processes to enhance nutrient uptake and efficiency, tolerance to abiotic stress, in addition to crop quality (Calvo, Nelson, and Kloepper 2014). Bio-stimulants govern a range of categories, in which classification varies, however Calvo *et al* settled on the following 5; (i.) microbial inoculants, (ii.) humic acids, (iii.) fulvic acids, (iv.) protein hydrolysates/amino acids, and (v.) seaweed extracts (Calvo, Nelson, and Kloepper 2014). These products are gaining increased attention due to the sustainability that they can offer, with market growth rate forecast to increase by around 12% annually (Calvo, Nelson, and Kloepper 2014).

The microbial inoculants category utilizes naturally occurring, soil dwelling microbes such as *Rhizobium* spp., Azotobacteria and *Pseudomonas fluorescens* to help contribute to crop

productivity, stress resilience, crop defence and an environmentally benign system (Bhattacharyya and Jha 2012). Microbial inoculants have immense potential for an increased role in agricultural practices, however they have often yielded inconsistent results (Kinkel 1997; Joyner and Lindow 2000). This lack of reliability has naturally seen interest in bio-inoculants fade. The inherent variability of agricultural systems, in addition to the high complexity of the soil biology means that predicting inoculant use in different agricultural settings is difficult. Therefore, a better understanding of soil microbial communities and their interactions with crops is vital in assessing the real potential for bio-inoculants in future agricultural systems.

1.2. Soil-microbe interactions in agriculture

1.2.1 The microbiome

The microbiome refers to the total microbial community associated with an environment. This includes all bacteria, fungi, archaea and viral species found in different niches such as humans, animals, plants, water and soil. Many consider such host-microbiome relationships a product of evolutionary integration, resulting in the constitution of a 'superorganism' (Salvucci 2019). This extension of the host has been shown to be beneficial, whether that be through promotion of health and development in humans, animals and plants, or the ecosystem services provided by microbes in the environment. Focusing on plant health and development, the transfer of microorganisms from the soil can shape the microbiome present on plant surfaces in addition to plant internal tissues (Cordovez et al. 2019). Both the below and above ground parts of a plant can be influenced by the soil, with numerous niches for microbes to colonise, namely the rhizosphere, endosphere and phllyosphere (Bodenhausen et al. 2013, Frank et al. 2017). Although the focus here is on the soil microbiome and its relationship with the root microbiome of plants, soil microbiology is also of interest to human and animal health. The 'One Health' approach, considers the transfer of both pathogenic and non-

pathogenic microbes between humans, animals, and the environment (Trinh et al. 2018). The food system offers a multitude of opportunities for such transfers to arise, including the transfer of microbes present on the surface of fruits and vegetables into the human gut (Al-Kharousi 2018). Thus, the use of microbial inoculants should be approached with care, since manipulation of the soil ecology for agricultural purposes could have consequences for the human gut microbiome.

1.2.2 Soil heterogeneity

To better understand how to utilise the soil microbiome, the complexity of soil first needs to be considered. The global soil map in figure 1.3 shows the distribution of the 12 soil orders according to the United States Department of Agriculture (USDA), which groups soils with distinct characteristics and ecological significance. The map highlights the scale of soil variability, which can be influenced by many factors including parent material, topology, climate, biota and time (Egli, M et al. 2018).

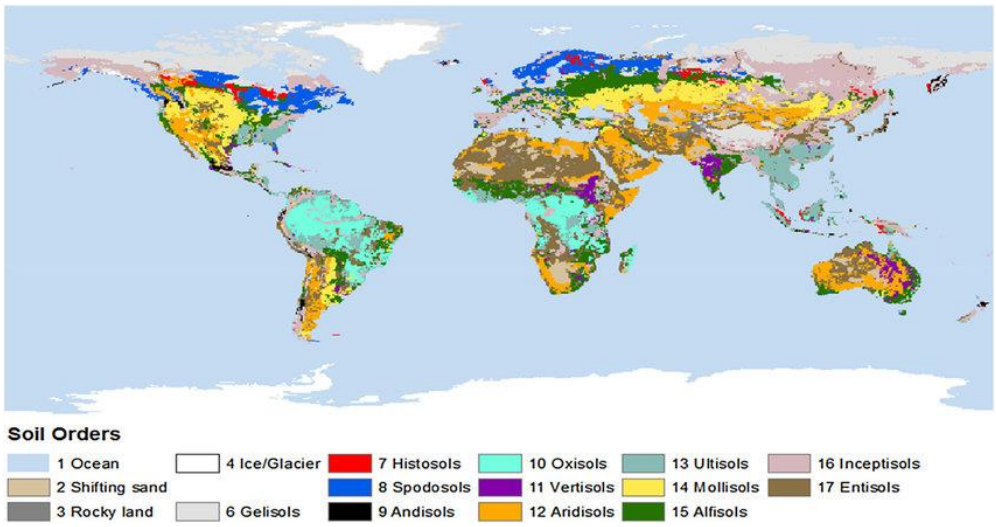


Figure 1.3: The global soil map: The Global soil map is based on a reclassification of the FAO-UNESCO Soil Map of the World combined with a soil climate map. The soil map shows the distribution of the 12 soil orders according to Soil Taxonomy (FAO-UNESCO 2005)

Not only is variability observed laterally across the globe, but a soils vertical profile can also

differ significantly, with layers defined by distinct horizons. Amongst the various ecosystem services soils can provide, perhaps the most important layer of soil from an agricultural perspective is that of the A horizon (topsoil). Here, crop productivity relies heavily on the vast chemical, physical and biological interactions. These properties constantly fluctuate both temporally and spatially with differences observed not only globally, but also regionally and even across the same field (Sun, Zhou, and Zhao 2003). This is because of the complex and non-static nature of soils which can be shaped by numerous environmental and anthropogenic factors, including season and land management. Soil is consequently a dynamic and interconnected ecological system which is incredibly difficult to unravel from both a short and long-term perspective. When trying to manipulate the soil microbiome to enhance agricultural benefits, it's difficult to know what factors encourage or discourage beneficial traits. Various studies have therefore focused on trying to understand how the soil microbiome can differ both structurally and functionally across soils with different properties (Hirsch et al. 2009, Lundberg et al. 2012, Seaton et al. 2019).

1.2.2 The rhizosphere, rhizoplane and endosphere.

A hotspot for microbiome research within agriculture is the rhizosphere. The rhizosphere can be defined as soil which is in close proximity to plant roots and is thereby rich in plant rhizo-deposits (Bakker et al. 2013). The inherent diversity across plant genotypes means that there is no singular definable size or shape for the rhizosphere, rather it is a gradient in chemical, biological and physical properties which change along the root (Fageria and Stone 2006). It is estimated that 5-20% of a plants photosynthetically derived carbon is exuded via the roots. thus providing a rich environment that can attract and sustain a range of soil dwelling organisms (Hutsch, Augustin, and Merbach 2002; Marschner 1995). A plant is therefore capable of partially shaping its own root microbiome via root secretions. To date the rhizosphere is perhaps the most studied niche compartment of importance to plant-microbe-

interactions, however increasing attention is now focused on the rhizoplane (root surface) and the endosphere (internal root) as seen in figure 1.4 (Van der Heijden and Schlaeppi, 2015, Fernández-González et al. 2019). These niches can be considered to host microbial communities that are more closely associated to the root and thus the plant itself. Additionally, endophytes have been found colonising internal tissues in the aerial parts of plants, in addition to the plant surface- known as the phyllosphere (Yao et al. 2019). The soil microbiome and endophytes present in seeds, can migrate over the surface of growing seedlings to then colonise the above ground areas, thus playing a role in shaping the phllyosphere microbiome (Frank et al.2017). Microbes which have co-evolved with plants to enable such colonisation include pathogens, symbionts and commensals alike. Hence, the soil microbiome can have deleterious, beneficial or a neutral impact on plant health.

The concept of the microbiome has received much attention in recent years, with many studies moving away from single organisms, and attempting to identify the structural and functional properties of the soil ecology. Metagenomic analysis has proved extremely insightful, showing clear changes in microbiome structure from bulk soil though to the endosphere (Edwards et al. 2015; Lundberg et al. 2012; Schlaeppi et al. 2014; Gottel et al. 2011). Generally, studies have found that bulk soil comprises lower bacterial species abundance, but higher levels of diversity compared to the rhizosphere and rhizoplane microbiome of different crops; conversely eukaryotic diversity tends to increase in the rhizosphere compared to bulk soil (Turner et al. 2013; Poole 2017). Despite lower bacterial diversity the rhizosphere is more active, with copiotrophic bacteria such as those belonging to the genus *Pseudomonas* and *Bacillus*, being prolific colonisers of the nutrient dense environment (Timm et al. 2015; Pandey and Palni 1997; B-.J. Koo 2005). On the other hand, the slow growing oligotrophs that grow optimally in low nutrient environments, tend to be outcompeted in the rhizosphere due to their low reactivity to nutrient bursts (Lopez-Guerrero et al. 2013). Despite decreases in diversity compared to bulk soil, it is estimated that one gram

of rhizosphere can contain up to 10^4 bacterial species with an abundance of 10^9 bacteria in total (Poole 2017; Turner et al. 2013; Weinert et al. 2011). That is not to forget the diverse archaeal and eukaryotic populations which include fungi, nematodes and mites that also thrive on plant exudates. Conversely, the endosphere has been shown to comprise microbial species in less abundance, with dominant phyla being less diverse compared to bulk soil, rhizosphere and the rhizoplane (Turner et al. 2013). Endophytic bacteria are thought to be a sub-population of the rhizosphere microbiome however some have distinct characteristics, suggesting that a specialized selection takes place (Hardoim, van Overbeek, and van Elsas 2008). This likely includes mechanisms needed to pass the physical barriers of the root and to survive the direct threat of immune attack by the plant. Additionally, endophytic bacteria when inside their hosts may change their metabolism, thereby becoming more adapted to the internal root environment (Monteiro et al. 2012; Compant, Clement, and Sessitsch 2010; Sessitsch et al. 2012)

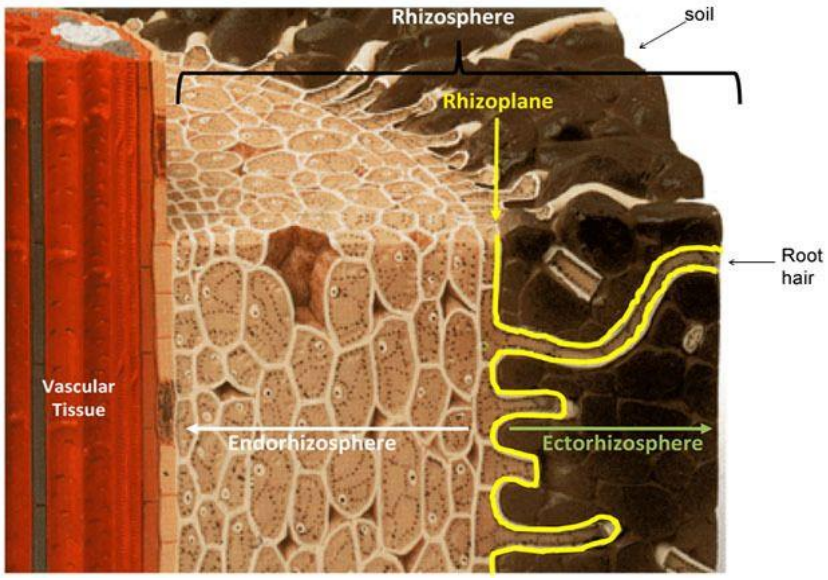


Figure 1.4: Niche compartments of the rhizosphere: Schematic of a root section showing the structure of the rhizosphere. Here the endosphere and rhizosphere as described in the main text is referred to as the endorhizopshere and ectorhizosphere respectively (McNear Jr. 2013).

1.2.2.1 Plant genotype and root architecture

Various studies agree that soil properties appear to be the dominating factor that drive alterations in microbiome assembly, with plant genotype associated with small but significant community shifts (Peiffer et al. 2013; Bulgarelli et al. 2012; Micallef, Shiaris, and Colon-Carmona 2009). Variations in plant exudation concentration and composition, as well as root structure are thought to shape such changes in the microbiome. Firstly, plants exude a variety of compounds including organic acids, amino acids, proteins, sugars, phenolics and other secondary metabolites which are considered low molecular weight compounds, in addition to high molecular weight compounds such as cellulose (McNear Jr. 2013). A portion of these exudates can act as rhizo-attractants, whereas others can act as antimicrobials and quorum sensing inhibitors, thereby excluding certain microbial genera from colonisation. The molecules exuded by plants can differ in addition to the quantity and rate at which they are released. This can be influenced according to plant genotype, developmental stage, edaphic factors and climate (Nuccio et al. 2016).

Root exudates are not the only component of rhizodeposition. The sloughing of root cells and the release of mucilage deposits a large amount of material into the rhizosphere, including plant cell wall polymers such as pectin which can again influence the microbiome (Dennis, Miller, and Hirsch 2010; Turner et al. 2013). Additionally, the physical exploration of roots in soil can also play a role in the rhizosphere and root microbiome. Root architecture can vary greatly between different plant genotypes, which can thereby impact the volume of soil explored. As soil is heterogeneous, the point of contact of a growing root with soil microbes is likely random. Therefore, microbial root colonisation can be limited to temporal and spatial elements of the surrounding soil biota, where rapid proliferation of species occurs from those present and able to capitalise on nutrients being exuded at the time (Pinton 2007). On the other hand, roots better capable of searching for nutrient fluxes or exploring deeper into the

soil for pockets of water are more likely to encounter hotspots of microbial life, thereby indirectly shaping their root microbiome further (Bao et al. 2014). Understanding differences in the structure and stability of soil microbial communities in the rhizosphere and root compartments may offer indicators of plant health in addition to plant disease progression. This is useful information when considering microbial inoculant application and whether such a product will be able to establish, persist and provide a beneficial function to a crop, within a predefined soil ecology.

1.3 Agricultural inputs and the soil microbiome

1.3.1 Fertiliser application

Farming inputs intended to increase crop productivity include fertiliser applications to enhance the nutrient content of soil. Although intended to promote crop growth, such applications can dramatically change soil properties, including the soil microbiome. Various macro and micro nutrients are applied to agricultural land to encourage optimal crop growth, but Nitrogen (N), Phosphorous (P) and Potassium (K) application are perhaps the most well-known. Use of inorganic NPK fertilizers have been associated with increases in soil microbial biomass, which is thought to be a result of increases in soil organic carbon (SOC) promoted through increased plant exudation (Zhu, Vivanco, and Manter 2016). Organic fertilisers are also associated with increased soil microbial abundance, but they can additionally promote increases in species richness, diversity and overall enzyme activity compared to inorganic fertiliser applications (Wang et al. 2016; Garcia-Ruiz et al. 2008; Moeskops et al. 2010). Nutrient fluxes in the soil alter the microbiome both structurally and functionally but perhaps the most well studied application altering the soil microbiology is N fertilisation.

Although N additions to soil have been shown to increase microbial biomass, studies have found negative shifts in the microbiome under high levels of N, with reduced microbial richness and diversity in both the rhizosphere and bulk soil (Kavamura et al. 2018; Dai et al.

2018). Such shifts in community structure can result in the domination of species specialised in multiple aspects of the N cycle. Indeed, the abundance of N-cycling genes have been shown to increase significantly with nitrogen application in a dose-dependent manner (Zhu, Vivanco, and Manter 2016). Denitrifying bacteria tend to be more numerous in soil than any other functional groups involved in the N cycle and can include up to five percent of all soil bacteria (Philippot, Hallin, and Schloter 2007; Clark et al. 2012). High denitrification activity can be of concern due to decreases in soil available N for crop uptake but also due to the release of nitrous oxide-a greenhouse gas contributing to climate change. Increasing N fertilizer applications have been reported to favour abundance and diversity of the denitrifying gene *nirK* in agricultural soils (Jones and Hallin 2010; Smith and Ogram 2008). Although studied extensively, there is currently no unified view on the effects of N application and the soil microbiome due to inconsistent patterns in the negative/positive shifts observed. This is likely due to various factors such as soil type, management regime, crop type, fertilizer application rate, and other biotic and abiotic factors such as soil pH (Hartmann et al. 2015; Lupwayi et al. 2011).

1.3.2 Soil pH

Obtaining an optimal soil pH appropriate for crop genotypes can be vital for food productivity. There are various factors contributing to differences in soil pH, for example acidification can occur as a result of protons (H+) being released during the transformation and cycling of C, N, and other fertilizer reactions (Bolan et al. 2015). Farmers can try to counteract decreases in soil pH through techniques such as liming. Conversely, soil alkalinity can occur from over-liming in addition to alkaline water applied via irrigation systems, but this can be adjusted with applications of sulphur or acidic organic material. Differences in soil pH not only affect crop growth but are a consistent driver in shifts within microbial communities. Various studies have found that changes in β-diversity and in most cases richness across soil microbiomes, can be

partly attributed to soil pH at a national and global scale (Delgado-Baquerizo et al. 2018; Lauber et al. 2009; Fierer and Jackson 2006; Dequiedt et al. 2011). Higher microbial diversity is often observed in neutral pH soils, with lower diversity found in acidic and alkaline soils that select for specialised species capable of adapting to such conditions (Fierer et al. 2007; Buee et al. 2009; Bates et al. 2013). The effect of pH changes on non-specialised bacteria has been shown to induce stress response regulons and alteration in motility, thereby impacting potential plant-microbe interactions. A low pH has been found to accelerate acid consumption and proton export in bacteria, whilst co-inducing oxidative stress and heat shock genes (Maurer et al. 2005). A high pH on the other hand, can accelerate proton import, in addition to repressing flagellar and chemotaxis genes (Maurer et al. 2005). Limitation in bacterial motility under different pH levels in soil, can therefore impact accessibility to nutrient fluxes and impede rhizosphere colonisation.

1.3.3 Soil moisture content

Soil moisture content can vary dramatically in different soils and is an important factor for crop productivity. Depending on the climate, season and precipitation rates of an area, farmers may need to irrigate land to ensure an appropriate soil moisture content is maintained throughout the growing season. The consequences of low water content can result in drought stress, with both short and long periods of drought negatively impacting crop productivity and the soil microbiome alike (Naylor et al. 2017). Perturbations in the environment alter plant physiology and metabolism which can further affect plant-microbiome interactions in the rhizosphere. In general, total bacterial biomass has been observed to decline under periods of drought, with diversity remaining relatively stable (Hueso, Garcia, and Hernandez 2012; Alster et al. 2013; Acosta-Martinez et al. 2014). A recurring finding in various studies is an enrichment of the bacterial taxa Actinobacteria in drought-treated soils across a range of environments (Bouskill et al. 2013; Bouskill et al. 2016;

Kavamura et al. 2013; Taketani et al. 2017). This could be a result of differing life strategies, specifically, the spore-forming ability of Actinobacteria, which allows entry into a stable and quiescent state during periods of environmental stress (Naylor et al. 2017). Interestingly, Actinobacteria are commonly found to hold amino-cyclopropane carboxylate deaminase (ACCd) genes, encoding for an enzyme that breaks down amino-cyclopropane carboxylate (ACC)-the immediate precursor to ethylene (plant stress hormone) (Nascimento et al. 2014). ACC exudation can occur under a variety of stress related processes, such as in drought and salt stressed environments which both reduce soil water availability. ACC can thereby act as a chemoattractant for ACCd bacteria in the rhizosphere under stress related conditions.

1.3.4 Tillage practices

Mechanical tillage of soil is a conventional farming practice used to reduce topsoil compaction and thereby change the structure and aeration of soil for crop production. It can also reduce the need for herbicides as crop residues and weeds are ploughed back into the soil. A growing body of research now suggests that minimising soil disturbance by no till or minimum till practices is sustainably beneficial since it can decrease soil erosion, nutrient runoff and reduce the energy needed to power heavy vehicles used to till the land (DeFelice, Carter, and Mitchell 2006). Compaction of arable soils has been associated with wheel traffic, where heavy machines are used in unfavourable conditions such as when a soil is wet (Hakansson 1994). Such practices are usually associated with increases in subsoil compaction whereas no tillage can see increases in topsoil compaction along with increased herbicide use (Gersmehl 1978; Hakansson, Voorhees, and Riley 1988). Soil properties can change vastly between no till and tilled soils and so too this can alter microbial communities through habitat modifications including pore space, the loss of connectivity of species and the disruption of physical networks of nutrient passage (Young and Ritz 2000). When looking at the soil microbiome, no-till farming practices have been shown to result in higher microbial biomass within the top soil

layer when compared to standard tillage (Govaerts et al. 2007; Madejon et al. 2007; Liu et al. 2016). Additionally, bacterial community structure can change with tillage, shifting microbial communities towards fast growing copiotrophs, while no-till practices have been found to support slow growing oligotrophs (Schmidt et al. 2018). This is likely due to nutrient fluxes released under tillage, which copiotrophs quickly exploit. Additionally, the diversity of bacterial species has been shown to increase under no till practices, whist conventional tillage appears to reduce diversity in line with a selection for copiotrophic bacteria (Schmidt et al. 2018).

1.3.5 The Highfield experiment

The Highfield experiment at Rothamsted Research UK, is a long-term experiment initially set up to look at the effects of converting permanent grassland into arable (1949) and bare fallow (1959) on soil properties. The bare fallow plots have been maintained plant-free for over 50 years by regular tilling and the occasional use of herbicides, whilst the arable plots have been cultivating winter wheat for over 60 years receiving regular NPK inputs in addition to herbicides, liming and tillage. Marked declines in C, aggregate structure and microbial and mesofaunal abundance has been demonstrated in the bare fallow and arable plots compared to the grassland treatments (Coleman et al. 1997; Watts et al. 2001; Hirsch et al. 2009) . Additional findings include declines in soil pH, N and P in both arable treatments and bare fallow compared to continuous grassland. Although the long-term effects on SOC across contrasting treatments was apparent, little was known on the short-term changes of SOC. In 2008, reversion subplots on previously managed permanent grassland, arable and bare fallow treatments at the Highfield experiment were each converted into the two alternatives, to better understand the short-term effects of land change. Four years after conversion, SOC in arable and bare fallow soils converted to grassland had increased significantly, whilst decreasing in plots converted to bare-fallow and arable from the previous grassland treatment

(Hirsch et al. 2017). The Highfield experiment is a valuable resource to investigate the impacts of land use intensity on the soil microbiome, independent of soil type and climate.

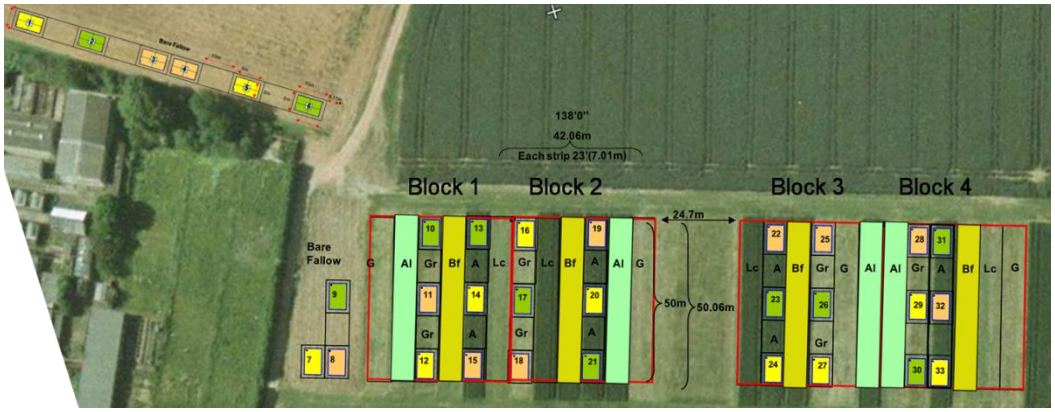


Figure 1.5 The random block design of the Highfield experiment- Rothamsted research

The Highfield experiment at Rothamsted Research is split into four blocks, with individual plots randomised within this block design. Additional bare fallow plots were established in 1959 to the left of the four block design, along with the plots in the Geescroft field (far left).

1.4.0 Plant and microbial phytohormones

Root secreted phytohormones are increasingly being realised as potential rhizosphere attractants for the associated soil microbiome, although to date studies have mostly focused on carbon sources and other exudates that attract microbes. Since plants are sessile organisms, they are required to rapidly perceive and adapt to changing environmental conditions. This utilises complex signalling systems, which elicit various adaptations to help combat abiotic and biotic stresses or encourage optimal growth and development when favourable conditions arise (Egamberdieva et al. 2017). Phytohormones modulate multiple physiological and biochemical processes at low concentrations and thereby play a critical role in enabling a plant to adapt to such changes. There are five “classical” hormones including auxins, abscisic acid (ABA), gibberellins (GA), cytokinins (CK) and ethylene (ET) (Li, Li, and Smith

2017) with more recent characterisation of other compounds including brassinosteriods, jasmonates, salicyclic acid and strigolactones (Li, Li, and Smith 2017). Phytohormones play multiple physiological roles *in planta*, with effects varying across plant species, developmental stages and environmental conditions. Despite generalised functions associated with individual hormones, the importance of crosstalk between these compounds on the whole plant system is now becoming more apparent. Typically, the five classical phytohormones have been characterised as either growth promoters or growth inhibitors as shown in figure 1.6. To combat various environmental stresses, phytohormone engineering could be a potential to improve crop productivity since they are key regulators of plant growth (Wani et al. 2016). Various microbial species also produce phytohormones as secondary metabolites and are therefore a focus for microbial inoculant development, as well as targets for fermentation processes to synthesize phytohormones on a large scale (Egamberdieva et al. 2017).

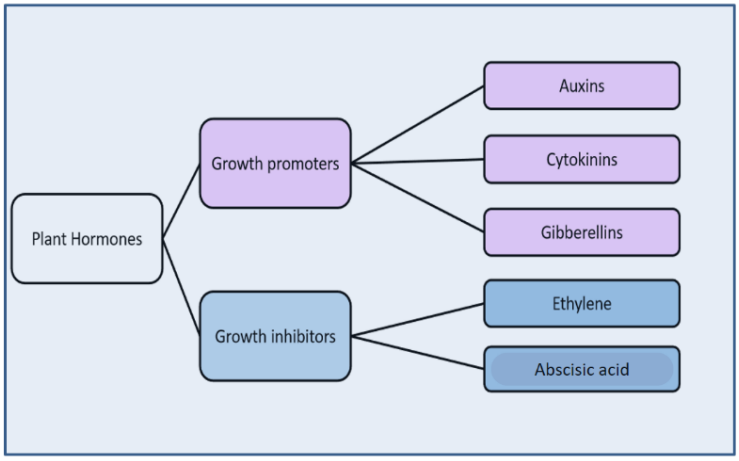


Figure 1.6: Classical plant hormones as growth promoters or growth inhibitors. Generally, phytohormones have been characterised for their activity as either promoters or inhibitors of plant growth. Despite this, new research suggests that expanded roles exist beyond these singular characterisations.

1.4.1 Auxins

Auxin was the first phytohormone to be identified within plants where its importance in the signalling of leaf curvature towards light (phototropism) was confirmed (Whippo and Hangarter 2006). Auxins are generally known to promote growth and development and include compounds such as indole-3-butyric acid, phenyl acetic acid, and 4-chloroI acetic acid, however the best characterised compound is the primary auxin, indole-3-acetic acid (IAA) (Ma, Grones, and Robert 2018). IAA is associated with various functions, influencing cell division, cell elongation and cell differentiation, in addition to greatly impacting the final shape and function of cells and tissues in higher plants (Ljung 2013). There are at least five known biosynthesis pathways of IAA, including the indole-3-acetamide (IAM) pathway, the indole -3-pyruvic (IPy) pathway, the indole-3-acetonitrile (IAN) pathway and the tryptamine (TAM) pathway which all utilize the amino acid precursor tryptophan. Enzymes utilized in each of these pathways have been observed in various microbial species (Spaepen and Vanderleyden 2011b). A fifth pathway that is independent of tryptophan utilisation has been identified-however the genes and proteins involved in this pathway have yet to be determined (Woodward and Bartel 2005). This is more commonly associated with plants, however it has been suggested that a strain of *Azospirillum brasilense* is capable of producing IAA via this pathway (Prinsen et al. 1993; Spaepen and Vanderleyden 2011b).

It has been assumed that >80% of cultured rhizosphere bacteria can synthesize IAA, often resulting in a genetic redundancy of IAA biosynthesis in some microbes (Patten and Glick 1996). Although a large majority of microbes appear capable of utilizing the IPY, TAM and IAN pathways, phytopathogens are better characterised for auxin production via the IAM pathway. Phytopathogens such as *Agrobacterium* spp. and *Pseudomonas syringae* pathovars contain genes on virulence plasmids for tryptophan-2-monoxygenase (*iaaM*) and indole-3-acetamide hydrolase (*iaaH*)- key enzymes in the IAM pathway (Aragon et al. 2014). Generally, these pathogens produce high levels of IAA and specific type 3 secretion system effectors such as

AvrRpt2, which can disturb normal plant auxin levels causing tumour and gall formation that contributes to their pathogenicity (Cui et al. 2013).

IAA is often reported as a PGPR trait capable of promoting crop growth (Idris et al, 2007). Conversely, there is evidence that IAA production does not necessarily exert positive effects on root architecture. Dobbelaere *et al.,* showed that wild type IAA-producing strains of *A. brasilense* Sp245 and Sp7 when applied to wheat resulted in a strong decrease in root length but an increase in root hair formation. (Dobbelaere et al. 1999). However, applying KO mutants of these strains (disruption of the *ipdC* gene encoding for indole-3-pyruvate decarboxylase-a key enzyme in the IPy pathway) increased wheat root length and decreased root hair formation (Dobbelaere et al. 1999). This indicates that efficient concentration of bacterial auxin may be in a narrow range (Schwachtje et al. 2012). Overall, microbial IAA production and its effect on plant physiology is varied, this seems to depend on various factors including the pathway of biosynthesis, the concentration of auxin produced and in the case of phytopathogens, certain effector proteins which further alter the balance of plant IAA.

1.4.2 Cytokinins

Cytokinins were first discovered when an adenine derivative, known as 6-furfurylaminopurine, was found to stimulate the proliferation of cultured tobacco pith cells in the 1950s; the molecule was named kinetin (Kieber 2002; Miller et al. 1955). This study inspired the search for a naturally occurring molecule similar in activity, which was later found in the extract of immature endosperm of *Zea mays* in 1960 (Kieber 2002). This compound was identified and named Zeatin in the 1970s which is now known as *trans*-zeatin. In addition to the role that CKs play in cell proliferation, these molecules promote differentiation of plant cells, delay senescence, control root/shoot balance, increase crop productivity and are involved in the transduction of nutritional signals (Eckardt 2003). Following the initial discovery of trans-zeatin, numerous molecules have since been characterised for their CK activity. Naturally

occurring CKs are adenine derivatives, which can have either an isoprene derived or an aromatic side chain, known as isoprenoid CKs and aromatic CKs respectively (Kaminek et al. 2000). In both groups the physiological significance of small variations in these side chains has not yet been fully determined (Kieber 2002). Originally it was thought that CKs were only produced in the roots and then translocated to other areas of the plant via the xylem; more recently it has been shown that CKs can be synthesized in aerial parts of the plant and transported to the root through the phloem (Kudo, Kiba, and Sakakibara 2010).

Since CKs exhibit long-distance communication, it's possible that CK producing bacteria can influence plant physiology in different tissues. Perhaps the best studied bacterial CKs are those produced by phytopathogens, in which the phytohormone has been suggested to alter host physiology to facilitate maximum access to nutrients during early interactions (Grosskinsky et al. 2011). Alteration of CKs by microbes and insects have also been identified to cause green island formation, galls, growth abnormalities and modulation of primary carbon metabolism (Lara et al. 2004; Morris et al. 1991; Grosskinsky et al. 2011). Conversely, other studies have looked at CK production in beneficial bacteria and identified multiple CKs within cell free cultures in addition to growth promotion when applied as inoculants (Garcia de Salamone, Hynes, and Nelson 2001; Sturtevant and Taller 1989; Patel and Saraf 2017). Despite this, the elucidation of CK biosynthesis and regulation pathways in bacteria is currently not well characterised, with few studies directly showing CK bacterial-plant interactions *in planta.* Großkinsky *et al* studied cytokinin-deficient mutants of beneficial *Pseudomonas* spp. G20-18, which exhibited impaired biocontrol activity against the pathogen *Pseudomonas syringae* in *A. thaliana* when compared to its wildtype (Grosskinsky et al. 2016). The authors restored biocontrol function within the G20-18 strain by re-introducing cytokinin biosynthetic genes into the bacteria. This study highlights the inoculant potential for CK producing PGPRs, however it's clear that more research is needed to confirm the role of such bacteria in promoting crop benefits.

1.4.3 Gibberellins

The function of gibberellic acid (GA) was initially discovered in 1912 as a secondary metabolite produced by the pathogenic fungi *Gibberella fujikuroi,* which causes *Bakanae* or foolish seedling disease in rice plants (Salazar-Cerezo et al. 2018). Notably, the pathogen was responsible for inducing uncontrollable growth in rice, in which the plant eventually falls under its own weight resulting in death (Sawada, 1912). It wasn't until the 1930s, that the compound from fungi was purified and thereby received the name of Gibberellin (T. Yabuta 1938). GAs were later found to be ubiquitously present in higher plants where they can have various physiological effects. Perhaps their most well-known function is the stimulation of organ growth through enhancement of cell elongation and in some cases cell division, but they are additionally involved in processes such as starch hydrolysis during germination and fruit maturation (Hedden and Thomas 2012). Collectively, there are 136 GAs currently identified. However the true number is likely to be higher since the low abundance and difficulty in chemical characterisation can make identification of new GAs a rarity (Hedden and Thomas 2012).

Gibberellins have also been found in bacteria thought to offer plant growth benefits. The production of GAs and growth promotion by bacteria in plants is still very much in its infancy compared to other phytohormones, with few studies highlighting plant benefits. Symbiotic nitrogen-fixing and legume-associated rhizobia, including *Bradyrhizobium japonicum* and *Sinorhizobium fredii* contain a putative GA biosynthetic operon which encodes the enzymes necessary to produce GA9 (Nett, Dickschat, and Peters 2016). Rhizosphere and endophytic bacteria such as *Enterobacter ludwigii* GAK2 and *Bacillus amyloliquefaciens* RWL-1 both isolated from rice, also produce various GAs in different quantities (Shahzad et al. 2016; Gutiérrez-Mañero et al. 2001; Lee et al. 2019). When these bacterial strains were applied as inoculants to rice plants, plant growth increased. Additionally, Shahzad *et al*, found significant

up-regulation in plant endogenous GA1, GA4, GA7, and GA9 compared to the positive (chemical GA3 application) and negative (water) treatments (Shahzad et al. 2016). It's unfortunate that the growth promotion results obtained in both of these studies are slightly undermined by a lack of appropriate controls, in which the negative standard of water is not comparable to a bacterial culture that can provide nutrients and stimulate a multitude of signalling responses within plants. Although it is interesting that non-pathogenic bacteria are able to produce GAs, more research is clearly needed to identify the role of GAs interacting with the plant phytohormone system and potential growth benefits.

1.4.4 Abscisic acid

The discovery of abscisic acid (ABA) occurred in the 1960s, where it was first linked to leaf abscission in fruits and leaves (although it was later discovered that the primary signal regulating leaf abscission initiated in most plants is by ethylene, not ABA) (Fosket 1994). Nevertheless, the phytohormone is involved in several important aspects of plant growth and development including the initiation of seed and possibly bud dormancy, the control of stomatal closure and the initiation of senescence (Fosket 1994). Unlike the phytohormones discussed previously, ABA is often associated with plant stress, as it is synthesized in response to decreased cell turgor under a variety of environmental stresses such as drought, salinity and extreme temperatures. Root-sourced ABA is considered a long-distance chemical signal that triggers physiological responses, including stomatal closure and decrease in leaf growth for example in response to soil drying (Tardieu, Lafarge, and Simonneau 1996; Zhang and Davies 1990). Conversely, shoot sourced ABA can regulate physiological responses in roots (Holbrook et al 2002, McAdam et al 2016). Again, this makes microbial species capable of ABA production and colonising the root potential modulators of physiology in aerial parts of the plant.

As with GAs, the production of ABA by bacteria is not as extensively studied compared to other phytohormones. The first discovery of microbial production of ABA occurred in 1977 in *Cercospora rosicola* (Assante et al. 1977), since then there have been other examples of fungi producing ABA, namely those that are plant pathogens. Elevated ABA levels in rice plants have been associated with increased disease severity of rice blast caused by the fungus *Magnaporthe oryzae* as well as bacterial blight caused by *Xanthomonas oryzae* (Spence et al. 2015; Koga, Dohi, and Mori 2004; Jiang et al. 2010; Xu et al. 2013). Spence *et al* reported the biosynthesis of ABA by *M. oryzae* in knockout mutants was impaired and thus unable to form lesions on rice compared to the wildtype (Spence et al. 2015). It is thought that endogenous fungal ABA affects plant defence by acting antagonistically on salicylic acid, jasmonic acid, and ethylene, which are key signals required in plant immune responses. Belimov *et al* isolated ABA metabolising bacteria, which increased shoot growth and decreased primary root growth (Belivmov *et al* 2014). Despite this, more research is required to better elude to a clear role for microbial ABA metabolism in promoting beneficial plant-microbe interactions (Shahzad et al. 2017).

1.4.5 Ethylene

Ethylene is a simple hydrocarbon present in gaseous form within plants, making it freely able to diffuse across membranes (Bleecker and Kende 2000). Unlike other phytohormones, it is thought to be synthesized at or near its site of action (Dongdong Hao 2017). The biological activity of ethylene was first discovered within illuminating gas, which caused premature senescence and defoliation of plants in greenhouses and trees near gas lines; in 1901 the active component in illuminating gas was identified as ethylene (Abeles F. B. 1992; Schaller and Kieber 2002). The phytohormone regulates many aspects of plant life including seed germination, root initiation, root hair development, flower development, sex determination, fruit ripening, senescence, and responses to biotic and abiotic stresses (Lin, Zhong, and

Grierson 2009). Although ethylene is produced by cells during various stages of plant development, the highest rates of ethylene production are associated with meristematic, stressed, or ripening tissues, which makes the manipulation of this hormone interesting in agricultural applications (Abeles F. B. 1992). Similarly to ABA, ethylene is most studied for its role as a growth inhibitor under plant stress responses, but evidence is accumulating that ethylene can also promote growth. Pierik *et al* proposed a biphasic model of ethylene, with low levels of ethylene promoting growth and high levels inhibiting growth (Pierik et al. 2006). The exact range of stimulatory or inhibitory concentrations of the hormone is thought to be a result of environmental conditions, internal signals (e.g. other hormones) and species-specific characteristics (Pierik et al. 2006). This means that modulation of ethylene by microorganisms could be in a narrow range and needs to be extensively studied before release within a bioinoculant.

Along with IAA, modulation of plant ethylene by microorganisms is commonly cited in the literature for potential PGPR traits. Although some microbes produce ethylene, the enzyme amino-cyclopropane carboxylate deaminase (ACCd) has been most studied (Cristescu et al. 2002). ACCd can modulate plant ethylene levels through degradation of amino-cyclopropane carboxylate, which is the immediate precursor to ethylene in its biosynthesis pathway (Honma and Shimomura 1978). Various microorganisms possess the ACCd structural gene *acdS*, however to enable optimal activity the regulatory gene *acdR* is thought to be essential (Grichko and Glick 2000a). It is thought that microorganisms utilize plant ACC as N and C sources, which gives a competitive advantage over non-ACCd producers in the rhizosphere. Indeed, enrichments of ACCd bacteria surrounding the root have been observed, particularly under stressed conditions such as that imposed from drought, where plant ACC exudation may be higher (Nascimento et al 2014, Timmusk et al. 2011). Microbial inoculation with ACCd-containing bacteria has alleviated the growth inhibition stimulated by plant stress (Penrose

and Glick 1997; Ali and Kim 2018). The root colonizing bacteria *Pseudomonas putida* GR12-2 and *Pseudomonas* sp. UW4 were no longer able to promote canola root elongation after their *acdS* gene was knocked out (Li et al. 2000; Glick et al. 1994). Additionally, Chen *et al* inoculated ACCd producing *Variovorax paradoxus* 5C-2 onto *A. thaliana* wild type and several ethylene-related mutants (*etr1-1, ein2-1* and *eto1-1)* (Chen et al. 2013). Bacterial inoculation promoted leaf area and shoot biomass along with enhanced floral ignition of wild type plants by 2.5 days, whilst inoculation of the ethylene insensitive mutants displayed no growth promotion after inoculation with *V.paradoxus* 5C-2. Despite these results, it remains unclear whether application of ACCd bacteria could help alleviate symptoms of plant stress in the field. What does appear to be more certain, is the influence of plant ACC exuded from roots on the structure of the soil microbiome, particularly under environmental factors causing plant stress (Bouffaud *et al* 2018).

1.5.0 *Pseudomonas fluorescens*: a candidate for microbial inoculants

1.5.1 The importance of the *Pseudomonas* spp. genus

The taxonomic class of the gamma proteobacteria comprises a multifarious and large repertoire of environmentally and medically important bacterial members, isolated from a range of environments. The genus *Pseudomonas* of the family Pseudomonadaceae is taxonomically very diverse and the classification of species within this genus has a complex history. The number of species being assigned to the genera grew rapidly to an unmanageable number, until the 1960s-80s when reclassification of the *Pseudomonas* genus began with the advent of biochemical characterisation and DNA technology (Stanier, Palleron.Nj, and Doudorof.M 1966; Palleroni 1984). Members that are still classified as belonging to *Pseudomonas* include an array of functionally diverse bacterial species, which have been isolated from various environmental habitats including soil, water, animals, insects and

humans. Some isolates have been found in the low temperatures of Antarctic ice in addition to the high-altitude soils of Gangotri (Dziewit et al. 2013; Kumar et al. 2019). *Pseudomonas fluorescens* is best known agriculturally for the beneficial role it can play in promoting crop growth and health. Although in rare occasions it has been implicated in human infections in addition to being identified at low levels in the indigenous microbiota of the human body (Scales et al. 2014). *P.fluorescens* species have been found to perform a range of benefits when viewed in terms of their plant inoculant potential including bio-fertilization, bio-regulation, bioremediation and bio-control. Figure 1.7 highlights the genetic complexity of this species, where Garrido-Sanz *et al* found 9 subgroups through phylogenetic analysis, they named this the *P. fluorescens* complex (Garrido-Sanz et al. 2016).

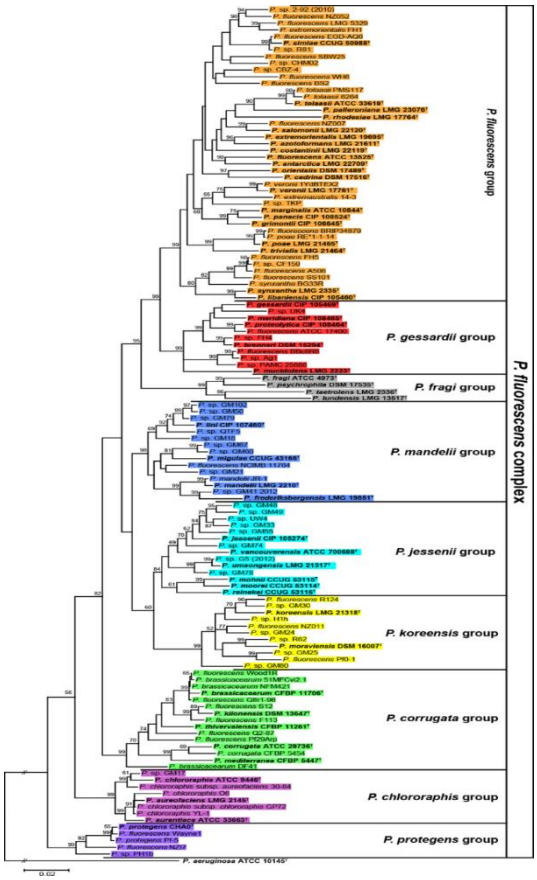


Figure 1.7. Phylogeny of the P. fluorescens complex inferred by MLSA: A phylogenetic tree of 127 sequenced and type strains belonging to the *P. fluorescens* complex based on concatenated partial sequences of the 16S rRNA*, gyrB, rpoD* and *rpoB* genes (Garrido-Sanz et al. 2016).

1.5.2 Efficient colonisation of the root and rhizosphere

Although PGPR traits are of importance when formulating microbial inoculants, bacterial candidates must also be capable of establishing themselves in the species rich and competitive rhizosphere. An appropriate population density and close vicinity to roots of the target crop is needed to achieve growth benefits. *P.fluorescens* strains are abundantly present within rhizosphere soil and can also be found on both the root surface and in endophytic compartments; however, colonisation ability can vary across strains and under different environmental conditions. Flagella can be considered as an early stage colonisation factor, facilitating a bacterium to actively seek out favourable conditions such as in the rhizosphere (Rossez et al. 2015). Alsohim *et al* (2014) found that flagella presence is essential for horizontal root surface migration by *P. fluorescens* SBW25, but the bio-surfactant viscosin which increases surface spreading over the plant root is also required for optimal colonisation (Alsohim et al. 2014). In addition to motility, positive taxis towards a chemoattractant is also needed, this occurs via stimulation of the sensory Methyl Acceptor Proteins (MCPs), which are responsible for the detection of various chemotactic ligands. Stimulation of MCPs in turn results in the expression of *Che* genes, which can initiate flagella assembly and alter flagella rotation, playing an important role in the 'run' and 'tumble' features that direct bacteria towards chemo-attractants (Magariyama et al. 2005). A study by Muriel *et al* highlights this with competitive assays of the wild type *P. fluorescens* F113, against its motile but non-chemotactic *CheA1* mutant (Muriel et al. 2015). They found that the *CheA1* mutant was displaced from the rhizosphere, indicating the importance of the chemotactic system for colonisation. *P. fluorescens* strain F113 upon rhizosphere colonisation from bulk soil, has also been found to undergo a genetic phase variation in response to environmental signals, which in turn stimulates hypermotility. These variants usually harbour mutations in the Gac two-component system (Rivilla 2013). In contrast, other studies have found that motility has an

insignificant role in the movement from bulk soil towards roots, suggesting that motility serves a more prominent role in the movement along roots, perhaps due to a hindrance from soil structure (Toyota and Ikeda 1997). In addition to motility and growth density, adherence to seeds or to plant roots is thought to be an important factor, particularly for retaining microbial species on the seed after inoculation. Certain bacterial species can achieve this through flagella mediated adherence or from the presence of pili (Rossez et al. 2015).

The ability to detect and utilize a range of root exudates, naturally plays some role in determining rhizosphere competence. Various *P. fluorescens* strains have been shown to metabolise a range of different carbon sources, highlighting their ability to colonise a variety of different plant species (Simons et al. 1997; Timm et al. 2015). As pseudomonads are known copiotrophs, opportunistic increases in population density within the rhizosphere may play a beneficial role in outcompeting other microbes. Additionally, siderophores which have a high affinity for iron and are produced by various *Pseudomonas* strains, may be of importance in rhizosphere competence through the chelation of insoluble iron and its sequestration (Sharma and Johri 2003). Iron is a vital nutrient and is utilised in various biological processes; its sequestration by *P. fluorescens* therefore puts the bacterium at a competitive advantage in conditions of low iron availability. Similarly, various *Pseudomonas* spp. have been shown to utilise plant ACC and IAA as a carbon and nitrogen source as previously discussed (Scott, Greenhut, and Leveau 2013). This can additionally be an advantage for colonisation, particularly under stressed conditions in which the exudation of plant ACC is associated with. Although particular traits may be deemed as beneficial for colonisation, the context of the environment which promotes the expression of such traits must be appreciated before advantages in colonisation may be seen.

1.5.3 Biocontrol

Aside from indirectly competing with pathogens for nutrients, *P. fluorescens* has the ability to

actively compete with pathogens within the rhizosphere by producing antimicrobial secondary metabolites, such as hydrogen cyanide, phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol (DAPG) (Siddiqui et al. 2006; Mavrodi et al. 2007). By dominating the rhizosphere and suppressing pathogen colonisation*, P. fluorescens* can play a positive role in maintaining plant health against pathogenic invaders, hence the interest for its use in bio-control agents (Frapolli et al. 2012). For example, take-all disease which is caused by the soil borne fungus *Gaeumannomyces graminis* var. *tritici* is one of the most important root diseases of wheat worldwide. Suppression of this fungus in a phenomenon referred to as take-all decline, can be defined as the "the spontaneous decrease in take-all incidence and severity induced by continuous monoculture after a severe outbreak of the disease"(Weller et al. 2002; Weller et al. 2007). Pseudomonads have been associated with the natural suppression of fungal take-all disease in wheat, but only after a period of yield losses that can last between 4-6 cropping seasons. Some studies have directly correlated take-all decline to the build-up of *P. fluorescens* strains that produce the broad-spectrum antibiotic 2,4-DAPG (Raaijmakers and Weller 2001, 1998; Weller et al. 2007).

PGPRs are also known to interact more directly with the plant immune system to cause an induced systemic resistance (ISR); in which priming of the plants immune system produces a weak, transient and localized defensive response. This later provokes an enhanced expression of immune defence-related genes upon pathogenic recognition (Pieterse et al. 2014). The mechanisms as to how beneficial rhizobacteria induce ISR yet evade the host immune response are not well understood. There is a clear correlation between *P. fluorescens* colonisation and ISR with plant production of the phytohormones jasmonic acid and ethylene, which leads to activation of transcriptional co-regulator proteins important for switching on genes involved in immune defence (Wu et al. 2012). Additionally, Spence *et al* found that *P. chloraphis* EA105 reduced virulence of the pathogenic fungi *M. oryzae* by mechanisms that appeared to counteract the effects of pathogenic ABA biosynthesis (Spence et al. 2015). As

previously discussed, ABA production is often seen in pathogenic fungi and contributes to virulence. Other studies have focused on uncovering the mechanisms of plant immune evasion by colonising rhizobacteria. The possession of type III secretion systems (SS) found in beneficial *P. fluorescens* have been suggested to dampen the plant immune response after Microbial Associated Molecular pattern (MAMP) detection. This involves the injection of effector proteins that can modulate plant signalling (Mavrodi et al. 2011). It's clear that there are various ways in which beneficial PGPR can interact with plants to help in biocontrol of pests and pathogens, with various examples of such traits commonly found in pseudomonad strains.

1.5.4 Bioregulation

Many variations have been observed in the physiological responses of plants to different environmental stresses, this can make some plant species naturally more tolerant to stress than others. As previously discussed, phytohormones are produced by plants and function as regulators in crop growth. PGPRs capable of the production and metabolism of phytohormones are therefore of interest as potential bioregulators of plant physiology. So far, the only phytohormones characterised in pseudomonads are IAA, ACCd and cytokinins, with a lack of evidence to support GA and ABA production to date. The roles of these phytohormones have previously been discussed in section 1.4. Although more commonly identified biochemically in bacteria, studies have begun to characterise the genes involved in the biosynthesis of these phytohormones. ACCd was first characterised in *Pseudomonas putida* UW4, with the structural gene *acdS* and regulatory gene *acdR* found to be essential for the optimal functionality of the enzyme (Grichko and Glick 2000a). Identification of genes encoding for IAA production has proven to be more difficult, since there are 5 different biosynthesis pathways. It's thought that multiple IAA pathways exist within a single bacterium, in which a complex multi-route system protects against the loss of one particular pathway, via

the option of an alternate circuit (Lehmann et al. 2010). Duca *et al* studied *P. putida* UW4, and identified amino acid sequences *nit* and *nthAB* encoding for the enzymes indole-acetonitrilase and nitrilase respectively; both are involved in the IAN biosynthesis pathway (Duca, Rose, and Glick 2014). Studies have also suggested some PGPR pseudomonads have IAA genes more commonly associated with other bacterial species or pathogenic pseudomonads, such as the gene *ipdC* encoding for indole-pyruvate decarboxylase in the IPA pathway and *iaaM, iaaH* genes utilized in the IAM pathway (Patten and Glick 2002b; Kochar, Upadhyay, and Srivastava 2011). The genes involved in CK biosynthesis in bacteria are not well characterized, with the only known biosynthetic gene in *Pseudomonas* strains being *miaA*, encoding for tRNA delta (2)-isopentenylpyrophosphate transferase (Grosskinsky et al. 2016). Although pseudomonads possessing the ability to modulate plant hormone levels are of interest for inoculants, there is a need to better characterise pathways of biosynthesis further, whilst understanding the range in which bacterial phytohormones effect plant physiology under different variables.

1.5.5 Biofertilisation

Accessibility to nutrients can greatly benefit plant growth and one of the main ecosystem services provided by soil microorganisms is the cycling of nutrients. Pseudomonads capable of indirectly enhancing plant nutrition through the solubilisation of nutrients in soil are therefore of interest for inoculant development. Phosphorus (P) is an essential nutrient required by all living organisms and although organic and inorganic P is abundant within soils, the total P that is available for plant uptake is restricted due to poor solubility and P fixation, this can markedly reduce plant size and growth (Sharma et al. 2013). The ability to mineralize insoluble organic phosphates such as phytates, phosphomono-, di- and tri-esters and organophosphonates via the actions of various phosphatases, phytases and phosphonatase enzymes, has been studied in several *Pseudomonas* species (Rehm 2008). Miller *et al* studied

various *Pseudomonas* spp. and their ability to liberate soluble phosphate from the insoluble $Ca_3(PO_4)_2$ as the sole phosphate source and glucose as the sole carbon source (Miller et al. 2010). All *Pseudomonas* strains could solubilize $Ca_3(PO_4)_2$, however four of the *P. fluorescens* strains (Pf-5, CHA0, Pf153 and F113) were observed to be the most effective. When testing *in planta*, Samavat *et al* (2012) found that co-inoculation of *P. fluorescens* strains with *Rhizobium* spp. saw an increase in phosphate which correlated with an increase in plant growth and yield (Samavat et al. 2012). There is also some evidence to suggest that the production of siderophores is effective in nutrient solubilisation, by solubilising phosphate from the inorganic mineral $FePO_4$ (Ghosh, Rathinasabapathi, and Ma 2015). Utilisation of PGPRs capable of solubilising P would be of great benefit since it could improve efficiency of P fertilization, thereby increasing crop growth and reducing P application to help conserve phosphate rock reserves.

1.5.6 Bio-remediation

Bio-remediation is an *in-situ* technology for the clean-up of environmental pollutants that utilizes biological organisms such as plants or microorganisms. Soil and water can be contaminated with various toxic compounds particularly as a result of the rapid growth of industrialization and the use of aromatic compounds in dyestuffs, explosives, pesticides and pharmaceuticals (Singh and Jain 2003). Aromatic hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and their derivatives are highly toxic, mutagenic and/or carcinogenic to surrounding biology and also present health concerns for humans (Pashin and Bakhitova 1979). Microbes capable of degrading such toxic compounds are therefore of interest to agriculture. Various pseudomonads have been isolated and studied for bioremediation. *Pseudomonas rhizophilia S211* which was isolated from pesticide-contaminated agricultural soil, was found to have a wide spectrum of PGPR genes involved in biocontrol, biofertilization and rhizoremediation (Hassen et al. 2018). Siderophores produced

by *P. aeruginosa* RZS3 strains were shown to chelate various heavy metal ions including $ZnCl_2$, $CuCl_2$ and $CoCl_2$, with their bioremediation potential deemed superior over chemical ion chelators like ethylenediaminetetraacetic acid (EDTA) and citric acid (Patel, Shaikh, and Sayyed 2016). Another example includes *P. citronellolis* P3B5, in which genome exploration found genes encoding for enzymes that degrade long- and very long-chain alkanes, and terpenes making it a candidate for phyllo-remediation based bioremediation approaches (Remus-Emsermann et al. 2016). Additionally, *Pseudomonas* spp. strains GA07, GA09 and GC04 demonstrated degradation capabilities towards glyphosate, in which inoculation into glyphosate-treated soil samples showed 2-3 times higher rate of glyphosate removal compared to non-inoculated soil (Zhao et al. 2015). Since these toxic compounds can contaminate the food system through water and soil, the use of bacteria able to degrade such compounds is of great interest within agriculture to promote food safety.

1.6.0 Challenges in microbial inoculant efficacy

The benefits of microbial inoculants to enhance crop productivity has been a concept for over a century, with the first patented bio-inoculant of the diazotrophic *Rhizobium* developed in 1896 (Nobbe 1896). Since then, the wealth of information gathered for various microorganisms to perform several crop benefits has expanded dramatically. This has led to other microbial species with lifestyles not as well understood as rhizobia to be developed as inoculants. It is often found that these have low efficacy and give unpredictable results in the field. It additionally becomes apparent in the literature, that many studies looking at the growth promoting effects of PGPR have not used appropriate controls, which can undermine claims of plant growth benefits observed. This can include 'negative' controls containing water, which doesn't account for the nutrient sources that a rich microbial inoculum can provide to a plant, along with the multitude of immune signalling responses that inoculants

can elicit in plants. Asides from poor controls, studies have often focused on singular bacteria whilst overlooking the symbiotic and antagonistic capability of the microbiome that can affect application. By failing to take into consideration possible trophic cascades within the ecology of the rhizosphere, along with co-evolutionary responses and the overall resistance and resilience of the native soil community, many inocula fail to establish in the field (Gaba.S 2014). Successful microbial inoculant development is a challenging task, since the inherent variability affecting soil microbial communities makes understanding, predicting and controlling beneficial traits difficult. Despite some successes, there are still major challenges to improve bio-inoculant efficacy. This includes gaining a comprehensive understanding of the root-soil interface and how inoculant application affects the dynamics of such a system.

In recent years, key studies have emerged that address the need for the analysis of population dynamics within the plant-rhizosphere microbiome, via the use of high throughput metagenomic analysis. This has provided new and comprehensive insights into the structure of soil microbial communities, enabling comparison of microbial diversity within microbiomes under different environmental variables. A study by Lungberg *et al* (2012) utilized pyrosequencing to compare and characterise the microbiome associated with different *Arabidopsis* genotypes, their findings displayed differences in genera associated with bulk soil, rhizosphere and endophytic bacterial communities in addition to different plant genotypes (Lundberg et al. 2012). Another approach utilising PhyloChip-based metagenomic analysis was used by Mendes *et al* (2013) to identify bacterial taxa associated with suppressive soils against the fungal root pathogen *Rhizoctonia solani* (Mendes et al. 2011).

These studies give valuable insight into the composition and diversity of the microbiome across different variables; however, although beneficial genes can be screened for within the composition of these communities, the information gained can only indicate the potential of that bacterial species to express a particular gene, rather than show if it is functional. Newer

studies are building on the information from metagenomic studies by the incorporation of metatranscriptomic analysis, to determine whether a gene of interest is transcribed into mRNA, hence an indication that it is functional. Chaparro *et al* (2014) carried out meta-transcriptomic analysis on the rhizosphere of Arabidopsis at four different time points, revealing 81 unique transcripts that were significantly expressed during different stages of plant development (Chaparro, Badri, and Vivanco 2014). These included genes involved in streptomycin synthesis which were induced at bolting and flowering stages and presumably play a role in disease suppression (Chaparro, Badri, and Vivanco 2014). The capture of genetic structure and function throughout individual microbiomes is a major step forward in unlocking the vast and intricate multi-trophic interactions occurring within soil. However, despite these relatively recent advances within the field, the use of meta-genomics and meta-transcriptomics cannot offer the same functional data provided by meta-proteomics and metabolomics.

Genomic and transcriptomic studies have increased more rapidly over the years mainly due to the lower costs and simpler handling (Metzker 2010). However, without proteomics and metabolomics, only a partial understanding of the root-microbial system can be achieved, this is because not all genes that are transcribed are translated into functional gene products. Furthermore, post translational regulation plays an extremely important role on a microbe's lifestyle, and is required for quick adaptation of metabolism in response to coping with environmental changes. Temporal scales of translation, protein turnover and metabolite formation may strongly diverge, which can impact the understanding of beneficial interactions occurring, not only between plant roots and specific microbes but also between players of the microbiome itself (Feussner and Polle 2015). Additionally, whole genome sequencing of isolates can provide a valuable resource, since it provides an opportunity to screen for genes whilst also being able to test the functionality of isolates-again providing information that meta-genomics and meta-transcriptomics cannot offer. What is clear, is that a comprehensive

model which embodies the assessment of plant and microbial responses at different hierarchical levels is required. The collective information gained from the study of the genome, transcriptome, proteome and metabolome of PGPR's together with plant genotypes sampled at different time points, will help to further understand and construct better microbial inoculants.

The development of inoculants in the root environment have typically failed and a likely factor contributing to this, is the study of organisms *in vitro* rather than *in vivo*, resulting in the application of microbes into ecologically unsuitable environments. One of the major constraints is the inconsistent colonization of roots after microbial inoculant application, which traditionally has been difficult to study due to the many fluctuating variables associated within a natural setting (Deacon and Berry 1993). Pffeifer *et al* (2013) studied rhizosphere microbial communities associated with 27 maize genotypes, grown in five different fields, each with unique soil types and within 2 distinct climatic regions within the US (Peiffer et al. 2013). They found that soil type was the main driver of microbial community composition, whilst the two climatic regions studied did not have a significant effect on bacterial OTUs. They also found that plant genotype has a small but significant effect on community composition. This highlights the difficultly of disseminating microbial inoculant products nationally or globally, as they are introduced into soil communities that naturally differ in composition. Soil properties are extremely complex and can vary dramatically across different regions with marked physicochemical heterogeneity in pH, water content, hardness, oxygen levels and nutrient concentrations (Watt, Silk, and Passioura 2006). Not only this, but the soil is comprised of many different lifeforms which can include fungi, viruses, nematodes, mites etc. that can each contribute to interactions that shape soil microbial communities. It's estimated that less than 1% of the soil bacterial community can be recovered from soil using current microbiological techniques, making the non-culturable microbes more difficult to study (Delmont et al. 2011).

However, a relatively recent development is the isolation chip (iChip) which places bacterial cells taken from varying environmental samples into diffusion chambers, that are then returned to nature for incubation (Berdy et al. 2017). This can increase cultivable microbial recovery from 5 to 200 fold and provide access to a unique set of microbes that are inaccessible by standard cultivation.

Overall there are many unknowns in terms of biological interactions, both within the microbiome associated with different plants and across trophic levels within the soil. Unravelling the intricate and complex interactions of soil biology is not just of benefit for addressing inoculant efficacy; global intensive cereal systems that account for a large proportion of total human calorie consumption are suffering from a decline in crop yield, mainly as a result of undiagnosed biological soil factors (Hol, Bezemer, and Biere 2013). Collectively, these issues indicate the complex nature of soil biology and the need for a focus on soil-crop-microbe interactions, to better understand the factors required not only for better performing inoculants but also for a more productive food system.

1.7 Aim and objectives

1.7.1 Overall aim

It's clear that there are many problems to address with current agricultural practices. Tailoring a system to meet the supply demands of >9billion people, on less land, with more efficient input use and a higher resilience to environmental stress is an extremely challenging task. This is compounded by the additional need for a food system that is more environmentally benign. Substantial evidence indicates that manipulating the soil microbiome could have real potential in helping to move towards such a system. Although many advances have been made to help understand microbial inoculant efficacy, a better understanding of factors contributing to microbiome structure and function is still required before consistent and reliable results can be achieved. The Highfield experiment based at Rothamsted Research- Harpenden UK provides an ideal opportunity to investigate the impacts of soil properties on plant-microbe interactions. The overall aim of this project is to better understand how agricultural practices can impact beneficial soil pseudomonad communities, in relation to plant growth promoting ability, with a focus on plant-bacterial phytohormone interaction.

1.7.2 Chapter aims

1. To assess the impacts of crop domestication on *Pseudomonas* community structure within the rhizosphere of wheat

The domestication of wheat has a complex evolutionary history which has resulted in a variety of genetic and phenotypic differences between modern and ancestral species. Modern wheats have reduced genetic diversity, altered root exudation and altered root architecture compared to their wild relatives, which has been suggested to potentially alter the root associated microbiome. *Pseudomonas fluorescens* is a PGPR which can offer many benefits to a crop. Investigating the community structure of Pseudomonad communities associated with ancestral and modern wheats grown in soil sampled from the grassland plots at the Highfield

experiment, could give insight into the impacts of agriculture and whether the ability to recruit a community of beneficial pseudomonads has been altered.

2. To assess root phytohormone composition and bacterial *acdS* distribution in the rhizosphere of field grown wheat from different land managements

Different land managements have been associated with vast chemical, physical and biological soil alterations which can have marked effects on plant physiology and thereby alter plant productivity. Phytohormones are major signalling components in the adaptation of plant physiology towards their surrounding environment. Determining the root phytohormone profile of wheat grown under 3 land management histories sampled from the Highfield experiment, could indicate the impacts of varying practices on plant physiology. Further to this, root phytohormone changes can alter the soil microbiome. Therefore, understanding the distribution of the bacterial *acdS* gene in the rhizosphere together with root phytohormone status, could offer valuable insights into the impacts of land management on soil-plant-microbe interactions.

3. To assess the impacts of land management on pseudomonad phytohormone gene abundance and community selection

Little is known about how changes in microbial community structure translates into altered microbiome functioning. There are many beneficial PGPR traits including nutrient solubilisation, pathogenic competition and induced systemic response. One area of interest that seems to span each of these traits and can directly interact with and alter plant physiology is the production of bacterial phytohormones. The roles of phytohormones are varied but they can promote growth and alleviate symptoms of stress including low nutrient availability, water deficit, salt stress and biotic stress. Gaining a better understanding of the distribution of pseudomonad phytohormone genes across different land managements may offer information on farming practices that encourage beneficial plant-microbe interactions.

4. To screen the genetic and functional potential of *Pseudomonas* spp. for plant growth promoting activity

Genome sequencing can be a great resource to screen and compare genomes of microbes for various traits including beneficial genes involved in PGPR activity. One of the attractions of using microbes as inoculants rather than purifying and applying active molecules from the organisms is that they are responsive to their environments and so can adapt and respond appropriately with a range of beneficial traits rather than one stand-alone purpose. Screening the genomes of various pseudomonads for beneficial traits is a relatively quick way of finding candidates with multiple genes of interest, which could be utilized in multi-purpose microbial inoculant products. Identifying and understanding an inoculant which helps alleviate symptoms of stress or promote crop growth would be of great potential and value towards a more sustainable future.

2.0 Assessing the impacts of wheat domestication on pseudomonad community structure within the rhizosphere

2.1 Introduction

2.1.1 Wheat and its domestication

Triticum aestivum-commonly known as wheat, is a grass species belonging to the *Poaceae* family. It is the most widely cultivated crop globally, providing around a fifth of the total calories consumed by humans in addition to the most protein when compared to other food sources (United Nations. 2017, Appels et al. 2018). Today, around 95% of cultivated wheat is hexaploid (bread wheat), with the other 5% tetraploid (durum wheat) (Shewry 2009). Modern wheats have a complicated evolutionary history, resulting in a large hybrid genome made up of three separate sub genomes (AA-BB-DD). Due to this complexity, it is only relatively recently that high-quality sequencing of the genome of a modern wheat cultivar- *Chinese spring*- has been successfully completed. Humans have played a dramatic role in shaping the genome of modern hexaploid wheats through the process of crop domestication.

The domestication of wheat began around 10,000 years ago, when the nomadic lifestyle of hunter-gather communities transitioned towards agrarian settlements (Doebley 2006) . The switch from gathering wild crops along migratory trajectories towards targeted cultivations, has given rise to crop descendants with phenotypes centred around taste, yield and ease of management (Chen, Gols, and Benrey 2015). Ultimately this crude selection for desirable phenotypes has resulted in a suite of traits which differ between domesticated crops and their wild relatives, a term known as the domestication syndrome (Hammer 1984; Beleggia et al. 2016). Domestication syndrome traits have mostly been studied in crops belonging to the *Poaceae* family, including the previously mentioned polyploid wheats *Triticum durum*, durum wheat (4n=28, AABB) and *T.aestivum,* bread wheat (6n=42, AABBDD) (Meyer, DuVal, and Jensen 2012). These were independently domesticated, with AABB genomes thought to

originate from the hybridization of the diploid *Triticum monococcum* (2n=14, AA) and an unknown species which closely resembles *Aegilops speltoides* (2n=14, SS (BB?)) (Charmet 2011). Hexaploid wheat with AABBDD genomes were the last domesticated through hybridisation of the domesticated tetraploid species *Triticum turgidium-diccocum* (4n=28, AABB) and *Aegilops tauschii* (2n=14, DD)-see Figure 2.1.

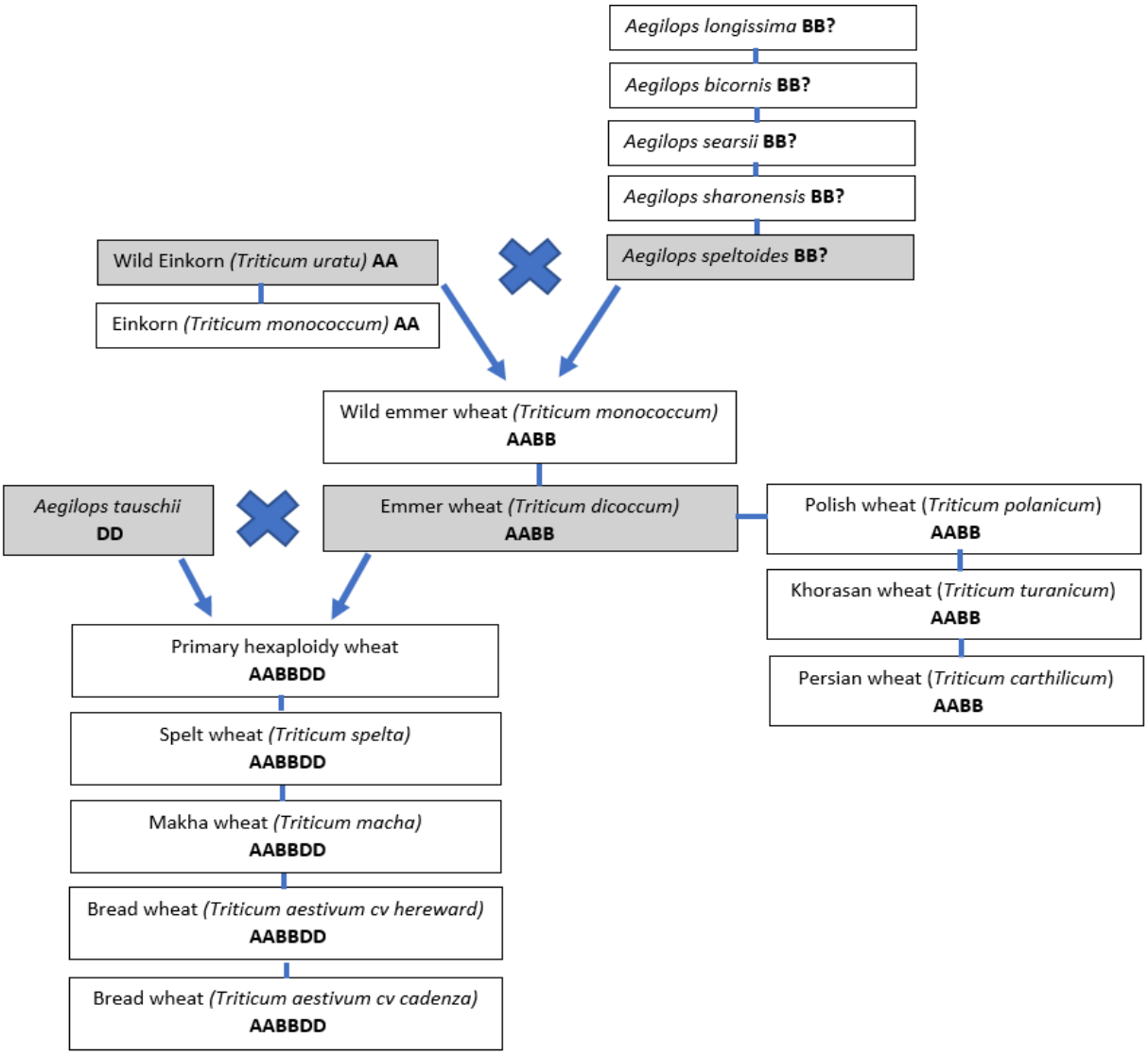


Figure 2.1: Schematic of hybridisation events associated with the domestication of wheat. It's theorized that the AA genome of wild einkorn (*Triticum uratu*) crossed with a BB genome donor-suspected to be a close relative of the goat-grass *Aegilops speltoides*. This first hybridisation event resulted in the tetraploid wheat wild emmer (*Triticum turgidium* subsp. *diccocoides* AABB). A second hybridisation event occurred between cultivated emmer wheat

(*Triticum turgidium* subsp. *diccocum*) and the DD genome donor *Aeliops tauschii*, resulting in the bread wheat *Triticum aestivium* (AABBDD) commonly grown in our agricultural systems today.

2.1.2 Phenotypic traits of ancestral and domesticated wheat

The complex evolutionary history along with the large size of the wheat genome has made exploration of domestication traits difficult. Despite this, some morphological, physiological, and genetic modifications have been identified. The first phenotypes likely selected for by the early Neolithic farmers include a larger grain size, loss of seed dispersal mechanisms, loss of seed dormancy, in addition to a decrease in bitter substances in edible structures (Purugganan and Fuller 2009). These common domestication traits are easily detected and are not limited to wheat species. Common phenotypes associated with wild wheats include a brittle rachis- which leads to spikelet shattering thereby dispersing seeds, in addition to tougher glumes. In contrast, domesticated wheats have softer glumes and a non-brittle rachis, thereby improving threshing efficiency and ease of harvest. The tools equipping the crop breeders of today have allowed more precise selections, typically centred around improving productivity. Perhaps the most successful example of wheat breeding to date can be attributed to the incorporation of reduced height (*rht*) semi-dwarfing alleles during the Green Revolution. The resulting high yielding genotypes have diminished stem elongation, allowing a higher proportion of photosynthate to be partitioned to the grain, thereby helping to prevent lodging and increasing grain number within the spikelets (Thomas 2017).

The birth of modern agriculture is no doubt one of the major successes contributing to today's civilisation, but despite crop improvements tailored for human demands, domestication comes with its problems. One of the main observations of the domestication syndrome is a loss in genetic diversity of modern day genotypes when compared with their wild relatives. Anthropogenic selection of desirable traits, in combination with relatively small progenitor

population sizes, are thought to have decreased the genetic diversity in domesticates (Haudry et al. 2007). The full extent to which this genetic bottlenecking affects phenotype is not currently known, but studies have found that wild wheat appears better adapted to tolerating environmental stresses than modern wheats which better utilise resource inputs (Grime 1977; Matson et al. 1997; Chen, Gols, and Benrey 2015). Domesticated wheats invest a large proportion of energy in above ground biomass rather than below-ground, with shallower rooting depths and finer roots compared to their wild ancestors (Roucou et al. 2018). Irrigation and the application of fertilizer may have contributed to this, as deeper rooting in search of valuable nutrients and water isn't needed under anthropogenic management. Although this offers improved productivity, shifts in resource allocation between competing physiological processes often results in a phenotypic trade-off, bringing into question the resilience of modern domesticates.

2.1.3 Crop domestication and the soil microbiome

A relatively under-explored trait potentially affected by domestication, is the ability of crop species to effectively interact with soil microbes. The root microbiome provides beneficial services to plants in various ways (Backer et al. 2018). Multiple factors can affect microbiome assembly and function including soil properties, climate and plant genotype, but little data has been collected on the impact of domestication. Modern day agronomic inputs such as fertilizers and irrigation can profoundly affect the root system, with crops investing less in below ground biomass and thereby reducing the area of soil explored. Not only do domesticated cultivars display differences in root architecture, but they've also been shown to have altered root exudation (Iannucci et al. 2017). Root exudates are key mediators in interactions between organisms sharing soils including other plants, microbes, and invertebrates (Pangesti et al. 2013). Since these interactions can provide plant benefits, there appears to be a biological importance in sustaining carbon costs through exudation, hence

differences between domesticates and wild relatives could have affects on the rhizosphere biota (Uren 2007). In addition, the movement of wild crop species taken from their native habitat and cultivated in contrasting environmental conditions under human management, could have had negative consequences on plant-microbe interactions- assuming that crops have co-evolved with their native soil biology. It has therefore been suggested that modern domesticated cultivars may not be as harmonised with their microbiome compared to wild progenitors (Perez-Jaramillo, Mendes, and Raaijmakers 2016).

2.1.4 Phylogenetic analysis of microbial communities

Phylogenetics is the study of evolutionary relationships that has greatly contributed to the field of microbial evolution and ecology. In the past, morphological data was used to create phylogenies but with the advent of molecular techniques the use of nucleotide and amino acid sequences has provided valuable and reliable comparisons of organisms, in a relatively cheap and easy way (Lee et al. 2015). The basic principles of phylogenetics relies on the process of Darwinian evolution, in which organisms undergo descent with modification, driven by mutation and selection. Variations in DNA can arise through the loss or gain of genes, for example as seen in obligate symbionts which can become reliant on host genes to provide vital nutrients (Waterworth et al 2020, Norman et al. 2009). Conversely, horizontal gene transfers can occur in which whole genes can move between organisms by mobile genetic elements for example via plasmids or bacteriophages. A more common source of variation is seen with single polynucleotide (SNP) mutations, in which the substitution of a single nucleotide base with another occurs. A SNP can result in a synonymous substitution, in which the corresponding amino acid does not change. Alternatively, a SNP can result in a nonsynonymous substitution in which an amino acid change does occur, which can ultimately impact protein function (Yates et al. 2013).

The comparison of SNPs within a gene common across species, can be a powerful tool in

taxonomy in addition to identifying differences in population structure. This is a common technique utilised in microbial ecology and has been well studied using the bacterial 16S rRNA gene (Větrovský and Baldrian, 2013). The 16S rRNA gene is referred to as a housekeeping gene since it is conserved across all bacteria, encoding for the 16S subunit of the ribosome that is essential for bacterial protein translation. More recently, phylogenetic studies of bacteria have begun to focus on a collection of housekeeping genes in attempts to gain better genetic insights, that more reliably distinguish differences between species. Multi-locus sequence analysis (MLSA) is a method commonly used, in which several gene sequences (e.g. *16S, gyrB, rpoB, rpoD*) are concatenated before phylogenetic relationships are inferred (Glaeser and Kämpfer, 2015). Despite this, some studies have found that the study of other singular housekeeping genes can sufficiently offer better resolution between closely associated species over the 16S rRNA gene. The *gyrB* gene is conserved across all bacterial species and encodes for DNA gyrase subunit B, an enzyme essential during DNA replication, by catalysing the ATP-dependent negative super-coiling of double-stranded closed-circular DNA. The *gyrB* gene has been suggested as a superior marker due to its higher molecular evolution rate compared to 16S rRNA, meaning that its variable regions can be larger and thereby help to distinguish between closely related species (Kasai et al. 1998; Anzai et al. 2000; Yamamoto and Harayama 1995).

2.2 Aims and objectives

2.2.1 Overall objective

To assess pseudomonad community abundance and diversity within bulk soil and the rhizosphere compartment of 17 different wheat species ranging from ancestral diploid (AA, BB, DD) and tetraploid genomes (AABB) through to the modern day domesticated hexaploid wheats (AABBDD) when grown in soil sampled from the low intensity grassland treatment at the Highfield experiment. Identifying and understanding differences in a crops ability to attract and sustain PGPRs, could enable improvements in crop genetics and microbial inoculants to allow manipulation of the rhizosphere microbiome. Future proofing crops to be more resilient to the predicted issues of the future is of current importance, in which a rhizosphere-based breeding program may help to contribute a solution.

2.2.2 Specific objectives

In this chapter I aim to:

1. Assess the abundance of culturable *Pseudomonas* spp. isolated from bulk soil and the rhizosphere of ancestral and domesticated wheats grown in a grassland managed soil.
2. Create a culture collection of *Pseudomonas* spp. isolated from bulk soil and the rhizosphere of ancestral and domesticated wheats grown in a grassland managed soil.
3. Sequence the *gyrB* gene of isolates in the culture collection.
4. Identify isolates by sequence comparison of the *gyrB* gene using BLAST.
5. Assess community phylogeny of the isolates associated with bulk soil and the rhizosphere of ancestral and domesticated wheats grown in a grassland managed soil.

2.3. Methods

2.3.1 Pot experiments

2.3.1.1 Soil sampling and crop genotypes

Soil was collected at a depth of 20-30cm from the permanent grassland managed treatment (plots 10, 17, 26 and 30) at the Highfield experiment-Rothamsted Research in Harpenden UK (see figure 1.6 in chapter 1). Soil was sieved (2mm gauge) and subsequently used in pot experiments. The soil texture at Highfield is defined as a silty loam over clay (Batcombe series), a Chromic Luvisol by FAO criteria, which has been maintained as grassland for over 200 years. The grassland plots utilised in this experiment were maintained by mowing twice yearly.

2.3.1.2 Crop genotypes

2.3.1.2.1 Ancestral and domesticated grasses

A total of 19 different grass species were cultivated in pots, including wheat species of either diploid, tetraploid or hexaploid genomes in addition to diploid goat-grass species and two oat species plus a bulk soil control. Details of individual species are shown in table 1.1.

Name	Family	Tribe	Species	Genome	Ploidy
Einkorn wheat	*Poaceae*	*Triticeae*	*Triticum monococcum*	AA	Diploid
Wild Einkorn wheat	*Poaceae*	*Triticeae*	*Triticum urartu*	AA	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops longissima*	SS (BB?)	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops bicornis*	SS (BB?)	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops searsii*	SS (BB?)	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops sharonensis*	SS (BB?)	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops speltoides*	SS (BB?)	Diploid
Goatgrass	*Poaceae*	*Triticeae*	*Aegilops tauschii*	DD	Diploid
Emmer wheat	*Poaceae*	*Triticeae*	*Triticum dicoccum*	AABB	Tetraploid
Khorasan wheat	*Poaceae*	*Triticeae*	*Triticum turanicum*	AABB	Tetraploid
Persian wheat	*Poaceae*	*Triticeae*	*Triticum carthlicum*	AABB	Tetraploid
Polish wheat	*Poaceae*	*Triticeae*	*Triticum polanicum*	AABB	Tetraploid
Wild emmer wheat	*Poaceae*	*Triticeae*	*Triticum dicoccoides*	AABB	Tetraploid
Makha wheat	*Poaceae*	*Triticeae*	*Triticum macha*	AABBDD	Hexaploid
Bread wheat	*Poaceae*	*Triticeae*	*Triticum aestivum* cv. Hereward	AABBDD	Hexaploid
Bread wheat	*Poaceae*	*Triticeae*	*Triticum aestivum* cv. Cadenza	AABBDD	Hexaploid
Spelt wheat	*Poaceae*	*Triticeae*	*Triticum spelta*	AABBDD	Hexaploid
Wild oat	*Poaceae*	Aveneae	*Avena fatua*	AACCDD	Hexaploid
Oat	*Poaceae*	Aveneae	*Avena sativa cv English berlie*	AACCDD	Hexaploid

Table 1.1. Domesticated and ancestral crop genotypes: A total of 17 different grasses species relating to the evolutionary history of wheat (see figure 2.1) and two grass species relating to

oat were utilised in pot experiments. The two oat species are both hexaploidy however *Avena sativa* is a common oat commercially grown globally, whilst *Avena fatua* is a closely related wild oat considered to have little economic value.

2.3.1.2.2 Crops from taxonomically distinct family

A small study was set up after the initial experiment looking at ancestral and domesticated grasses related to wheat, in attempts to help further clarify results obtained. As the initial crop genotypes were all from the *Poaceae* family, four crop genotypes from taxonomically distant families, as shown in Table 1.2, were utilised in repeat pot experiments.

Name	Family	Species
Bread wheat	*Poaceae*	*Triticum aestivum* cv. Cadenza
Tomato	*Solanaceae*	*Solanum lycopersicum* cv. Ailsa Craig
Oil Seed Rape (OSR)	*Brassicaceae*	*Brassica napus* cv. Makro
Pea	*Fabaceae*	*Pisum sativum* cv.

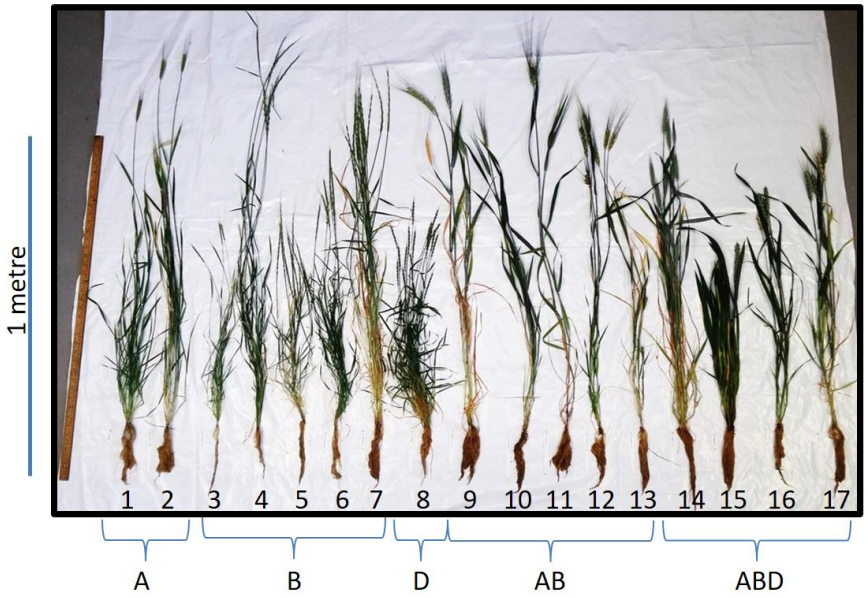
Table 1.2: Crop genotypes

Four economically valuable crop genotypes from the taxonomically distinct families *Poaceae*, *Solanaceae, Brassicaceae* and *Fabaceae* were utilised in a smaller scale repeat study.

2.3.1.3 Cultivation of crops and rhizosphere sampling

All seeds were surface sterilized (75% ethanol wash for 30 seconds followed by 3% sodium hypochloric acid for 10 min then washed thoroughly with sterile water) and sown in seedling trays containing grassland sampled soil. The winter wheat *T. aestivum* cv. Hereward required the longest vernalisation with a period of 12 weeks, therefore all 19 of the grass species were germinated and subjected to the same 12-week vernalisation period at 4 ^{o}C, before being transferred to pots and cultivated under glasshouse conditions. In the second experiment comprising different crop families, seeds were sown directly into pots containing grassland sampled soil, since none of the varieties required vernalisation. Rhizosphere soil was sampled

at the flowering stage for each of the species (see figure 2.2). This was collected by gently shaking and breaking away the bulk soil to reveal soil closely adhering to the root system, which was shaken from the roots into a sterile 50ml Falcon tube.



1. *T. uratu*; 2. *T.monococcum*; 3. *A. bicornis*; 4. *A. longissima*; 5 . *A. searsii*; 6. *A. sharonensis*; 7. *A. speltoides*; 8. *A. tauschii*; 9. *T. polonicum*; 10. *T. dicoccum*; 11. *T. turanicum*; 12. *T. carthlicum*; 13. *T.dicoccoides*; 14. *T. macha*; 15. Hereward; 16. Cadenza; 17. *T. spelta*.

Figure 2.2: 17 ancestral and modern grass genotypes at the early flowering stage

The rhizosphere of the ancestral and domesticated grass genotypes studied were all sampled during the early flowering period as photographed. Here, some of the phenotypic variation across the different species is visibly apparent.

2.3.2 Isolation of *Pseudomonas* spp. from soil

2.3.2.1 Culture medium

All cultured Pseudomonad isolates were selected from soil using *Pseudomonas* Selective Agar (PSA) which was prepared by dissolving 24.2g of *Pseudomonas* Agar base (Thermo Fischer Scientific, USA) in 500ml of distilled water followed by 5ml of glycerol (Thermo Fischer Scientific, USA). The pH was adjusted to 7.2 and the media was sterilized by autoclaving at 121 °C for 15 minutes at 100kPa. Molten agar was allowed to cool to ~ 50°C before adding

Pseudomonas selective agar CFC supplement (Thermo Fischer Scientific, USA) which contained Cetrimide 10mg/ml, fucidin (10µg/ml) and cephaloridine 50µg/ml that was dissolved in ethanol prior to use. 20ml of the prepared PSA was poured into sterile Petri dishes (Thermo Fischer Scientific, USA) within a sterile laminar air flow cabinet and stored at 4°C once set. After selection from soil, isolates were routinely cultured on Lysogenic Broth (LB) lennox agar (Thermo Fischer Scientific, USA) 40g was added to 1L of distilled water and the pH adjusted to 7.2 before sterilization by autoclaving and pouring into Petri dishes as above.

2.3.3.2. Soil dilutions and isolation of pseudomonads

Homogenised rhizosphere soil (1 g) was taken for each sample, diluted in 10 mL of sterilised distilled water and vortexed for 10 minutes to ensure bacterial cells were dislodged from the soil particles and in suspension. Serial dilutions were carried out to a dilution factor of 10^{-6} and suspensions spread onto PSA agar to select for pseudomonads. The plates were incubated at 28^{o}C for 24-48hours.

Random selection using the online random number generator (https://www.random.org) was used to select isolates from each treatment to subsequently create a *Pseudomonas* culture collection. Five isolates from each replicate of ancestral or domesticated grasses (20 total per treatment) and three isolates from each replicate of crops from different taxonomic families (15 total per treatment) were randomly selected to create a culture collection and for further phylogenetic analysis. 1ml of overnight cultures grown in LB broth were mixed with 1ml of 80% glycerol, tubes were inverted and promptly placed into the -80°C freezer for storage.

2.3.3 DNA analysis

2.3.3.1 DNA extraction, PCR and purification

MicroLYSIS-PLUS (Microzone) was used to release DNA from the isolates as per the manufacturer protocol. The housekeeping *gyrB* gene was amplified for all isolates in the culture collection via Polymerase Chain Reaction (PCR), since its more variable regions can

better distinguish between closely related strains. One isolate from each ancestral and domesticated grass species treatment was selected for 16S rRNA gene amplification as a reference. Each PCR reaction mixture was 24 µL in total and consisted of 10 x BioLine reaction buffer (2.5µL), BioLine dNTP mix 25 mM each (0.5 µL), Bioline $MgCl_2$, 50 mM (0.75µL), forward and reverse primers (both at 0.1 µM), BioLine DNA polymerase (0.25 µL), microLYSIS-PLUS DNA extract as a template (1 µL) and 18 µl of nuclease-free water. The PCR conditions were as below:

Degenerate primers and PCR programs for amplification of the *gyrB* gene were as follows:

Forward (UP1): CAYGCNGGNAARTTYGA (Yamamoto and Harayama 1995)

Reverse (UP2r): CCRTCNACRTCNGCRTCNGTCAT (Yamamoto and Harayama 1995)

94°C for 5mins followed by 30 cycles of 94°C for 1min, 60°C for 1min and 72°C for 2mins and a final elongation of 72°C for 5mins.

Primers and PCR programs for amplification of the 16S rRNA gene were as follows:

Forward (341f): CCTAGGGGAGGCAGCAG (Ferris, Muyzer, and Ward 1996)

Reverse (534r): ATTACCGCTGCTGC (Ferris, Muyzer, and Ward 1996)

94°C for 5mins followed by 45 cycles of 94°C for 15s, 55°C for 15s and 72°C for 30s and a final elongation of 72°C for 5mins

PCR products were examined on a 1.5% (w/v) agarose gel in 1x Tris-borate-EDTA (TBE) and stained with EtBr (0.2ug ml-1) with 1x TBE as the running buffer. Bands of DNA were viewed under UV light to identify fragments of the correct size which were then purified using the MinElute PCR purification kit (Qiagen) according the manufacturers guidelines.

2.3.3.2 DNA quantification and sequence analysis

DNA concentration and purity were analysed using the NanoDrop spectrophotometer ND-1500 (Labtech) following the manufacturer's protocol. Forward and reverse Sanger sequencing was carried out on PCR products by MWG Eurofins, a 10µL sample of DNA at a concentration

of 10ng/µL was prepared and sent to MWG Eurofins according to their instruction. Forward and reverse sequences were edited and aligned in the program Geneious. Consensus sequences were then multi-aligned using the MUltiple Sequence Comparison by Log- Expectation (MUSCLE) alignment tool and subsequently trimmed to result in a sequence length of 930bp for each of the sequences. Before phylogenetic construction, the J model test (2.1.10) was used to determine the best model fit for the alignment. A maximum likelihood tree was then constructed utilising a transitional model (TIM 012032) with 1000 bootstraps using the software PhyML (Guindon et al. 2010). The *gyrB* gene sequences of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* F113 were retrieved from the NCBI website and included in the phylogenetic analysis for reference. Sequences were subsequently exported into iTol (itol.embl.de/) for viewing and visual amendments. The NCBI BLAST tool was used to compare sequences from individual isolates to the NCBI database.

2.3.3.3 Statistical analysis

Four biological replicates were used for each treatment (n=4). When determining CFU, each sample had three technical replicates which were averaged to help minimise variation from the testing procedure itself. A one-way ANOVA was performed when comparing mean CFU across the different treatments (see chapter 9.1 for ANOVA table). Any significant results indicated were followed by the post hoc Tukey analysis, to identify which treatments differed significantly. All statistical analysis was performed in the software R studio.

2.4 Results

2.4.1 Abundance of *Pseudomonas* spp. associated with ancestral and modern grasses

The mean Colony Forming Units (CFU) of *Pseudomonas* spp. isolated from 1g of rhizosphere soil associated with each of the ancestral and domesticated genotypes was determined (Figure 2.3). All *Pseudomonas* CFU counts obtained from the different treatments were within 10^6 CFU g^{-1}. *T. monococcum,* (AA genome), *Ae. longissima* (BB genome) and *T. polanicum* (AABB genome) all had the highest CFU g^{-1} of rhizosphere soil which were each found to be significantly different compared to the three lowest CFU g^{-1} associated with *T.uratu* (AA genome), *T. macha* (AABBDD genome) and *T. aestivum*- Hereward (AABBDD genome) each had the lowest associated CFU g^{-1} of rhizosphere soil (p <0.02). A significant difference was additionally found between *T. polanicum* and bulk soil (p <0.04).

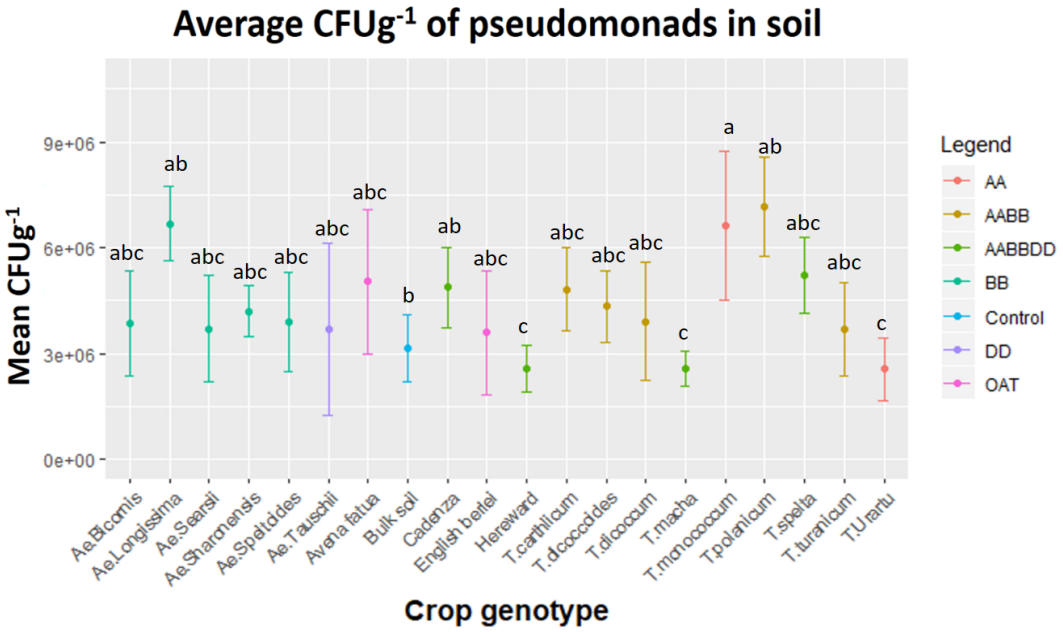


Figure 2.3 The abundance of *Pseudomonas* spp. isolated from rhizosphere soil associated with each crop genotype: The mean CFU g^{-1} ± standard deviation of *Pseudomonas* spp. isolated from rhizosphere soil associated with various wheat species belonging to the A, B, D, AB and ABD genomes in addition to oat hexaploid genotypes and a bulk soil control. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

Due to the significant results obtained, a smaller, repeat experiment with those genotypes highlighted as differing significantly was conducted to retrieve CFU counts only. On average the CFU was lower than the previous experiment at around 10^5 CFU (Figure 2.4). A one-way ANOVA found no significant difference in CFU g^{-1} across the genotypes in this experiment.

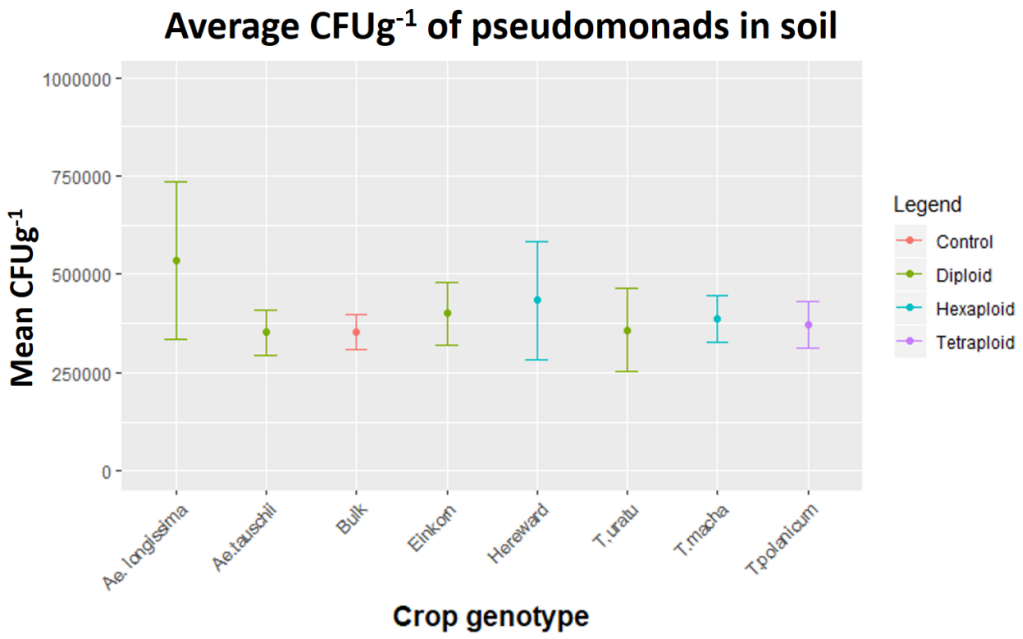


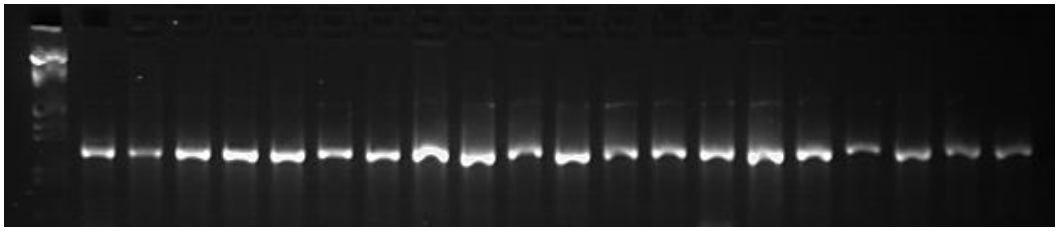
Figure 2.4 The abundance of *Pseudomonas* spp. isolated from rhizosphere soil associated with ancestral and domesticated grass genotypes: The mean CFU g^{-1} ± standard deviation of *Pseudomonas* spp. isolated from the rhizosphere soil associated with various grass species, grown in repeat experiments to test if previous significant differences held.

2.4.2 Identification of *Pseudomonas* spp. isolates

2.4.2.1 Gel electrophoresis identification of gene fragments

The *gyrB* gene with an expected size of ~1280bp was successfully amplified from each of the selected 400 isolates via PCR (Figure 2.5a). The 16S rRNA gene with an expected fragment size of 193bp was also successfully amplified from the 20 randomly selected isolates of each crop genotype (Figure 2.5b).

2.5 [a]



2.5 [b]

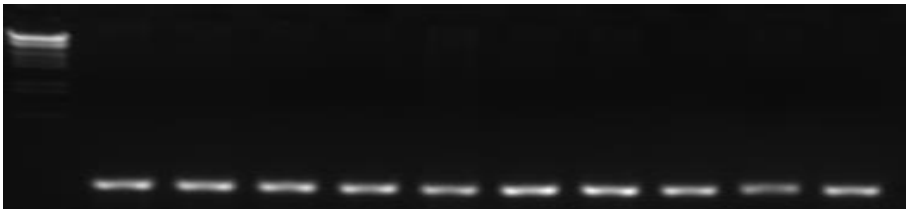


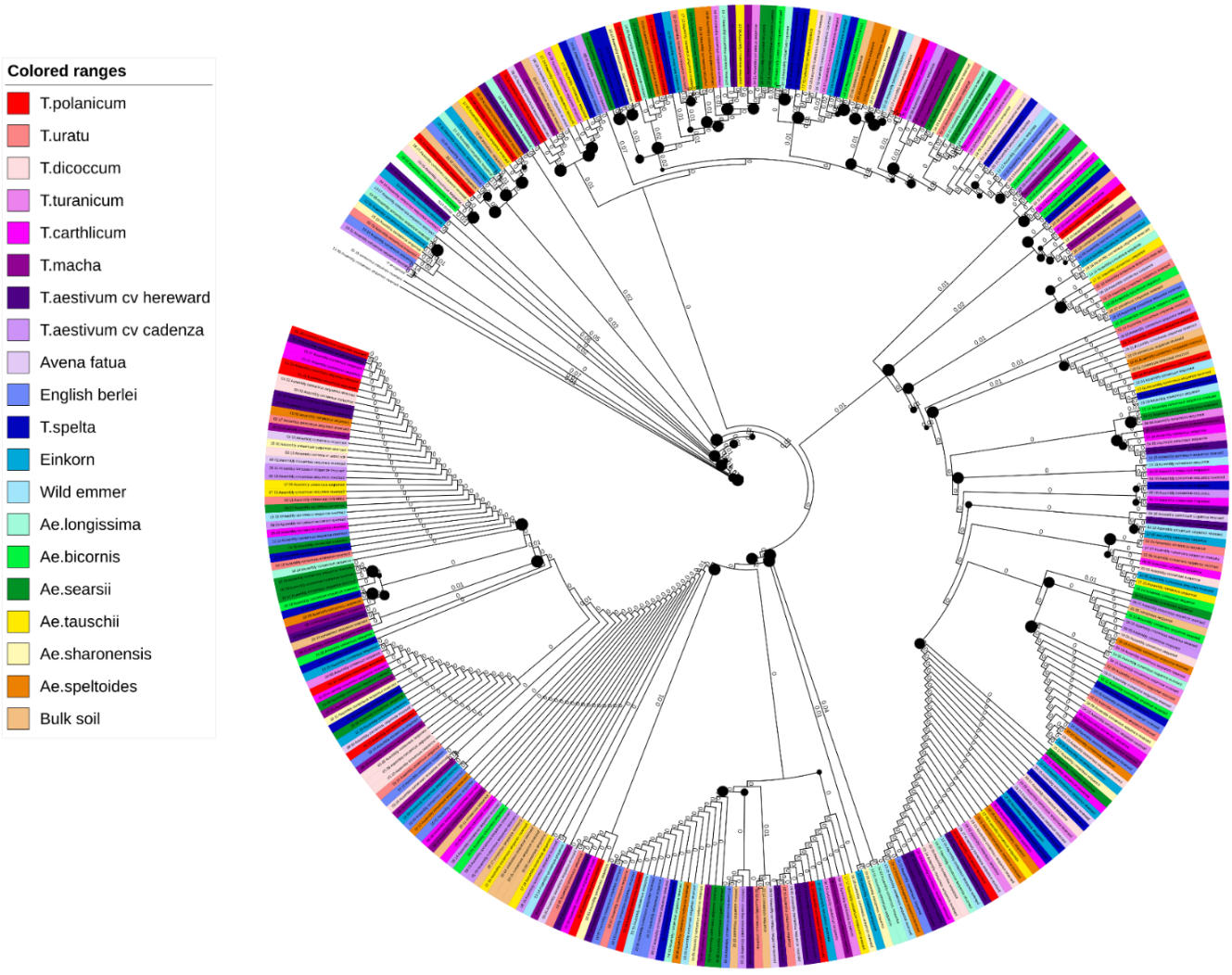
Figure 2.5 *gyrB* and *16S rRNA* DNA fragments amplified from potential *Pseudomonas* isolates: [a] Gel electrophoresis of PCR products amplified with degenerate *gyrB* primers (UP-1 and UP-2r). Lane L, DNA marker (lambda DNA-Digested with HindIII); lanes 1 to 20, amplified fragments of the *gyrB* gene from 20 *Pseudomonas* spp. isolates. **[b]** Gel electrophoresis of PCR products amplified with Muyzer 16S primers (341f- 534r). Lane L, DNA marker (lambda DNA-Digested with HindIII); lanes 1 to 10, amplified fragments of randomly selected isolates from 10 treatments.

2.4.2.2 *Pseudomonas* spp. diversity across ancestral and modern grasses

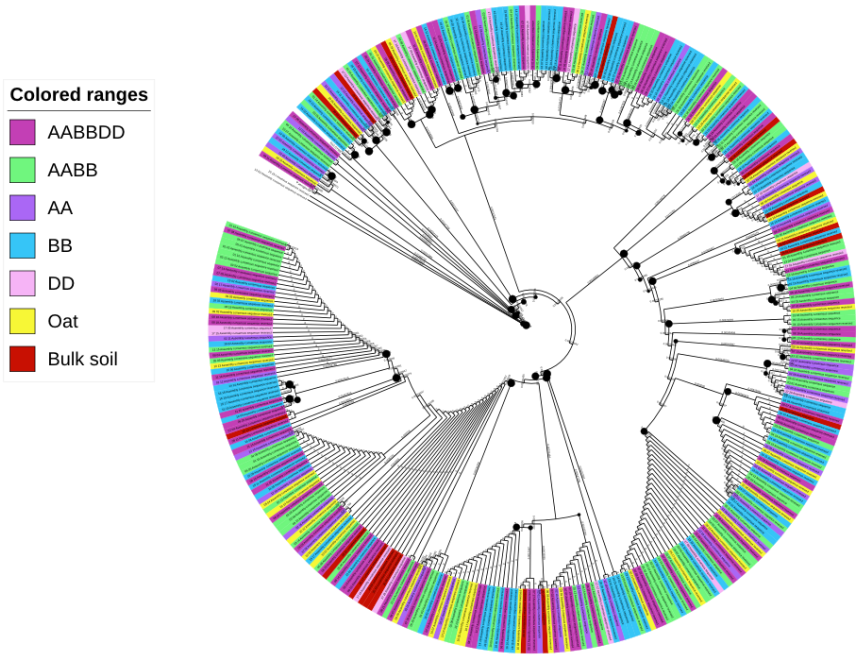
PCR amplification and sequencing of the *gyrB* gene resulted in 353 forward and reverse sequences that could be aligned together successfully. Phylogenetic analysis of the *gyrB* gene was carried out, with a tree constructed and colour coded by crop genotype and bulk soil, as shown in figure 2.6a below. There appears to be no grouping of isolates from one or a collection of treatments, indicating that *Pseudomonas* spp. diversity doesn't vary greatly across different grass genotypes when grown in a grassland managed soil. The same phylogenetic tree was constructed, and isolates colour coded according to the genome (AA, BB, DD, AABB, AABBDD) and ploidy level (diploid, tetraploid, hexaploidy) of wheat that the

isolates had originated from, as seen in figure 2.6b and 2.6c. Again, no obvious groupings of pseudomonad isolates could be visualised.

2.6[a]



2.6[b]



2.6[c]

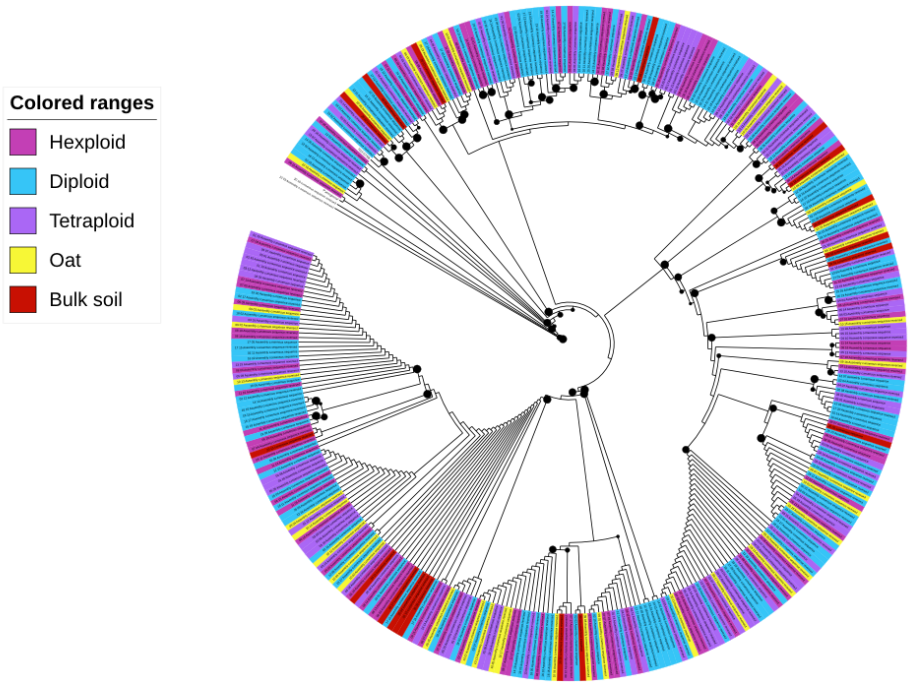


Figure 2.6. **Maximum likelihood tree based on *gyrB* nucleotide sequences from *Pseudomonas* strains associated with ancestral and domesticated crop genotypes: [a]** 353 isolates from a total of 17 ancestral and domesticated wheat genotypes along with 2 oat genotypes and a bulk soil control were phylogenetically analysed utilising an ML tree with a transitional model. Percentage bootstrap values higher than 70% of 1000 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to a:

Individual species **[b]** Genome of the species (AA, BB, DD, AABB, AABBDD) and **[c]** ploidy level of the species (diploid, tetraploid and hexaploidy).

2.4.2.3 BLAST identification

The consensus sequences generated for each of the isolates were cross referenced against the NCBI BLAST database to obtain a species identification. Overall 23 different strains were identified with the majority at ≥ 97% homology and all belonging to the *Pseudomonas* genus; a proportion of the isolates had BLAST hits matching *Pseudomonas* spp. but fell below ≥ 97% homology. There appears to be a higher proportion of isolates identified as *Pseudomonas* spp. R-41739 and *Pseudomonas* spp. URMO17WK12:|11, which were both found in the rhizosphere of each genotype and bulk soil, as shown in figure 2.7. *P. fluorescens* NCIMB 11764 also appeared to be more abundant in the majority of treatments, except for the rhizosphere of the hexaploid wheat *T. aestivum* cv. Cadenza and the oat cultivar *A.sativa* cv English berli. The crop genotypes which appeared to support a higher level of diversity within rhizosphere pseudomonad communities included the diploid (AA) *T. monococcum* and (BB) *Ae. speltoides* along with the tetraploid (AABB) *T. dicoccoides*. Those crops associated with lower levels of pseudomonad diversity included the hexaploid wheats (AABBDD) *T. aestivum* cv. Hereward and *T. spelta* in addition to the tetraploid *T. dicoccum.* The 16S rRNA gene sequences from randomly selected isolates associated with each crop genotype and bulk soil, were also identified against the NCBI BLAST database to cross reference with the *gyrB* results, with all isolates tested identified within the *Pseudomonas* genus.

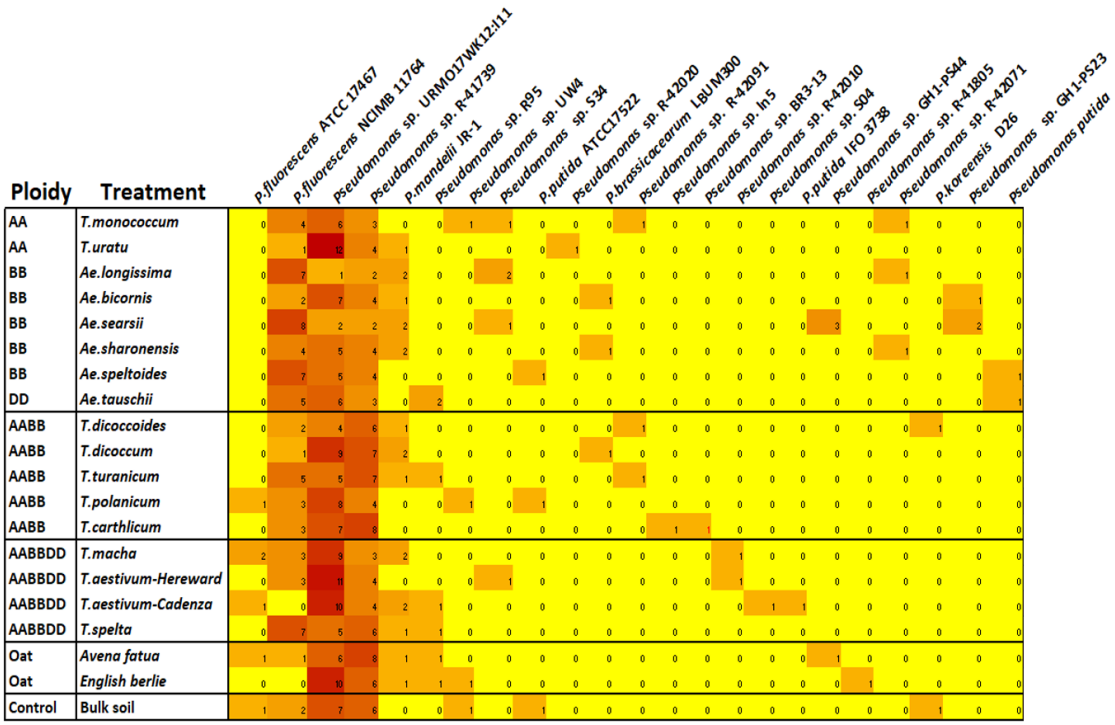


Figure 2.7. Identification of isolates based on *gyrB* gene sequences and compared against the NCBI BLAST database: A BLAST hit table showing strains isolated from the rhizosphere of each ancestral and domesticated wheat, in addition to two oat genotypes and a bulk soil control. Pseudomonad isolate number is represented in a heatmap to visualise differences associated with each crop species. Cells which are coloured red represent the highest and those coloured yellow represent the lowest number of pseudomonad species identified.

2.5 Discussion

2.5.1 Overview

Physiological and genetic differences have been identified in domesticated wheat species when compared to their ancestral progenitors, but investigations into the impacts of domestication on the rhizosphere microbiome is limited (Bulgarelli et al. 2015). Studies so far have shown some differences in microbial community structure and function; however, this varies depending on both the crop genotype and microbial taxa being investigated (Bulgarelli et al. 2015; Chen, Gols, and Benrey 2015; Iannucci et al. 2017). Such studies have focused on differences in rhizosphere microbiome composition across other grass species such as barley in addition to common bean, maize, sugar-beet and rice. In this study, the grass species related to wheat do not appear to be a significant driving factor for differences in pseudomonad community structure. This suggests that the chosen crop genotypes are similar in their ability to attract and sustain *Pseudomonas* spp. under the conditions imposed in this study. This is interesting when considering the range of grass species assessed and the variety of ploidy groups that they fall under. This could indicate the ability of pseudomonads to colonise a range of crops, since the genus *Pseudomonas* embodies multiple species that are genetically, ecologically and functionally diverse (Gomila et al. 2015; Garrido-Sanz et al. 2016; Spiers, Buckling, and Rainey 2000). Conversely, there are factors imposed in this study such as the grassland managed soil and glasshouse conditions which may have made crop phenotype selection on pseudomonads difficult to detect.

2.5.2 *Pseudomonas* spp. abundance in the rhizosphere

Differences in *Pseudomonas* community structure in the rhizosphere have been associated with various factors including soil type, plant developmental stage and root adhesion identified as strong drivers of change (Donn et al. 2015). Other factors such as crop genotype generally encourage small but significant changes, whilst other crops show no significant

difference (Wemheuer et al. 2017). Variation was seen in the abundance of pseudomonads across the ancestral and modern grass genotypes in this study. Although statistical analysis initially found these differences to be significant, all mean CFU g^{-1} counts were at 10^6. This suggests that the ability of the grass genotypes to attract pseudomonads to the rhizosphere was similar, particularly since a repeat experiment found no significant differences in abundance. Interestingly, the repeat experiment yielded a 10 fold decrease in pseudomonads associated with bulk soil and rhizosphere soil compared to the initial experiment. Since the soil used in the repeat experiment was sampled 1.5 years after the initial experiment, it is likely that the pseudomonad communities differed. Soils are not static and various abiotic and biotic changes are likely to have occurred over the 1.5 year period e.g rainfall, and temperature that may have influenced pseudomonad abundance. CFU counts can provide a quick and easy quantification of fast growing culturable bacteria, however there is inherent variability that comes with plate counts. For example, extra handling of samples by diluting with water, in addition to several species occurring in singular large clumps can underestimate the bacteria present in a sample (Ricchi et al. 2017). *Pseudomona*s specific qPCR avoids such issues and is therefore considered to produce more reliable and consistent results compared to culture analysis. Utilising qPCR in future work may provide a better route to assess pseudomonad abundance, albeit more expensive than traditional culture work.

Interestingly, the mean CFU g^{-1} of pseudomonads associated with bulk soil did not significantly differ from the rhizosphere, which is in contrast to results often reported in the literature. Since soil properties such as soil organic carbon (SOC) and pH strongly drive microbial changes, it's worth considering that the soil in this study was from a mixed grassland sward several centuries old, with physical and chemical properties shown to support higher levels of microbial biomass compared to more degraded agricultural soils (Hirsch et al. 2009). The grassland treatment at the Highfield experiment was originally chosen to present an

assortment of *Pseudomonas* species, allowing the crop to select from a diverse pool and thereby highlight rather than limit the detection of crop preferences. Retrospectively, this soil may have contained a *Pseudomonas* community adapted to grass rhizospheres from the previous land use. Therefore, small but significant differences due to genotype may not be easily detected. Additionally, it could indicate that the crops are selecting other microbial taxa which were not looked at in this study, in which metagenomic analysis would have proved greatly beneficial in better interpreting these results. Although many studies are now moving towards the sole use of molecular analysis of community structure and function, culturing techniques are still of importance, particularly where microbial inoculant development is concerned. Culturing of microbes provides a tangible resource, enabling further study into the metabolic and functional capability of isolates. Despite this, culture work can be biased and more prone to human errors when calculating microbial abundance.

2.5.3 *Pseudomonas* spp. identification and phylogeny

All isolates selected using PSA media were identified as belonging to the *Pseudomonas* genus from analysis of both the *gyrB* gene and *16SrRNA* gene. A variety of *Pseudomonas* species were detected via comparison to the BLAST database, but in both experiments particular strains were prevalent. Interestingly, isolates showing homology to *Pseudomonas* sp. R-41739 and *P. fluorescens* NCIMB 11764 were found in high abundance in both experiments, which could indicate that these species commonly thrive in soils, or specifically associate with the grassland managed soil used in this study. In the literature, few studies detail the ecological or functional relevance of these strains. Other pseudomonad strains associated with the rhizosphere of multiple ancestral and domesticated wheats included *Pseudomonas* spp. URMO17WK12:|11 and *Pseudomonas* sp. GH1-PS23 which appeared to be enriched across crops from taxonomically distinct families. Again, it was difficult to find further information regarding the functional capabilities of these two isolates, with none of the above

pseudomonad strains studied in detail compared to reference strains such as *P.fluorescens* F113 or SBW25.

The phylogenetic analysis of *gyrB* sequences from pseudomonads associated with ancestral and domesticated grasses revealed no obvious differences in community structure. This suggests that these genotypes had similar abilities to select for a variety of pseudomonads in the rhizosphere. Several studies have found significant differences in total microbial community structure when comparing modern and ancestral genotypes (Bulgarelli et al. 2015; Perez-Jaramillo et al. 2017; Zachow 2014; Shenton M 2016). In contrast, others have found that domestication did not impact rhizosphere bacteria, but instead fungal communities were significantly affected (Leff et al. 2017). Despite evidence showing that genotype can play a role in driving microbial community composition, when looking at the Proteobacteria genera that *Pseudomonas* spp. belong to, studies have similarly found no significant differences in diversity associated with the rhizosphere of wild and domesticated accessions (Bulgarelli et al. 2015; Perez-Jaramillo et al. 2017; Germida and Siciliano 2001). Since bulk soil communities also didn't appear to differ greatly in community structure to that of the rhizosphere, it suggests that soil properties may be the main driving factor for pseudomonad community structure in this study. As the initial experiment concerned grass species cultivated in a grassland managed soil, it could be possible that the soil was already adapted to that of grasses. However, studying more taxonomically distinct crops (*Poaceae, Solanaceae, Brassicaceae, Fabaceae*) again revealed no obvious differences in pseudomonad community structure (see supplementary S8.1). This further suggests that soil properties were the main driver of pseudomonad community structure in this study.

Temporal factors are also worth considering, as pseudomonad communities associated with the rhizosphere of different wheat genotypes evolve over time, with significant changes in diversity only detected after a second year of cropping (Donn et al. 2015). Additionally, the

more closely associated bacterial endophytes are more genotype-specific than bacteria in the loosely bound rhizosphere (Lundberg DS 2012; Donn et al. 2015; Bulgarelli et al. 2012). Therefore, the evolution of *Pseudomonas* communities under the influence of ancestral and modern varieties of domesticated wheat, may not be apparent upon initial potting experiments and may require investigations into the more closely associated root communities. When looking specifically at wheat domestication, differences in pseudomonad endophytic communities have been associated with wild and domesticated wheat, however these were not found to differ significantly (Germida and Siciliano 2001).

2.5.4 Future considerations

As previously mentioned, the grassland soil utilised in this study has been shown to promote microbial biomass compared to more degraded agriculture soils. Grassland soils are additionally associated with high soil organic matter, nutrient content and pore structure which can benefit crop growth (Hirsch et al. 2017). This, together with the glasshouse conditions that the crops were grown under is likely to encourage optimal plant growth. It has been shown that ancestral wheats may better tolerate different types of stresses compared to their domesticated relatives and may more tightly select their microbiome under such conditions (Merchuk-Ovnat *et al*, 2016). Since the arable and bare fallow plots at the Highfield experiment have been associated with more degraded soil properties, they may have been better soils to utilise when comparing the selection of pseudomonads to the rhizosphere of several wheat genotypes. Indeed, modern domesticated cultivars select for microbes with the ability to compete with phytopathogens, whereas ancestral species are better at selecting for those which can help tolerate abiotic stresses (Leff et al. 2017; Zachow 2014). This could reflect the stochastic wild versus intensive arable environments, of which different selection pressures may have shaped the co-evolution of a crop with its soil microbiome.

It's important to highlight that this study focused on cultivable pseudomonads which can present biases when determining bacterial community structure. Molecular approaches such qPCR to detect pseudomonad abundance and diversity may have been a better measurement for pseudomonad community structure. Although no clear differences were observed in pseudomonad core community structure, it is possible that community function may have been affected by domestication, which to date has not been well studied. The pseudomonad pangenome is extremely large, with high functional diversity seen in soil communities. Rhizosphere microbiome functional studies relating to domestication are few, but evidence so far suggests the main interactions affected by domestication are between crops and mutualistic symbionts such as rhizobia and mycorrhizal fungi (Mutch and Young 2004; Kim et al. 2014; Sangabriel-Conde et al. 2014; Hetrick, Wilson, and Cox 1992). This indicates that associative symbionts such as the pseudomonads, may not be the best microbial genus to target when looking to identify possible impacts of domestication on the soil microbiome.

2.5.5 Conclusions

Under glasshouse conditions, utilising a soil with qualities known to promote microbial biomass the selection for pseudomonads from ancestral and domesticated grasses appears to be similar. Together with the study of more taxonomically distinct crops, and other ancestral studies in the literature, this could highlight the versatility of pseudomonads in being able to colonise a range of crops. Going forward, investigating the recruitment of bacteria to the rhizosphere may be better studied under stressed conditions, to assess if ancestral or domesticated wheats have evolved to select beneficial PGPRs. Microbial functional traits in addition to colonising ability is also required, to identify beneficial relationships with microbes that can help ameliorate biotic or abiotic stressors. This will aid investigations into the impacts of crop domestication and help identify possible targets for a rhizosphere-based breeding

program. Overall, this work provides insights into the complexity of soil-plant-microbe interactions and an interesting starting point for further investigations.

3.0. The impact of land management on soil-plant-microbe interactions in bulk soil and the rhizosphere of field grown wheat

3.1 Introduction

3.1.1 Land management

Soils can offer many services but from an agricultural perspective, the most important function is arguably crop productivity. Despite this, concerns surround anthropogenic management of agricultural land since it can degrade soil qualities, thereby negatively impacting ecosystem services. Once critical soil functions are lost, they are potentially irrecoverable for millennia (Haygarth and Ritz 2009). Globally, intensive croplands have been expanding since the 1960s and have resulted in a reduction of forests and grasslands (Agren et al. 2013; FAO 2016). Intensive agriculture can quickly alter soil dynamics with practices such as monocropping, tillage and inappropriate fertiliser use, irrigation and pesticide application often promoting poor soil qualities (Kibblewhite, Ritz, and Swift 2008). Soil degradation through erosion and compaction in addition to altering nutrient cycling, depleting organic matter levels, weakening soil aggregate strength, salinization, acidification and pesticide pollution, is often associated with high intensity land-uses (Muhammed et al. 2018; Pretty and Shah 1997; Doran and Zeiss 2000; Commission 2002). These properties can have detrimental impacts on soil ecology and ecosystem services (Edwards 2002).

Conversely, low intensity land-uses such as grasslands, scrublands and forests are associated with good soil health. A diversity of plant species in these systems offer long term topsoil cover, along with a steady source of photosynthetically fixed C and organic matter inputs compared to high intensity managements (Piccolo, Andriulo, and Mary 2008). This can increase soil organic carbon (SOC) levels, better aggregate stability and pore structure in addition to increasing biodiversity of micro and macrofauna (Ding et al. 2013; Muhammed et al. 2018).

The Highfield experiment at Rothamsted Research UK provides an opportunity to study the impacts of different land use intensities on the selection of beneficial pseudomonads associated with wheat. Marked declines in C, N, P, aggregate structure and soil pH has been demonstrated in the bare fallow and arable plots compared to the grassland treatments- see table 2.1 (Coleman et al. 1997; Watts et al. 2001; Hirsch et al. 2009) . Wheat yields harvested from the continuous arable and conversion arable plots at the Highfield experiment significantly differed between treatments (Hirsch et al. 2017). The grassland to arable plots supported higher wheat yields compared to the continuous arable and the bare fallow to arable conversion plots. Whilst the three distinct soil managements have shown clear impacts on wheat yields, there is currently little data regarding the impacts of land management on plant-soil-microbe interactions.

	Density[a] / g cm^{-3}	pH[a] (H_2O)/-log ($g[H^+]L^{-1}$)	Organic Carbon mg g^{-1} soil	Free Organic Carbon[b] / mg g^{-1} soil	Intra-aggregate Organic Carbon[b] / mg g^{-1} soil	Nitrogen[a] / mg g^{-1} soil	NaOH-EDTA extractable Phosphorus[c]/ /mg g^{-1} soil
Grassland	0.99	6	3.9	4,690	3,010	390	662
Arable	1.3	5.8	1.3	370	490	150	517
Bare fallow	1.3	5.1	0.8	150	380	100	235

Table 2.1. Summary of the physical and chemical properties of soil from the Highfield Ley-Arable experiment: The table shows chemical and physical measurements of soils taken from the permanent grassland, arable and bare fallow plots of the Highfield experiment. The data shown has been adapted from Neal *et al* 2018 and displays results from a. Gregory *et al*. (2016). b. Hirsch *et al*. (2009) and c. Neal *et al* (2018).

3.1.2 Phytohormone signalling

Plant inputs have a clear role in shaping soil properties, particularly SOC which can positively impact soil health (Lal 2015). Conversely, changes in soil properties can have profound impacts

on plant physiology. Phytohormones play a major role in responding to environmental stimuli (Wani et al. 2016). Understanding root phytohormone changes of wheat grown in different land uses could offer further insights into the impacts of land management occurring at the Highfield experiment. Phytohormone signalling in plants is complex and can be influenced by various factors including plant genotype, developmental stage and the environment (Wang and Irving 2011). Conditions can vary dramatically over the course of a growing period, ranging from optimal to suboptimal. Plant stress can arise due to abiotic factors such as water and nutrient availability, in addition to biotic factors such as pathogenic or pest attacks (Cohen and Leach, 2019). These threats can occur simultaneously depending on the environment, meaning that plants must perceive and respond to multiple stresses at once. As sessile organisms, this requires various biochemical and physiological mechanisms to tolerate or prevent stresses to survive (Cohen and Leach, 2019).

3.1.2.1 Phytohormone signalling under favourable conditions

Under good resource availability such as favourable water, sunlight and nutrient levels, plants will initiate signalling to encourage growth. Auxins such as indole-3-acetic acid (IAA), cytokinins (CKs), gibberellins (GAs), brassinosteroids and strigolactones are well studied for their roles in growth promotion within different tissues and developmental stages (Todaka et al. 2017). Auxin, GAs and CKs play important roles in cell elongation of aerial parts of plants, for instance promoting growth in response to light and nutrient availability (Domagalska and Leyser 2011; Kurepin et al. 2007). Other important physiological responses to favourable conditions include shoot meristem differentiation, with CKs playing an important role in cell division alongside leaf growth and the delaying of senescence through stimulation of increased chloroplast activity (Werner et al. 2001; Polanska et al. 2007). Root growth is driven by elongation of cells generated by stem cell division in the root meristem, with auxin and brassionsteroids playing major regulatory roles (González-García et al. 2011). Auxin can trigger

root hair elongation and lateral root growth which can be crucial for water and nutrient uptake, in addition to root anchoring and interaction with soil microorganisms (Velasquez et al. 2016; Reed, Brady, and Muday 1998). Conversely, high auxin concentrations in roots has been associated with decreased primary root length through inhibition of cell elongation, whilst low concentrations of brassinosteriods and GAs increase root elongation (Bidadi et al. 2010; Mussig, Shin, and Altmann 2003; Friedrichsen et al. 2000). Collectively these physiological adaptations in response to resource availability leads to the investment of energy into crop growth, thereby encouraging increased photosynthesis in addition to nutrient and water uptake.

3.1.2.2 Phytohormone signalling under biotic stresses

Pathogens and pests can colonise and attack plants from above or below ground. Upon recognition of these threats, the plant innate immune system elicits responses to fight and protect against infection at its local source and in other tissues. The plant's immune system can perceive an invasion from the detection of proteins for instance pathogen associated molecular patterns (PAMPS) and nematode associated molecular patterns (NAMPS) (Noman, Aqeel, and Lou 2019). Following recognition, rapid initiation of signalling cascades stimulates the upregulation of genes involved in immune response and phytohormone signalling (Noman, Aqeel, and Lou 2019). The best studied phytohormones associated with defence against biotic attacks are JA, SA and ethylene. JA and ethylene act synergistically, whilst SA and JA act antagonistically. SA has generally been implicated in the activation of defence against biotrophic and hemi-biotrophic pathogens (Yan and Dong 2014; Fu et al. 2012), whilst JA and ethylene are responsible for defence against necrotrophic pathogens and herbivorous insects (Glazebrook 2005; Vlot, Dempsey, and Klessig 2009). Despite this, the upregulation of both SA and JA in plants under effector-triggered biotic stress has been found in some cases,

suggesting that both of these phytohormones can act simultaneously in different cells during infection (Betsuyaku et al. 2018). ABA can additionally play an important role in defence of above ground attack. ABA-induced stomatal closure can occur upon the sensing of PAMPS, helping to prevent pathogen entry into the leaf apoplast through open stomatal pores (Melotto et al. 2006; Melotto et al. 2017). Brassinosteriods are also implicated in biotic defence through the priming of innate immunity pathways and promoting trade-offs between growth and immunity (Yu, Zhao, and He 2018).

3.1.2.3. Phytohormone signalling under abiotic stresses

There are multiple abiotic stresses that affect plant physiology including nutrient stress, flooding, drought, salinity and high temperature. ABA has a central role in plant stress responses, with substantial accumulation found in roots and shoots of plants under various stresses. Water deficit stimulates ABA biosynthesis, which can discourage water loss through closure of stomata thereby preventing transpiration. This also limits the energy intensive process of photosynthesis by reducing CO_2 assimilation, allowing instead the allocation of resource to help protect against stress. ABA has been associated with the accumulation of osmolytes (e.g. sugars, polyamines), dehydrins and other protective proteins that can have important roles in protecting against stress related damage, including stabilisation of osmotic differences between cell surroundings and the cell cytosol (Dar et al. 2017; Chaves, Maroco, and Pereira 2003; Verslues et al. 2006). ABA inhibits seedling germination and restricts shoot and root growth under stress, in conjunction with ethylene.

Ethylene is considered to play diverse roles in plant stress responses and has been well studied for its role in changing root architecture (Bari and Jones 2009). High ethylene levels are associated with root growth inhibition under stress, however this is thought to occur via the stimulation of high concentrations of auxin which inhibits cell elongation in roots (Ruzicka et al. 2007). Additionally, ethylene has been implicated in up-regulating K and S transporters

under nutrient stress, along with the development of root hairs, root transfer cells, and cluster roots induced under Fe or P deficiency (Jung, Shin, and Schachtman 2009; Maruyama-Nakashita et al. 2006; Wang et al. 2014; Zhang, Lynch, and Brown 2003). More recently, cytokinin crosstalk with ethylene, ABA and JA has been recognized, with evidence suggesting a role for CKs in temperature and nutrient sensing under stress (O'Brien and Benkova 2013; Pavlu et al. 2018). It's clear that different responses can be regulated by the actions of phytohormones acting on different transduction pathways, in addition to acting in conjunction with other hormones and signalling substances to regulate responses. A currently underexplored area of research is the role of bacteria in modulating phytohormone levels from the biosynthesis of a range of phytohormones including IAA, cytokinins and ACCd. In relation to plant stress, ACCd producing bacteria are perhaps the most well studied for their potential to decrease plant ethylene levels and thereby promote root elongation.

3.1.3 Bacterial ACC deaminase and plant stress

ACC deaminase exists widely in bacteria and fungi (Nascimento et al. 2014; Bruto et al. 2014). Many studies have investigated the role of ACC deaminase-containing bacteria (applied as inoculants) in alleviating stress symptoms in a range of crops by modulating plant ethylene levels (Li et al. 2000). Despite this, the distribution of the ACC deaminase structural gene (*acdS*) across different environments is not as well studied. Additionally, the misidentification of the *acdS* gene with its homolog *dcyD* has led to an overestimation of ACC deaminase positive microbes, thus contributing to the misunderstanding of the *acdS* gene in microbial community composition and function (Nascimento et al. 2014). Amplification of the *acdS* gene by PCR with degenerate primers has been widely used for molecular identification of ACC deaminase producing bacteria, however those available in the literature are largely non-specific or conversely amplify genes from a narrow range of species (Shah et al. 1998; Ma, Guinel, and Glick 2003; Blaha et al. 2006; Hontzeas et al. 2005; Govindasamy et al. 2008;

Onofre-Lemus et al. 2009). More recently, primers have been designed for PCR and qPCR with better target gene amplification (Li et al. 2015; Bouffaud et al. 2018). Those studies that have looked at the ecology of ACC deaminase bacteria have found an increase of the *acdS* gene in rhizosphere populations compared to bulk soil or non-cultivated soils (Bouffaud et al. 2018; Marasco et al. 2012). The enzyme has also been associated with stressed environments, with a higher abundance found in water limited and high radiation environments (Timmusk et al. 2011; Siddikee et al. 2010). These findings highlight the validity of ACC deaminase as a potential marker for plant-microbe interactions under conditions that promote plant stress.

3.2 Aims

3.2.1 General aim

To assess the impacts of land management on characteristics of soil, wheat physiology and the microbial community of both bulk and rhizosphere soil. Different land managements have been associated with vastly different chemical, physical and biological soil properties, which can have marked effects on plant productivity. The time of sampling marked 10 years since the conversion plots were first incorporated into the Highfield experimental design, providing a unique opportunity to assess the short-term impacts of land management on soil microbial communities in addition to wheat root phytohormone levels. Understanding soil, wheat physiology and microbial community responses to changes in land management could offer valuable insights into soil-plant-microbe interactions and ecosystem services in agriculture.

3.2.2 Specific objectives

In this chapter I aim to:

1. Sample bulk soil, wheat rhizosphere soil and roots directly from the Highfield plots
2. Determine the impacts of land management on basic soil characteristics (Water content, % carbon, % nitrogen and pH).
3. Assess the impacts of land management on wheat growth in addition to root phytohormone levels from the permanent arable and conversion to arable plots
4. Assess total bacterial community abundance with the 16S rRNA gene across bulk soil and wheat rhizosphere soil from different land managements
5. Assess *Pseudomonas* sp. abundance via qPCR analysis of the 16S rRNA gene across bulk soil and wheat rhizosphere soil from different land managements
6. Assess total microbial abundance and pseudomonad specific abundance of the *acdS* gene in bulk soil and wheat rhizosphere soil from different land managements

3.3 Materials and methods

3.3.1 Experimental site

The Highfield experiment (00:21:48W, 51:48:18N) comprises 33 plots as seen in Figure 1.6 (chapter 1) . Plots are divided into 9 possible treatments outlined in Table 2.2, that are arranged in a randomized block design. Each treatment plot is 10 m with a 1 m buffer zone at each end and 0.5 m each side to reduce edge effects, resulting in an 8 x 5 m sampling zone. All plots except those remaining as permanent grassland were ploughed (standard depth 23 cm). At the time of sampling, arable plots had been under continuous arable with winter wheat (most recently *T. aestivum* cv Hereward-seed coated with an insecticide/fungicide treatment of Redigo/Deter, Bayer CropScience) and receiving ammonium nitrate fertilisation to provide approximately 220 kg N ha-1 annum-1, with an additional 250 kg K ha-1 and 65 kg P ha-1 every three years for the last 70 years. Conversion plots to grassland treatments were planted with a fescue/timothy grass/white clover mix, all grassland plots were mowed twice during summer. The bare-fallow plots were maintained by regular tillage at least four times per year to minimise weed growth.

Treatment	Plot numbers
Permanent Grassland	10, 17, 26, 30
Permanent Arable	14, 20, 24, 33
Permanent Bare-fallow	3, 4, 8
Grassland to arable	12, 16, 27, 29
Grassland to bare-fallow	11, 18, 25, 28
Bare-fallow to grassland	2, 6, 9
Bare-fallow to arable	1, 5, 7
Arable to grassland	13, 21, 23, 31
Arable to bare-fallow	15, 19, 22, 32

Table 2.2: Plots associated with each treatment at the Highfield experiment: 33 plots established at the Highfield and their corresponding treatment including the permanent and conversion plots.

3.3.2 Soil/ root sampling and processing

3.3.2.1 Bulk soil

All 33 plots were sampled for bulk soil at a depth of 10 cm using a 3 cm diameter auger, the top 2 cm of soil containing plant detritus was discarded. Ten cores of soil were sampled for each of the plots, which were then pooled together and mixed; the homogenised soil was sieved using a 2 mm sieve and placed into 50 mL falcon tube which was immediately placed into liquid nitrogen on the field site. The samples were later transferred into a -80 °C freezer for storage and freeze dried before analysis in the laboratory.

3.3.2.2 Rhizosphere soil and root

Wheat rhizosphere soil was sampled from the continuous arable plots in addition to the arable conversion plots (grassland to arable and bare-fallow to arable). Three wheat plants from each plot were sampled at the late flowering stage. Loosely bound soil was removed from the roots leaving the rhizosphere/rhizoplane, the roots were then cut from the stem. Rhizosphere soil was collected by a series of three washing steps (see figure 3.1). Roots were firstly placed into a 50 mL falcon tube that contained 20 mL of sterile distilled H_2O and shaken vigorously for 20 s. The roots were transferred with sterile (ethanol wiped) forceps into a 2nd tube containing 10 mL of H_2O and shaken vigorously for 20 s before being transferred to a 3rd tube containing 10 ml of H_2O for a final 20 s washing step. The washes with rhizosphere soil in each tube were pooled together to result in a 40 ml sample, the roots were transferred into a clean tube and both placed into liquid nitrogen on the field site. The samples were transferred into a -80 °C freezer for storage and later freeze dried. Freeze-dried roots were weighed and finely milled using a centrifuge miller (Retsch ZM-200, Germany) for further analysis.

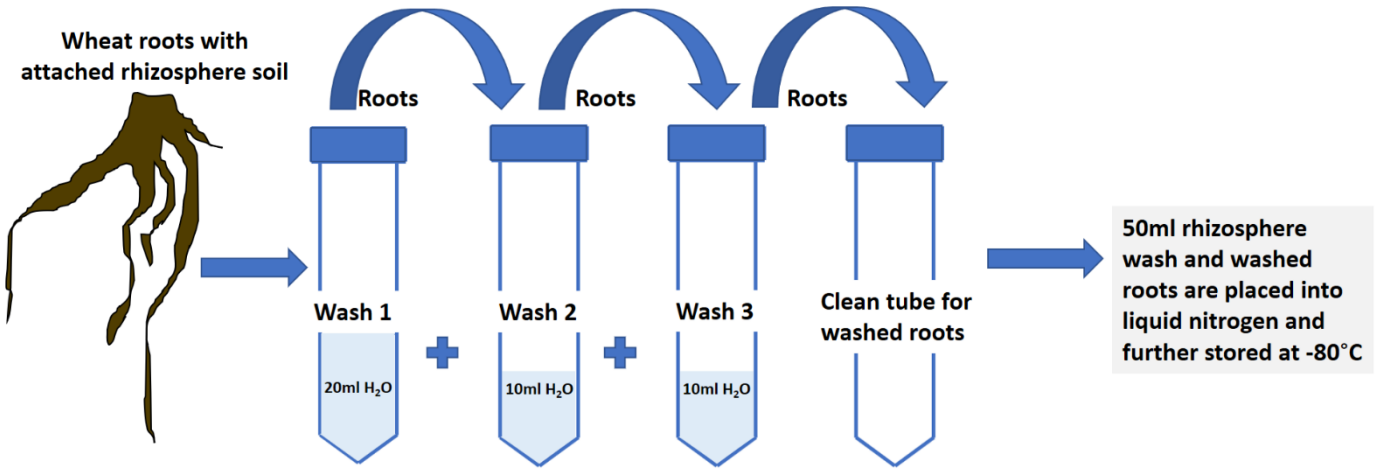


Figure 3.1 Rhizosphere soil and root sampling in the field: Wheat rhizosphere soil was collected in the field utilising a series of washing steps to help minimise the sampling time. Roots were placed into a tube containing sterile distilled H_2O and vigorously shaken for 20 s before being transferred to the next tube of sterile distilled water and repeating until 3 washes had been collected. The three rhizosphere washes were pooled together to create one sample and the clean roots were retained in a clean tube. Each were immediately placed into liquid nitrogen.

3.3.3 Soil measurements

Percentage C and N in bulk soil samples was measured using a LECO combustion analyser, performed by the analytical chemistry department at Rothamsted Research (https://www.rothamsted.ac.uk/analytical-chemistry-unit). Percentage water content of soils was measured by weighing 1g of sampled soil and placing in a 105°C oven for 48hours. Soils were immediately reweighed to obtain dry weight and percentage water content calculated. Soil pH was measured using a standard procedure where replicate 10 g aliquots of air-dried soil were suspended in 25 ml freshly-boiled deionised water.

3.3.4 Phytohormone analysis

Finely milled freeze-dried wheat root samples (50mg) were sent to the Department of Plant Nutrition, CEBAS-CIS (http://www.cebas.csic.es) for phytohormone extraction and analysis by HPLC. A total of 11 phytohormones were analysed including 1-aminocyclopropane-1-carboxylate (ACC), *trans*-zeatin (tZ), zeatin riboside (ZR), isopentenyladenine (iP), gibberellin A1 (GA1), gibberellic acid (GA3), gibberellin A4 (GA4), indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and salicyclic acid (SA).

3.3.5 DNA extraction

All soil samples were handled on dry ice whilst 250 mg of soil was weighed out for each sample. Soil community DNA was extracted using the DNAeasy power soil kit (Qiagen, USA). The manufacturer's protocol was followed excluding step 3-4, in which the FastPrep (MP biomedicals, USA) benchtop homogeniser with a program of 2x cycles of 30 s with a 30 s gap, speed 5.5 m/s was used to lyse cells and release DNA. Extracted DNA was quantified using the fluorometer Qubit® 2.0 dsDNA BR Assay Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Additionally, extracted DNA was analysed using the Nanodrop Microvolume UV spectrophotometer ND-1500 (Lab Tech) by its OD at 260 nm, the 260/280 ratio was used to determine DNA quality. Samples which had low DNA concentration or quality were discarded and the DNA extraction was repeated.

3.3.6 *In silico* testing of *acdS* primers in the literature

The amino acid sequence corresponding to the *acdS* gene in *Pseudomonas* sp. UW4 in addition to the homolog D-cysteine desulfhydrase encoded by the *dcyD* gene in *Escherichia coli* K12 were each retrieved from the NCBI database and used as reference sequences (see below).

>WP_015096487.1 1-aminocyclopropane-1-carboxylate deaminase [Pseudomonas sp. UW4]

MNLNRFERYPLTFGPSPITPLKRLSEHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEAIEQGCDTL

VSIGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAGFDIGIRP

SWEKAMSDVVERGGKPFPIPAGCSEHPYGGLGFVGFAEEVRQQEKELGFKFDYIVVCSVTGSTQAGMVVG

F AADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVELGREITEEDVVLDTRFAYPEYGLPNEGTLEAIR

LCGSLEGVLTDPVYEGKSMHGMIEMVRRGEFPDGSKVLYAHLGGAPALNAYSFLFRNG

>NP_416429.4 D-cysteine desulfhydrase [Escherichia coli str. K-12 substr. MG1655]

MPLHNLTRFPRLEFIGAPTPLEYLPRFSDYLGREIFIKRDDVTPMAMGGNKLRKLEFLAADALREGADTL

ITAGAIQSNHVRQTAAVAAKLGLHCVALLENPIGTTAENYLTNGNRLLLDLFNTQIEMCDALTDPNAQLE

ELATRVEAQGFRPYVIPVGGSNALGALGYVESALEIAQQCEGAVNISSVVVASGSAGTHAGLAVGLEHLM

PESELIGVTVSRSVADQLPKVVNLQQAIAKELELTASAEILLWDDYFAPGYGVPNDEGMEAVKLLARLEG

ILLDPVYTGKAMAGLIDGISQKRFKDEGPILFIHTGGAPALFAYHPHV.

The reference amino acid sequences were utilised in a Uniprot reference proteome search (https://www.ebi.ac.uk/Tools/hmmer/search/jackhmmer). The appropriate bit scores and sequence coverage were determined and used to identify sequences with high levels of homology to the target sequence, these were extracted as DNA FASTA sequences. DNA sequences were aligned using a MUSCLE alignment and a maximum likelihood tree was constructed utilising a Hasegawa, Kishino and Yano 1985 (HKY85) model with 100 bootstraps in the software PhyML (Guindon et al. 2010). Primers cited in the literature as amplifying the *acdS* gene were tested *in silico* (primers 3 and 4 in table 5.1), to determine the most appropriate primer set for quantitative PCR (qPCR).

3.3.7 Quantitative PCR

qPCR amplification was performed in 10 10 µl volumes containing 5 µl of QuantiFast (Qiagen, Manchester, UK), 2.8 µl of nuclease-free water (Severn Biotech, Kidderminster, UK), 0.1 µl of each primer (1 µM) and 2 µl of template DNA at 5 ng μl^{-1}, using a CFX384 Touch® Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK). The standards for each target were obtained using a 10-fold serial dilution of PCR products amplified from an environmental reference DNA (also used as positive control) and purified by gel extraction using the Wizard® SV Gel and PCR Clean Up System (Promega, Southampton, UK) following the

manufacturer's instruction and quantified by fluorometer Qubit® 2.0 dsDNA BR Assay Kit (Thermo Fisher Scientific). Standard curve template DNA and the negative/positive controls were amplified in triplicate. Amplification conditions for all qPCR assays consisted in two steps: first denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s that included annealing, elongation and reading. Each amplification was followed by melting curve (increase in temperature from 60 °C to 95 °C, with a reading every 0.5 °C) to assess the specificity of each assay.

	Target gene	**primer**	**Sequence 5'-3'**	**Product length**	**Reference**
1	Bacterial 16S rRNA	341F	CCT AYG GGR BGC ASC AG	465bp	Glarling *et al*. (2015)
		806R	GGA CTA CNN GGG TAT CTA AT		
2	*Pseudomonas* 16S rRNA	Pse434F	ACT TTA AGT TGG GAG GAA GGG	231bp	Bergmark *et al*., (2012)
		Pse665R	ACA CAG GAA ATT CCA CCA CCC		
3	Universal *acdS*	acdSf3	ATC GGC GGC ATC CAG WSN AAY CAN AC	683bp	Li *et al* (2015)
		acdSr3	GTG CAT CGA CTT GCC CTC RTA NAC NGG RT		
4	Universal *acdS*	acdSf8	GGC AAC AAG MYS CGC AAG CT	153bp	Bouffard *et al* (2018)
		acdSr5	CTGCACSAGSACGCACTTCA		

Table 2.3 Primer sequences: Primers used to assess microbial communities included 16S rRNA for bacteria and 16S rRNA for *Pseudomonas* sp. specific amplification, in addition to testing two universal *acdS* primer sets available in literature.

3.3.8 Statistical analysis

A one-way ANOVA compared the 9 different treatments. Data which did not follow a normal distribution was LOG10 transformed before ANOVA analysis (see chapter 9.2 for ANOVA results). Any significant results indicated were followed by the post hoc Tukey analysis, to identify which treatments differed significantly. All statistical analysis was performed in the software R studio.

3.4 Results

3.4.1 Basic soil measurements

Differences in soil properties were found across the nine treatments at the Highfield experiment (Figure 3.2). In the long-term treatments, soil moisture content varied considerably (p <0.001), with highest soil moisture in the permanent grassland plots, lowest soil moisture in the bare fallow plots, and intermediate values in the arable plots. Converting permanent grassland to arable or bare fallow significantly decreased soil moisture by 18% and 25% respectively. Converting arable soil to bare fallow decreased soil moisture by 11% but converting it to grassland had no significant impact ten years after conversion. Converting bare fallow to arable cropping had no significant effect on soil moisture but converting it to permanent grassland increased soil moisture by 35%.

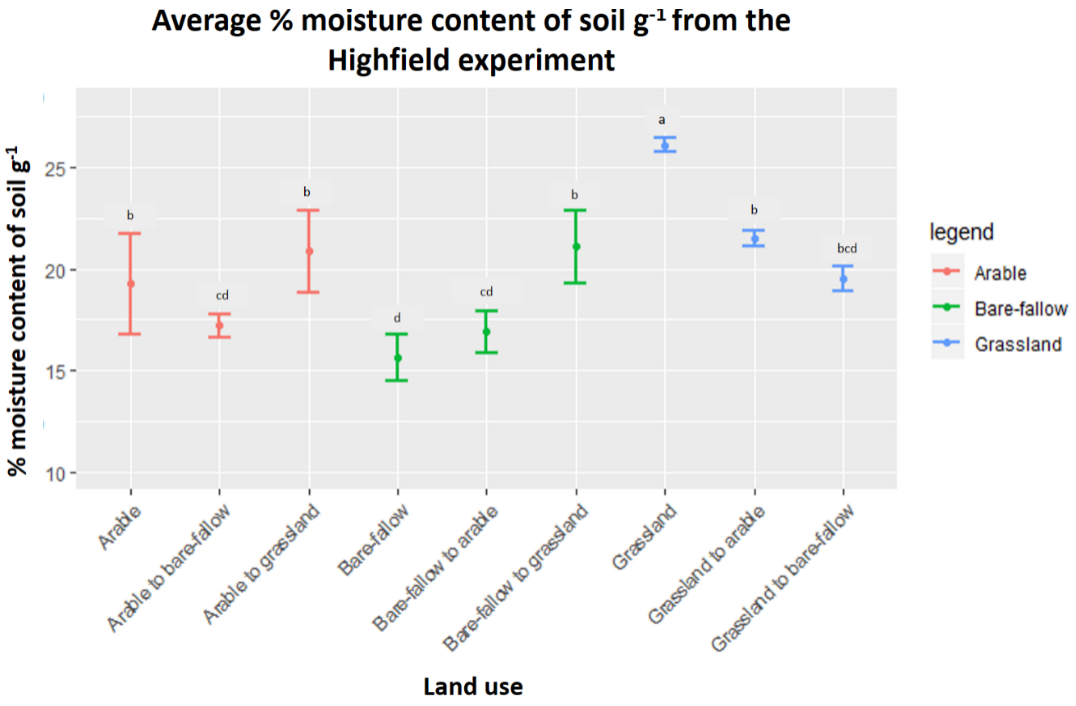


Figure 3.2: Percentage soil moisture associated with contrasting land management histories at the Highfield experiment: The average % soil moisture content per g^{-1} soil ± standard deviation. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

Percentage C content varied considerably in the long-term treatments (p <0.001), with highest C content in the permanent grassland plots, lowest in the bare fallow plots, and intermediate values in the arable plots (Figure 3.3). Converting permanent grassland to arable or bare fallow significantly decreased C content by 46% and 52% respectively. Percentage C in arable soil converted to bare fallow did not differ significantly whilst conversion to grassland significantly increased percentage C by 57% compared to permanent arable. Bare fallow converted to arable and grassland both significantly increased percentage C by 21% and 103% respectively.

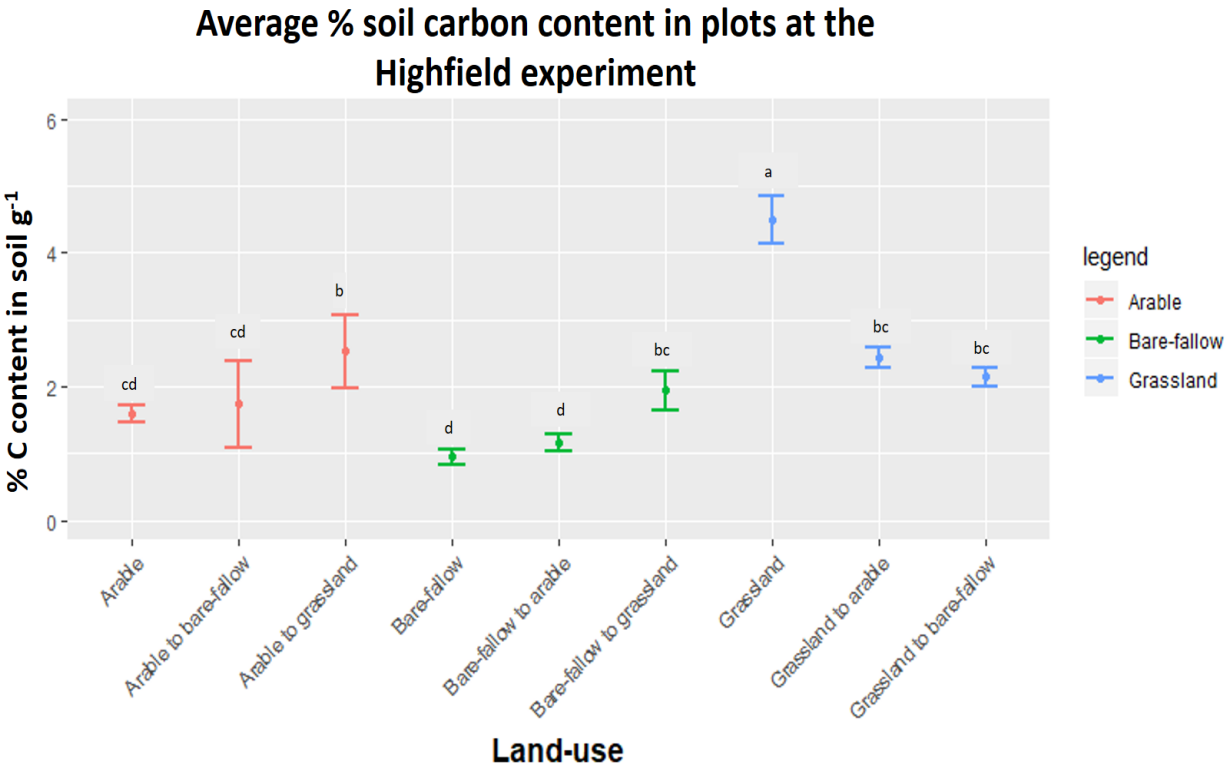


Figure 3.3: Percentage soil carbon associated with contrasting land management histories at the Highfield experiment: The average % soil carbon content per g^{-1} soil ± standard deviation. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

Percentage N content varied considerably in the long-term treatments (p <0.001), with highest N content in the permanent grassland plots, lowest in the bare fallow plots, and intermediate values in the arable plots (Figure 3.4). Converting permanent grassland to arable or bare fallow significantly decreased N content by 41% and 47% respectively. Percentage N in arable soil converted to bare fallow did not differ significantly whilst conversion to grassland significantly increased percentage N by 45% compared to permanent arable. Bare fallow converted to arable and grassland both significantly increased percentage N by 21% and 71% respectively.

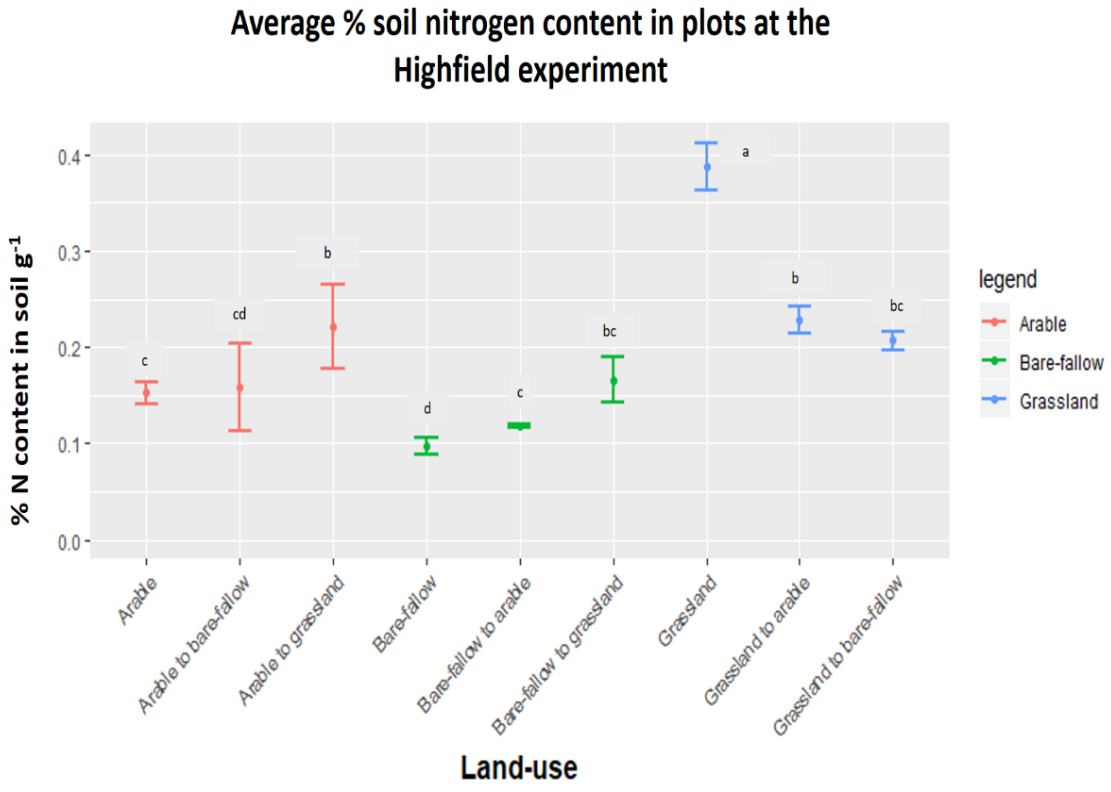


Figure 3.4: Percentage soil nitrogen associated with contrasting land management histories at the Highfield experiment: The average % soil nitrogen content per g^{-1} soil ± standard deviation. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

The average soil pH differed between treatments, ranging from pH 5.31 in the permanent bare fallow soil to pH 6.32 in the permanent grassland treatment. Despite this, results were not found to differ significantly across the treatments as seen in figure 3.5.

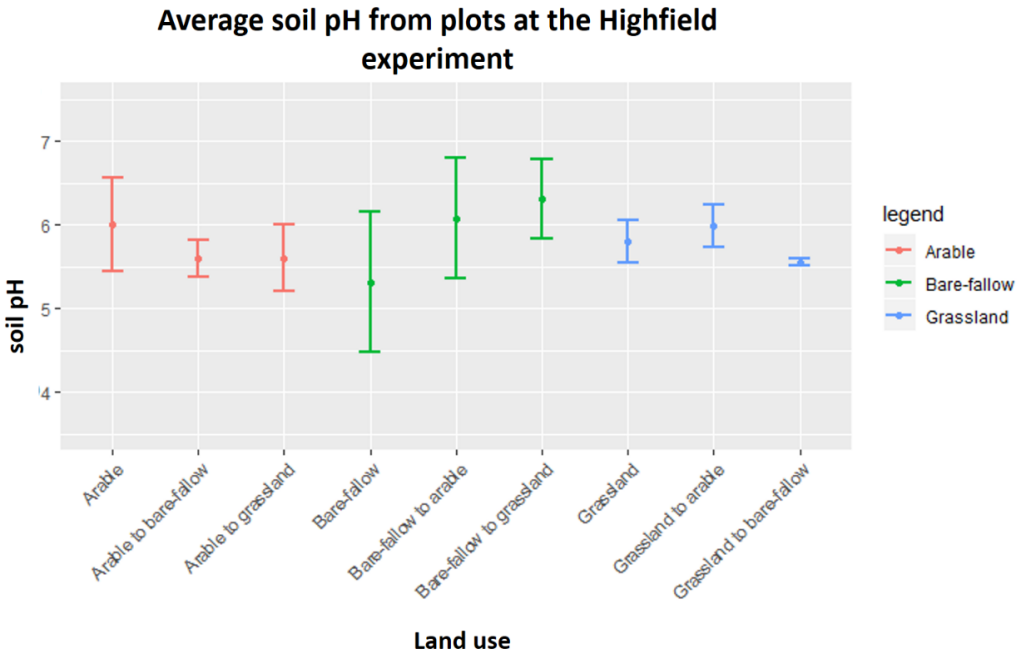


Figure 3.5: Soil pH associated with contrasting land management histories at the Highfield experiment: The average soil pH ± standard deviation.

3.4.2 Wheat measurements

3.4.2.1 Wheat yield and root dry weights

Wheat (grain) yields and root dry weight differed significantly according to previous land use (p <0.001 and p=0.027 respectively). Continuous arable plots and those converted to arable from permanent grassland had similar yields (averaging 5.5 and 5.3 t ha^{-1} respectively), whilst plots that were converted from bare fallow had significant yield reductions by 53% (averaging 2.6 t ha^{-1}) as seen in Figure 3.6[a]. Root dry weight of wheat grown in the continuous arable plots and those converted to arable from permanent grassland again were similar (averaging 1.07 g^{-1} and 1.06 g^{-1} respectively), whilst plots that were converted from bare fallow had significant reductions in root dry weight by 38% (averaging 0.67 g^{-1}) as seen in Figure 3.6[b].

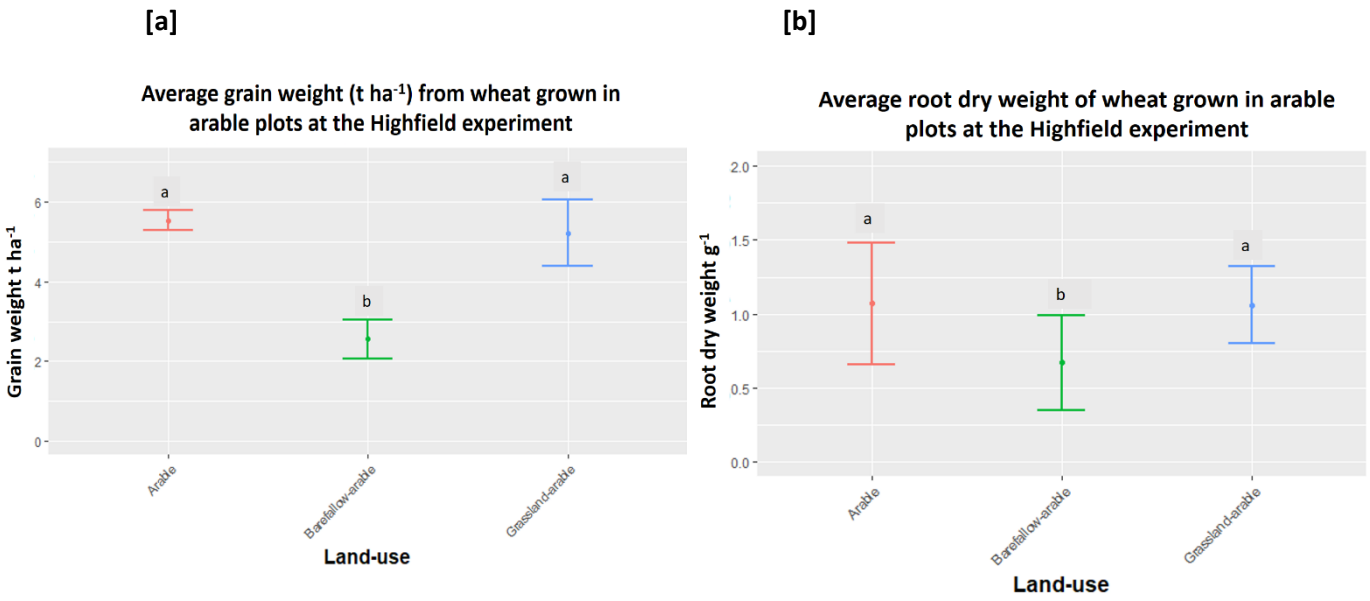


Figure 3.6: Yield and root measurements from wheat grown in soil from three distinct land management histories [a] The average grain weight (t ha^{-1}) ± standard deviation **[b]** average root dry weight ± standard deviation from wheat grown in soil with differing land-management histories. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

3.4.2.2 Root phytohormone levels

Of the eleven different phytohormones analysed, 10 were successfully detected (The ethylene precursor ACC, cytokinins (tZ and iP), gibberellins (GA1, GA3 and GA4), in addition to IAA, ABA, JA and SA) in wheat roots. The cytokinin ZR was only detected in one sample of wheat roots grown in the continuous arable plot and was therefore not included in further analysis. ACC, tZ, iP, GA1, GA4, IAA or SA levels in the wheat roots did not significantly differ with land use histories, (see Table 2.4). Conversely, JA, GA3 and ABA significantly differed between treatments. JA was significantly higher (p <0.001) in wheat roots grown in the permanent arable plots (averaging 158 ng g^{-1}) compared to those grown in the grassland to arable and bare fallow to arable conversions (averaging 115 and 81 ng g^{-1} respectively). Conversely, GA3

was significantly higher (p =0.003) in the roots of wheat grown in the bare fallow to arable conversions (averaging 13 ng g^{-1}) compared to permanent arable and grassland to arable plots (averaging 7.65 and 7.62 ng g^{-1} respectively). An ANOVA found ABA significantly differed across the treatments (p =0.05) with concentrations lower in wheat roots grown in the bare fallow to arable conversions (averaging 20.21 ng g^{-1}) and higher in the permanent arable and grassland to arable plots (averaging 32 and 30 ng g^{-1} respectively). Despite this, the Tukey post hoc analysis did not reveal any significances below the threshold p=0.05, with the bare fallow to arable conversion differing from the permanent arable plots with a p value of 0.058.

	Arable	Bare fallow	Grassland
ACC	4205±848	4200±1171	4593±910
ABA	31.78±11.01	20.21±8.49	29.97±10.64
tZ	857±250	880±227	1093±204
iP	6.49±2.94	7.06±2.90	6.74±3.84
GA1	0.32±0.21	0.53±0.69	0.42±0.25
GA3	7.62±2.28	13.14±3.17**	7.62±4.66
GA4	1.59±1.03	1.26±1.05	1.43±0.80
IAA	100±28	114±51	124±41
SA	248±129	152±51	217±79
JA	158.07±42.41***	80.67±27.81	114.77±22.12

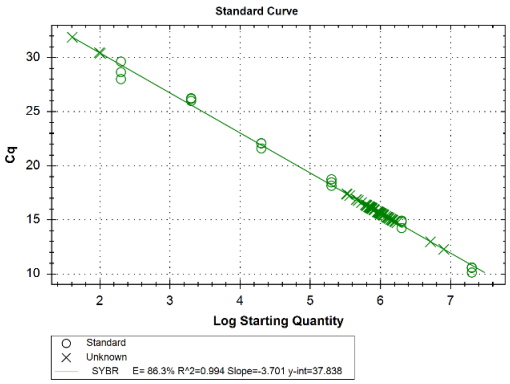
Table 2.4: Phytohormone content in wheat roots grown across three contrasting land management histories: The average phytohormone content of wheat roots (ng g^{-1} ± standard deviation) grown in the continuous arable, grassland to arable and bare fallow to arable plots at the Highfield experiment. The phytohormones amino-cyclopropane carboxylate (ACC), abscisic acid (ABA), trans-zeatin (tZ), isopentenyladenine (iP), gibberellin A1 (GA1), gibberellin A3 (GA3) gibberellin A1 (GA4), indole-3-acetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA) were analysed.

3.4.3 Bacterial abundance

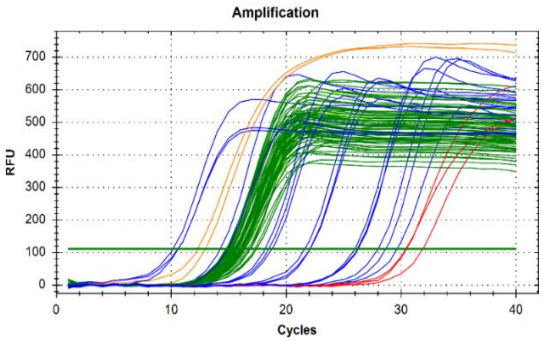
3.4.3.1 16S rRNA gene total community abundance

Bacterial abundance was successfully determined using qPCR to amplify the *16SrRNA* gene in bacteria (r^2= 0.994) as shown in figure 3.7[a,b]. Differences in the bacterial *16SrRNA* gene abundance were identified across the different land managements (Figure 3.7[c]). In the long-term treatments, bacterial abundance varied considerably (p <0.001), with the highest average abundance of the *16SrRNA* gene associated with the permanent grassland plots (1.85e+09 g^{-1}), lowest in the bare fallow plots (2.46e+08 g^{-1}), and intermediate values in the arable plots (6.78e+08 g^{-1}). Converting permanent grassland to arable or bare fallow decreased bacterial abundance but not significantly (averaging 1.30e+09 and 1.04e+09 g^{-1} respectively). Converting arable soil to bare fallow decreased bacterial abundance but not significantly (averaging 4.13e+08), whilst converting arable to grassland resulted in a two-fold significant increase in bacterial abundance (averaging 1.50e+09 g^{-1}). Converting bare fallow to arable cropping had no significant effect on bacterial abundance but converting to permanent grassland significantly increased abundance by six-fold (averaging 1.52e+09 g^{-1}).
Bacterial abundance utilising the *16SrRNA* gene was additionally assessed in rhizosphere soil of wheat grown in the permanent arable and arable conversion plots, with significant differences found compared to bulk soil (p <0.001). Significant two-fold increases in bacterial abundance from bulk soil to rhizosphere soil were detected in both the permanent arable and grassland to arable conversion plots. Conversely, a slight increase in the average bacterial abundance was observed from bulk soil to rhizosphere soil in the bare fallow to arable plots, but this was not significantly different (Figure 3.7 [d]).

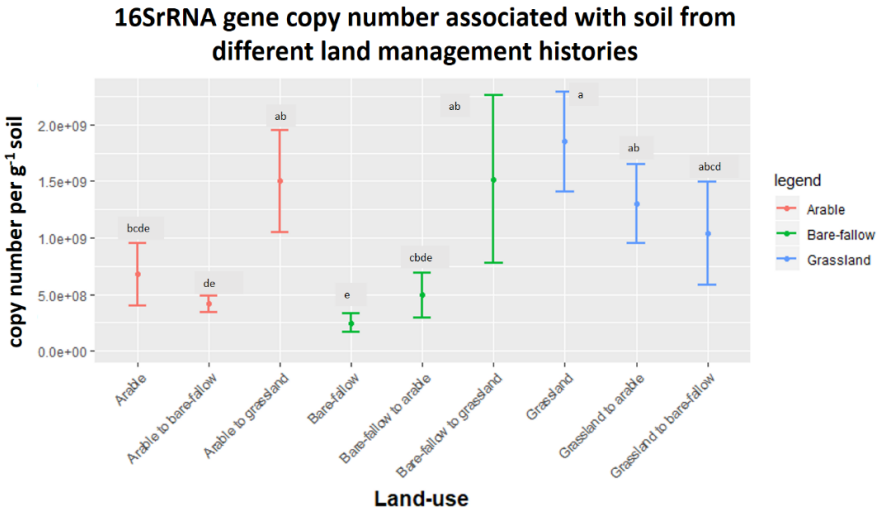
[a]



[b]



[c]



[d]

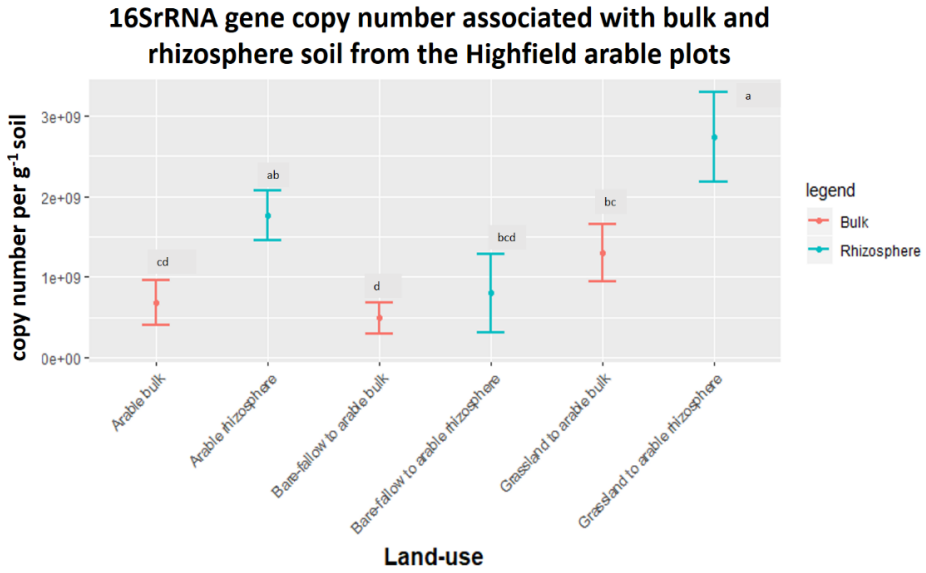


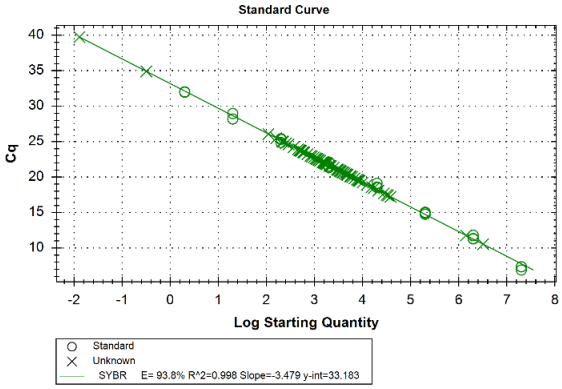
Figure 3.7: Bacterial *16SrRNA* gene quantification across bulk soil and rhizosphere communities of wheat grown in three contrasting land management histories: Quantitative PCR analysis of the bacterial *16SrRNA* gene across different land managements at the Highfield

experiment [a] qPCR calibration standard curve [b] qPCR amplification curve [c] bacterial 16S rRNA gene copy number ± standard deviation g^{-1} of bulk soil from all 33 plots at the Highfield experiment [d] bacterial 16S rRNA gene copy number ± standard deviation per g^{-1} of bulk soil and rhizosphere soil associated with wheat grown in the arable plots. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

3.4.3.2 16S rRNA *Pseudomonas* spp. abundance

Pseudomonas spp. abundance was successfully determined using qPCR to amplify the *16SrRNA* gene in bacteria (r^2= 0.998) as seen in figure 3.8 [a,b]. Differences in the average abundance of *Pseudomonas* spp. was observed across the different land managements, however these did not significantly differ across any of the treatments (Figure 3.8 [c]). Similarly, when looking at the abundance of pseudomonads in bulk soil and rhizosphere communities of wheat grown in the permanent arable and conversion to arable plots, there was no significant difference in abundance between the two niche compartments (Figure 3.8[d]).

[a]



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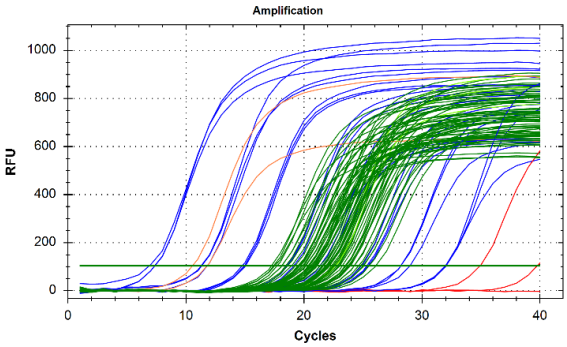

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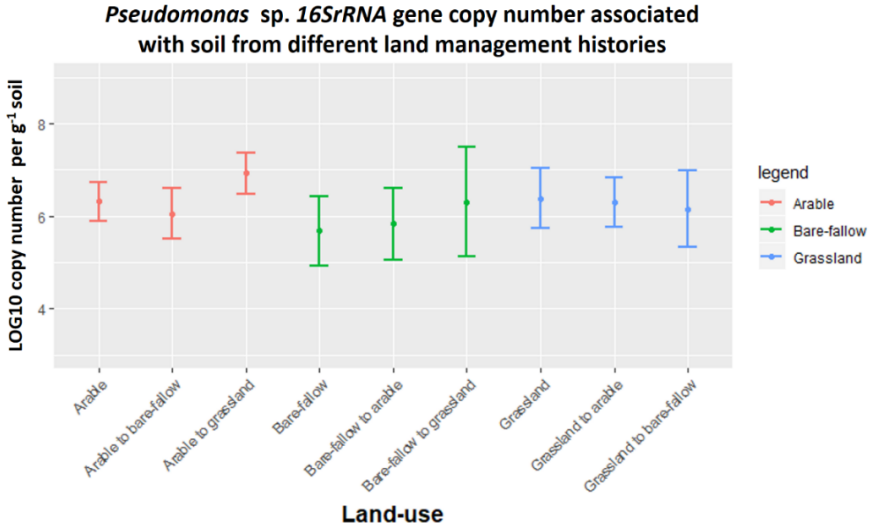


[d]

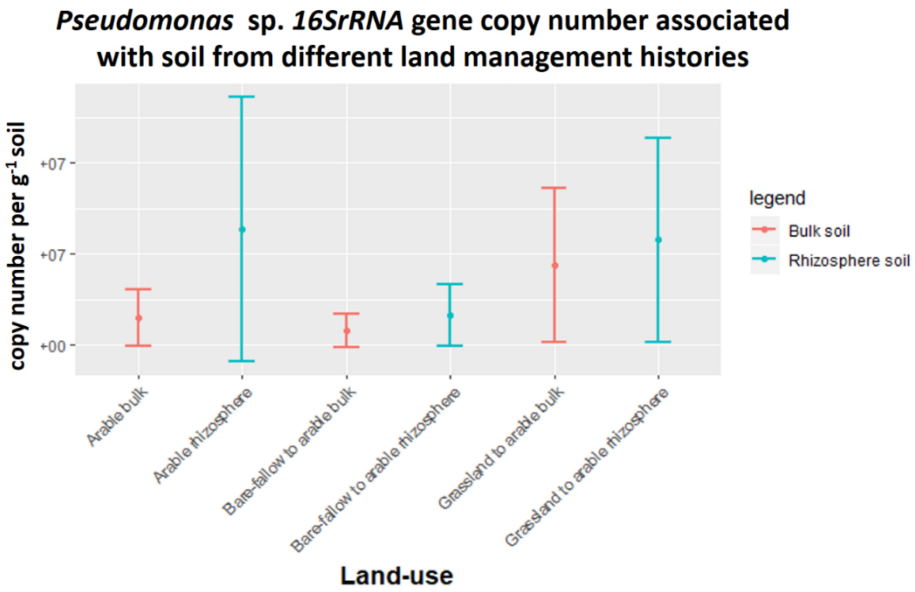


Figure 3.8: *Pseudomonas* spp. *16SrRNA* gene quantification across bulk soil and rhizosphere communities of wheat grown in three contrasting land management histories: Results from quantitative PCR analysis of the *Pseudomonas* spp. *16SrRNA* gene across different land

managements at the Highfield experiment [a] qPCR calibration standard curve [b] qPCR amplification curve [c] *Pseudomonas* sp. 16S rRNA gene copy number ± standard deviation per g^{-1} of bulk soil from all 33 plots at the Highfield experiment. [d] *Pseudomonas* sp. 16S rRNA gene copy number ± standard deviation per g^{-1} of bulk soil and rhizosphere soil from the continuous arable and arable conversion plots at the Highfield experiment.

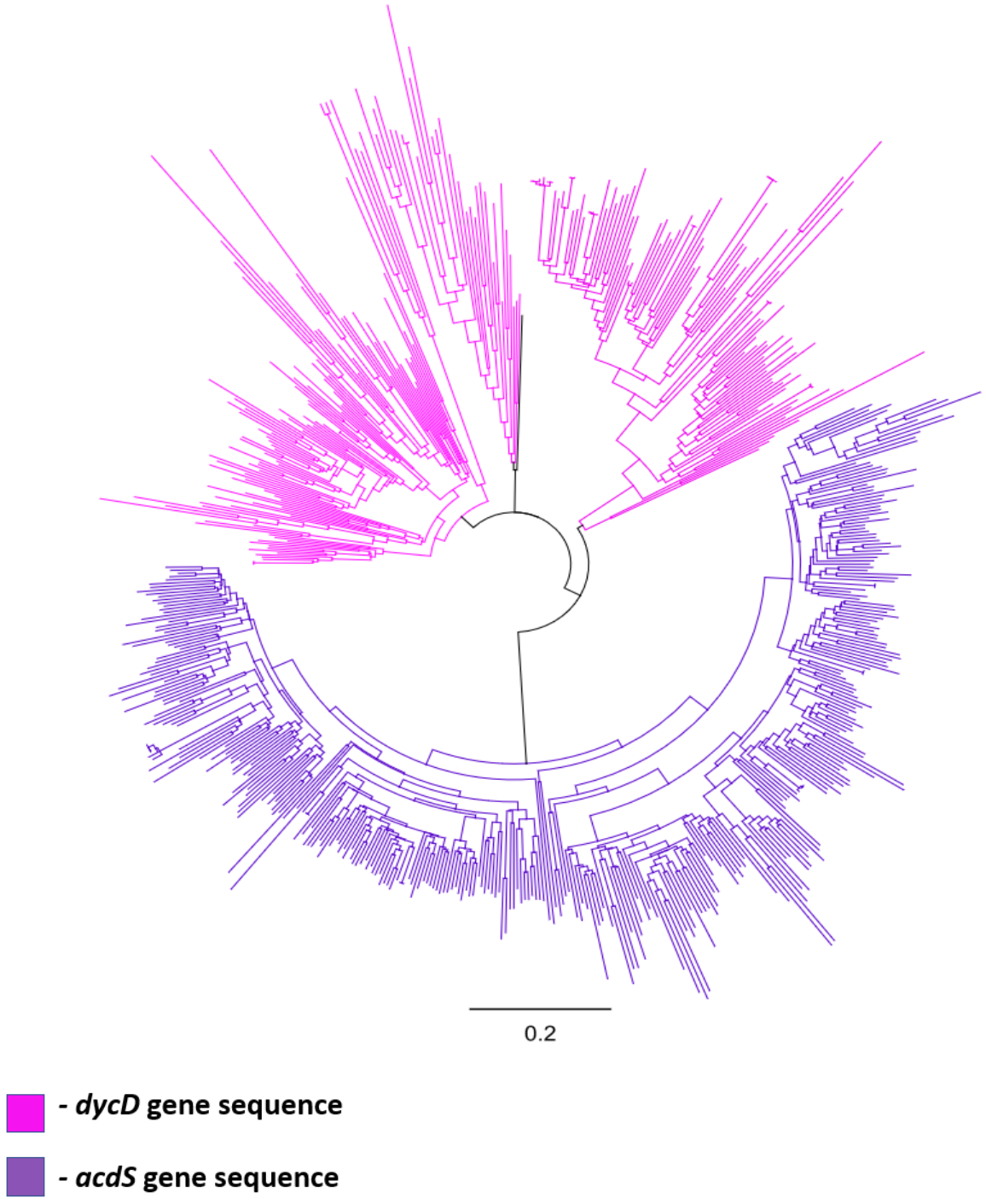
3.4.4 Abundance of the *acdS* gene

3.4.4.1 *acdS* primer analysis *in silico*

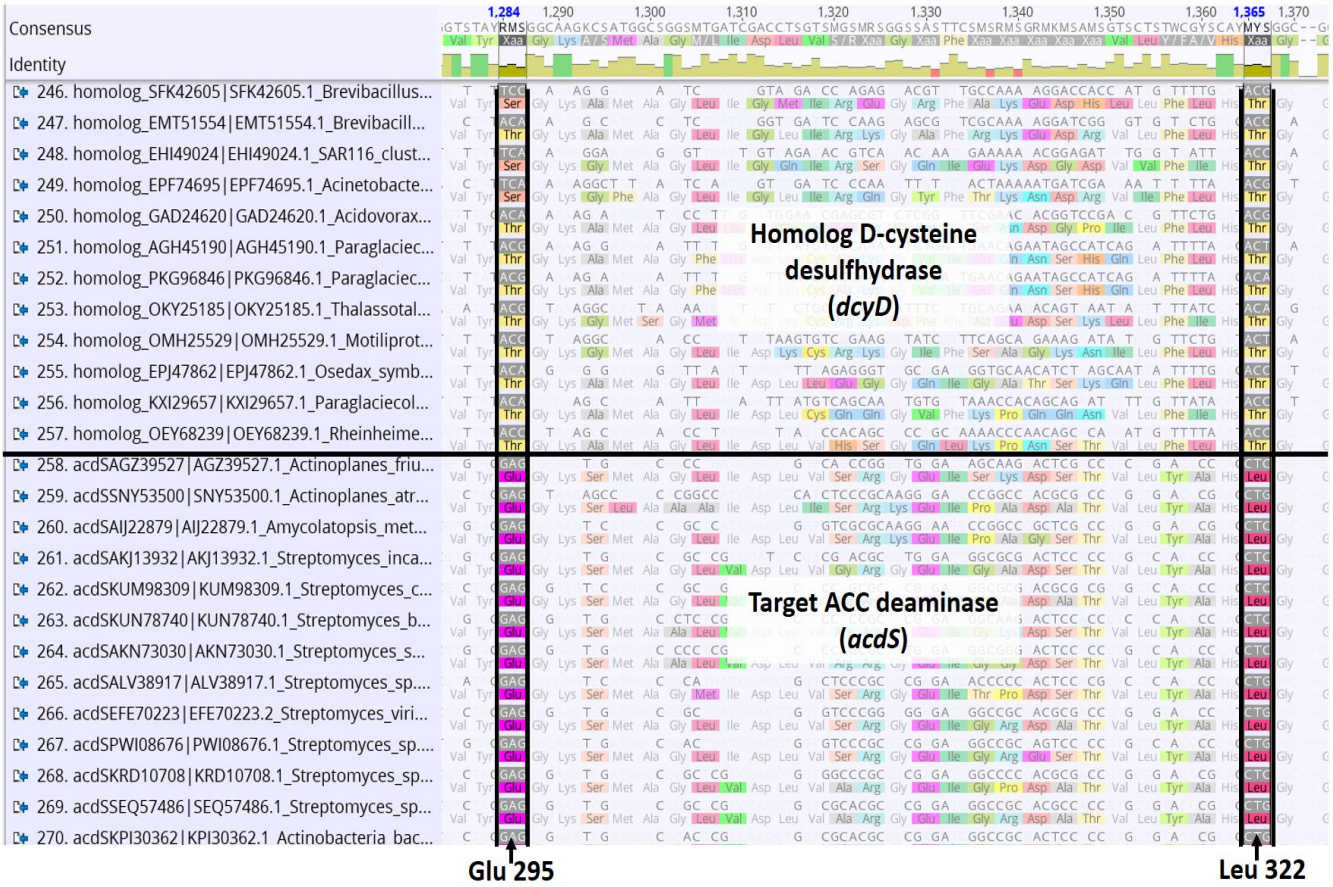
The *acdS* and *dcyD* DNA sequences downloaded from the uniProt database separated clearly from each other when visualised in a phylogenetic tree (Figure 3.9 [a]). Additional analysis of the DNA sequences for the key amino acids Glu 292 and Leu 322 (amino acid position in the *acdS* reference *Pseudomonas* sp. UW4) found that sequences annotated as *acdS* had these amino acids at the same aligned position, whilst the homolog sequences of *dcyD* did not have the corresponding amino acid in the aligned position (Figure 3.9 [b]). Collectively this evidenced that the annotated sequences downloaded as *acdS* were 'true' ACC deaminase genes, providing an ideal target to test *acdS* primers available in the literature. The *in-silico* testing of primers acdSF3/R3 and acdSF5/R8 on the alignment of 602 DNA sequences (345 *acdS* and 257 *dcyD*) found primer acdSF5 from Bouffard *et al.* had the highest percentage binding to *acdS* sequences (three mismatches allowed) with 97% target binding (Figure 3.9 [c]) (Bouffard et al. 2018). Target binding progressively decreased as the number of mismatches allowed got smaller with 56% target binding found at zero mismatches. Despite this, the primer was not specific to *acdS*, as it bound also to 80% of the *dcyD* sequences (three mismatches). The primer acdSR8 did not have problems with specificity, only binding to the *acdS* sequences at all levels of mismatches allowed. Conversely, both primers acdSF3/R3 taken from Li *et al.* showed high specificity to *acdS* sequences, with neither binding to sequences of the homolog *dcyD* (Li et al. 2015). Despite this, the primer pair had a maximum target binding

of 88% and 68% respectively for the total *acdS* sequences utilised in this alignment. The *in-silico* analysis implicates primer set acdSF3/R3 as a better candidate for qPCR analysis compared to the acdSF5/R8 primers.

[a]



[b]



[c]

Primer pair	Primer	*acdS* target binding				*dcyD* homolog binding			
		Mismatch number				Mismatch number			
		0	1	2	3	0	1	2	3
acdSF5/R8 (Bouffaud *et al* 2018)	acdSF5	56%	81%	92%	97%	30%	49%	68%	80%
	acdSsr8	13%	28%	46%	63%	0%	0%	0%	0%
acdSF5/R3 (Li *et al* 2015)	acdSF3	12%	25%	59%	88%	0%	0%	0%	0%
	acdSR3	3%	12%	37%	68%	0%	0%	0%	0%

Legend: High ↓ Low

Figure 3.9. The *in-silico* analysis of *acdS* primers available in the literature against *acdS* gene and *dcyD* (homolog) gene sequences [a] Phylogenetic analysis of *acdS* and *dcyD* gene sequences extracted from UniProt **[b]** *acdS* and *dcyD* gene sequence alignment highlighting the Glu295 and Leu 322 amino acid residues which are only found in the *acdS* gene and not the homolog *dcyD* **[c]** The percentage of *acdS* and *dcyD* sequences which the primers acdSF3/R3 and acdSF5/R8 bound to *in-silico*. A range of 0-3 primer mismatches were tested.

In addition to *in-silico* testing of the acdSF3/R3 and acdSF5/R8 primers they were also tested via qPCR. The primer set acdSF3/R3 showed primer dimerization, in both negative controls (H_20 and *acdS* negative organism)-see figure 3.10 [a]. The environmental reference sample and the *acdS* positive control organism both showed peaks that were slightly different, indicating that the primer set amplified products of slightly different size. Despite this, it's worth noting that gene size can vary across different species, therefore comparing an environmental sample consisting of various species to a singular reference species can result in slightly different sized products. The primers acdSF5/R8 amplified more similar product sizes in the environmental reference sample to the *acdS* positive organism, however a similar size product was also amplified for the negative control organism (Figure 3.10 [b]). Together with the *in-silico* analysis this suggested primer set acdSF3/R3 was more appropriate for use on the Highfield samples and was therefore used in further qPCR analysis.

[a] **[b]**

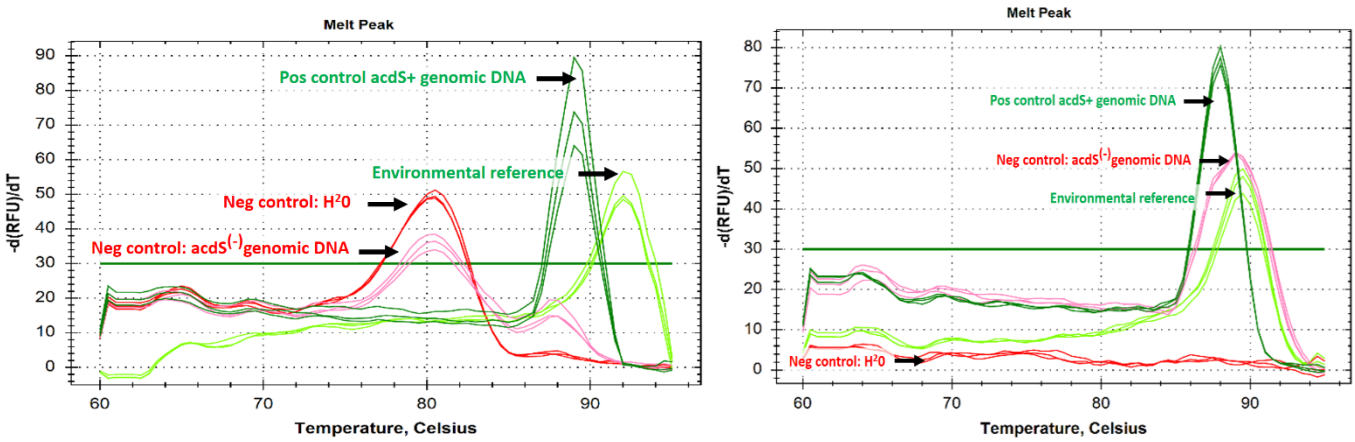


Figure 3.10. Quantitative PCR melt peak graph for the primer sets acdSF3/R3 and acdSF5/R8

Primer sets **[a]** acdSF3/R3 and **[b]** acdSF5/R8 were tested via qPCR utilising the genomic DNA of an organism known to have the *acdS* gene and a mixed environmental sample of DNA as positive controls. Genomic DNA from an *acdS* negative organism in addition to H_2O were used as negative controls.

3.4.4.2 *acdS* gene distribution

Microbial abundance of the *acdS* gene was successfully determined using qPCR with the primers acdSF3/R3 (r^2= 0.996) revealing significant differences across land managements (p <0.001) as seen in Figure 3.11. In the long-term treatments, *acdS* gene abundance varied considerably with the highest average abundance associated with the permanent grassland plots (6.0e+05 g^{-1}), lowest in the bare fallow plots (2.7e+04 g^{-1}), and intermediate values in the arable plots (2.5e+05 g^{-1}). Converting permanent grassland to arable or bare fallow decreased *acdS* abundance but not significantly (averaging 2.9e+05 and 3.2e+05 g^{-1} respectively). Converting arable soil to bare fallow decreased *acdS* abundance but not significantly (averaging 1.4e+05 g^{-1}), whilst converting arable to grassland resulted in a 2.7-fold significant increase in abundance (averaging 6.7e+05 g^{-1}). Converting bare fallow to arable cropping had no significant effect on *acdS* abundance but converting to permanent grassland significantly increased abundance by 15-fold (averaging 4.0 e+05 g^{-1}).

Investigation of *acdS* gene abundance was additionally assessed in rhizosphere soil of wheat grown in the permanent arable and arable conversion plots, with significant differences found compared to bulk soil (p <0.001). Four-fold significant increases in *acdS* abundance from bulk soil to rhizosphere soil were detected in both the permanent arable and grassland to arable conversion plots. Conversely, an increase in the average *acdS* gene abundance was observed from bulk soil to rhizosphere soil in the bare fallow to arable plots, but this was not significantly different (Figure 3.12 [a]).

When looking at the *acdS* gene abundance across the different land managements, the gene copy number follows a similar trend to that of the bacterial *16SrRNA* gene and may reflect the higher DNA levels found in the grassland and arable treatments. To better understand the distribution of the *acdS* gene and its selection in the rhizosphere, it's important to also consider the abundance as ng^{-1} DNA in which results have not been adjusted to soil dry

weights (Figure 3.12[b]). There were significant differences in the distribution of the *acdS* gene per ng^{-1} DNA compared to g^{-1} soil, with a higher abundance of the *acdS* gene found in the arable (6.59e+02) and bare fallow to arable (6.19e+02) plots compared to the grassland to arable plots (4.87e+02). Despite significant differences between bulk soil and rhizosphere, there was no significant difference found between the three rhizosphere treatments.

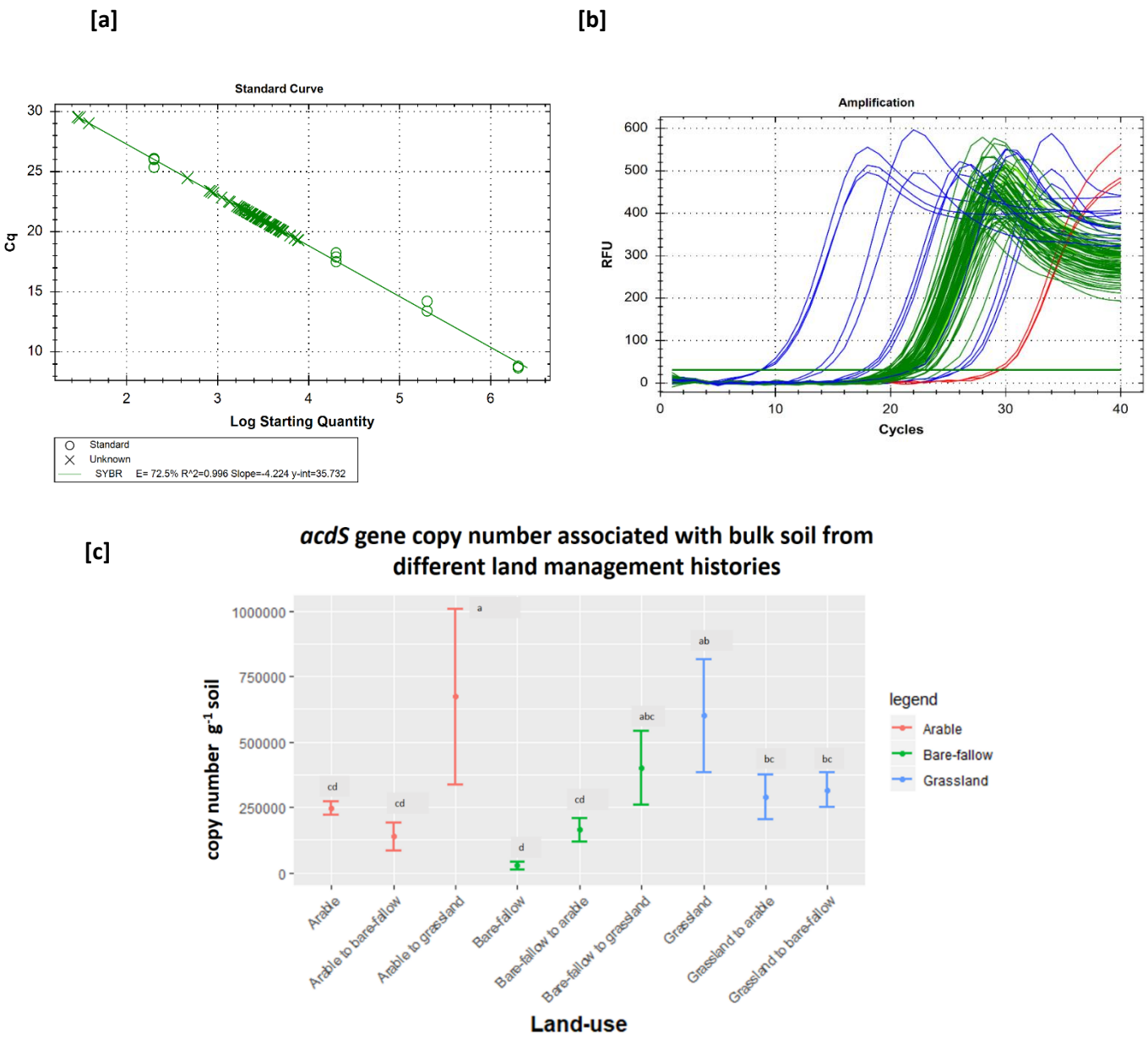
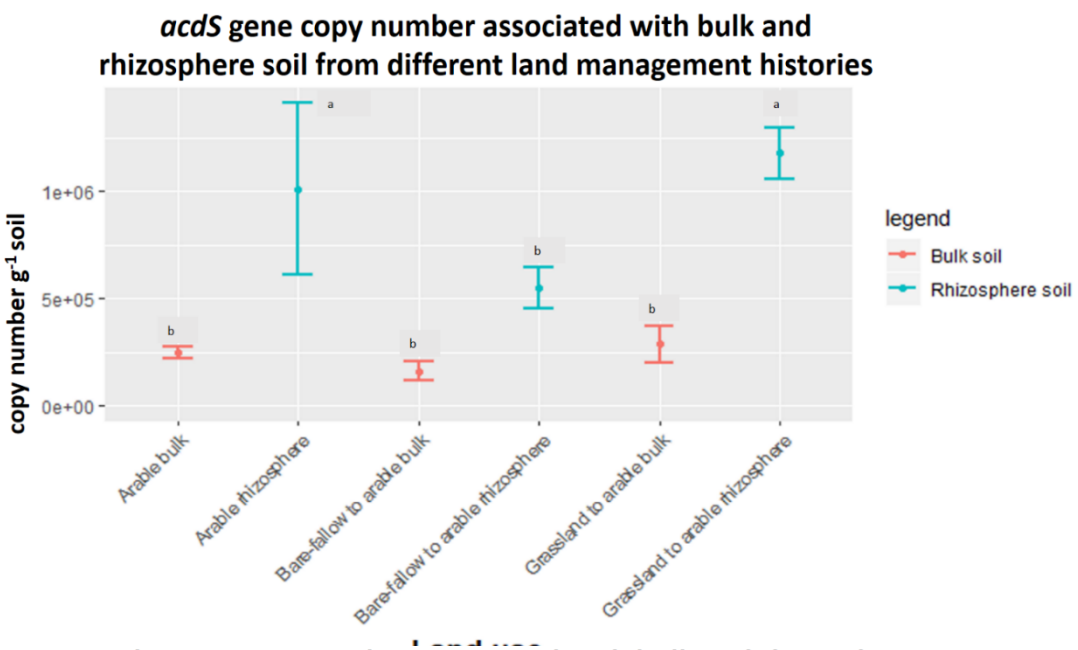


Figure 3.11. Bacterial *acdS* gene quantification across bulk soil communities in different land management histories: Quantitative PCR analysis of the bacterial *acdS* gene across the different land management plots at the Highfield experiment **[a]** qPCR calibration standard

curve **[b]** qPCR amplification curve **[c]** bacterial 16S rRNA gene copy number ± standard deviation per g^{-1} of bulk soil from all 33 plots at the Highfield experiment. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

[a]



[b]

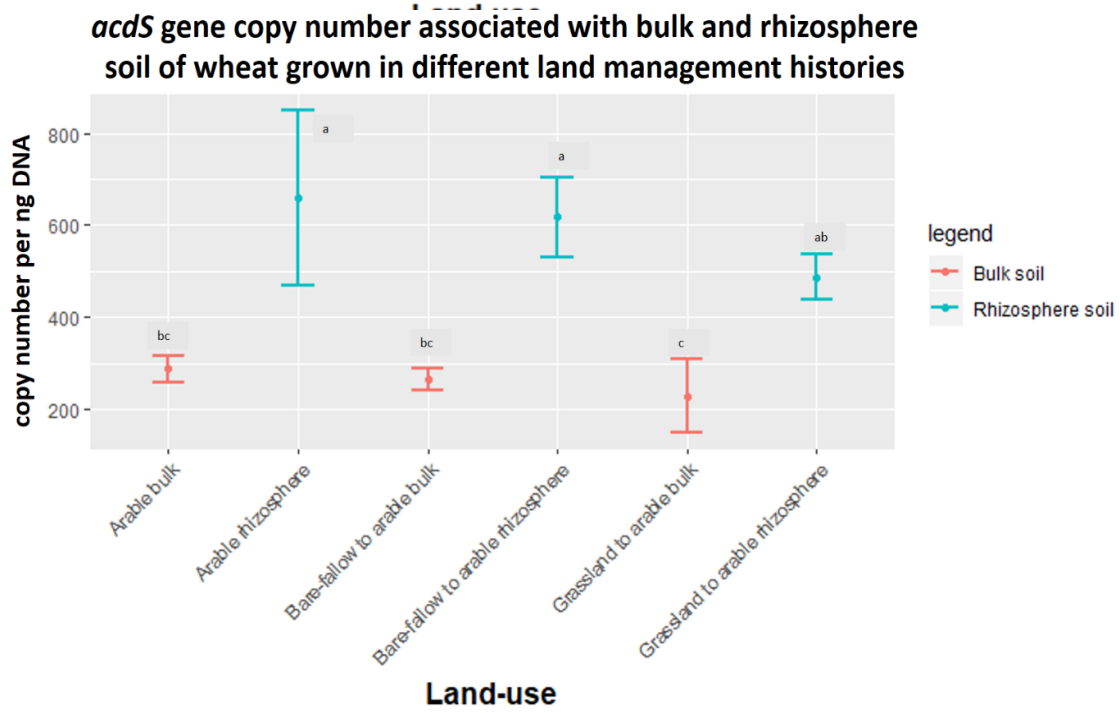


Figure 3.12. Microbial *acdS* gene quantification across bulk soil and rhizosphere communities of wheat grown in three contrasting land management histories: Quantitative PCR analysis of the microbial *acdS* gene across bulk soil and rhizosphere communities

associated with wheat [a] *acdS* gene copy number ± standard deviation per g^{-1} of soil [b] *acdS* gene copy number ± standard deviation per ng of DNA. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

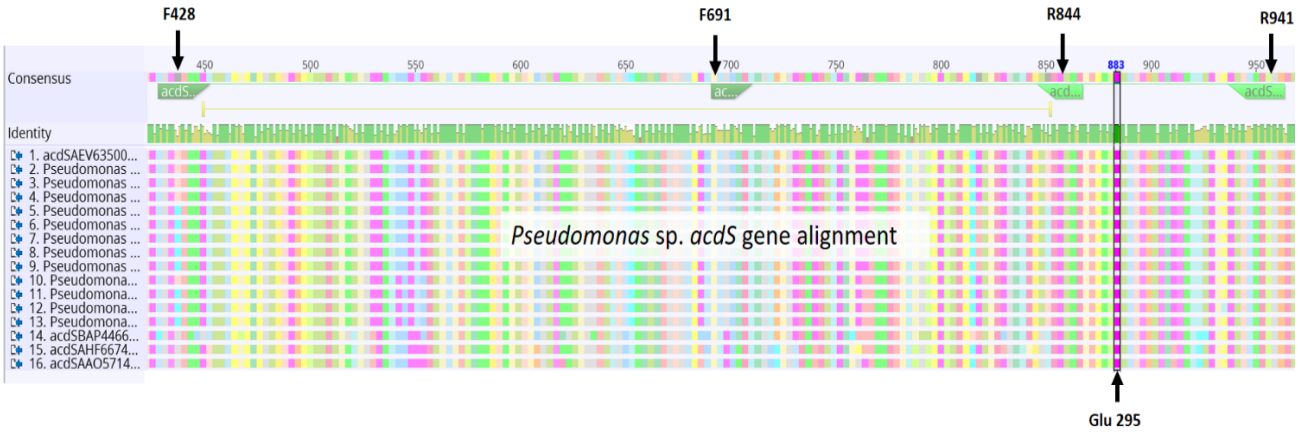
3.4.4.3 *Pseudomonas* specific *acdS* primers

Attempts were made to design *Pseudomonas* spp. specific primers for the *acdS* gene. Primers were designed *in silico* against the *acdS* genes of *Pseudomonas* spp. which resulted in four candidate primers shown in figure 3.13 [a, b]. The *in-silico* testing of all primers on the alignment of 602 DNA sequences (345 *acdS* and 257 *dcyD*) displayed specificity to the *acdS* gene with no binding observed against the *dcyD* homolog sequences- as seen in figure 3.13 [c]. Despite this, all primers displayed non-specific binding to the *acdS* gene of species other than pseudomonads, albeit this only accounted for a small percentage of the sequences tested (~1-5%). Target binding of the primers against *Pseudomonas* spp. ranged between 31-97% depending on the number of mismatches allowed.

[a]

Target gene	Primer	Sequence 5'-3'
acdS gene in *Pseudomonas* spp.	F428	AGGCSATGRRCGAKGTSGTSGGRCG
	F691	ACCAAGGCACAGATMCTGCG
	R844	CARCACRCCTTCAAGSCKGCCG
	R941	GTGTGCGTAAAGCACYTTSGARCCTTC

[b]



[c]

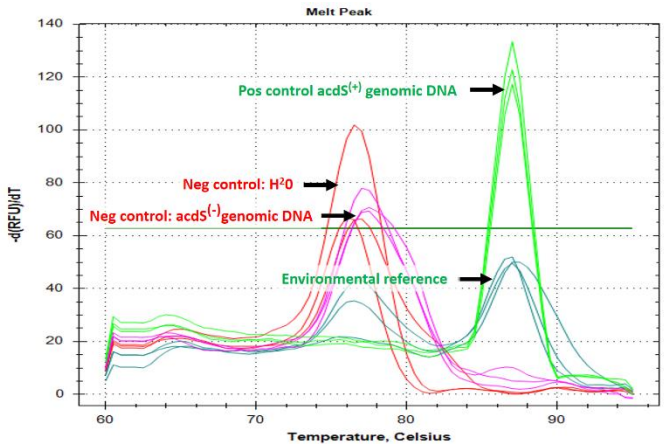
	acdS pseudomonad sequences				*acdS* bacterial sequences				*dcyD* sequences			
	mismatches				mismatches				mismatches			
Primer	0	1	2	3	0	1	2	3	0	1	2	3
acdSF428	0.00%	31.00%	81.00%	81.00%	0.00%	0.00%	0.30%	0.30%	0.00%	0.00%	0.00%	0.00%
acdSF691	69.00%	81.00%	81.00%	81.00%	0.00%	0.90%	3.00%	5.00%	0.00%	0.00%	0.00%	0.00%
acdSR844	50.00%	81.00%	88.00%	94.00%	0.00%	0.00%	0.30%	1.80%	0.00%	0.00%	0.00%	0.00%
acdSR941	81.00%	81.00%	88.00%	94.00%	0.00%	0.00%	1.20%	3.80%	0.00%	0.00%	0.00%	0.00%

Figure 3.13. The design and *in silico* analysis of *acdS* primers specific to *Pseudomonas* spp.

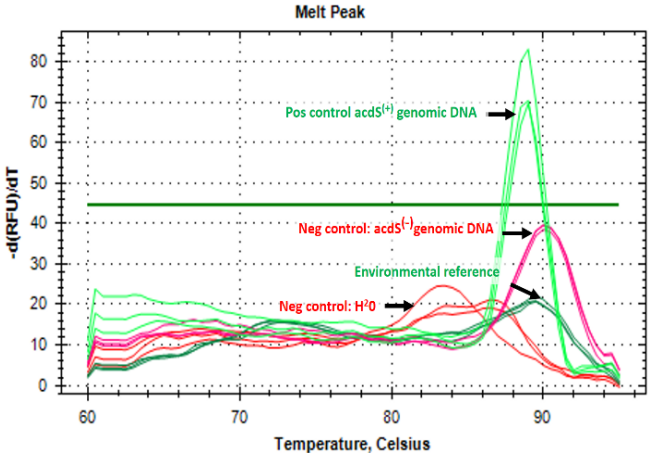
[a] Table of degenerate primer sequences designed with intention to amplify the *acdS* gene in *Pseudomonas* spp. only **[b]** Sequence alignment of the *acdS* gene in *Pseudomonas* spp. with primer binding shown on the consensus sequence - the Glu295 amino acid residue found in the *acdS* gene and not the homolog *dcyD* is additionally highlighted **[c]** The percentage of *Pseudomonas* spp. *acdS,* microbial *acdS* and *dcyD* homolog sequences that candidate primers bind to *in silico*. A range of 0 - 3 primer mismatches were tested against.

The candidate primers were tested in three qPCR reactions, with the melt peak data shown in Figure 3.14. The primer sets F481-F941 and F481-F844 showed strong levels of primer dimerization in negative controls whilst primers F481-F844 and F691-R941 displayed non-specificity with amplification of products in the *acdS* negative control DNA. The environmental reference sample and the *acdS* positive control DNA both showed peaks in similar positions using primers F481-F941, indicating that the primer set correctly amplified the same gene fragment in these samples. The F481-F941 primer set appeared to be the most target specific and was therefore utilised in a test qPCR analysis on the Highfield soil samples (see chapter 8.2.2). This analysis found that the *acdS* gene in pseudomonads was enriched in the rhizosphere of wheat grown in the permanent arable plots when compared to the bare fallow and grassland conversions to arable. Despite this, the levels of amplification in the environmental samples as shown in figure 3.16 were below the Cq threshold value. This indicates that further optimisation (and time- which was not available) is required to determine the efficacy of these primers for qPCR analysis.

[a]



[b]



[c]

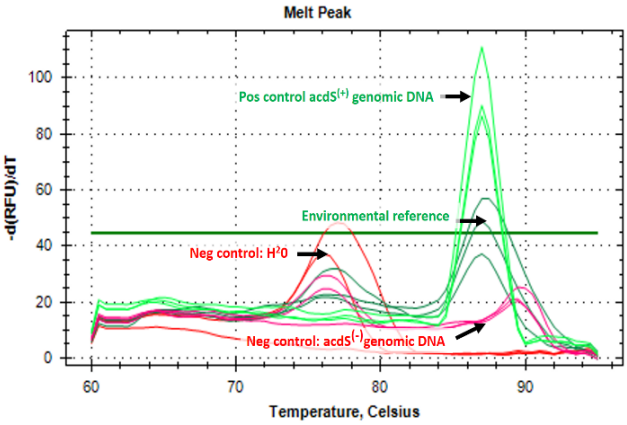


Figure 3.14. Quantitative PCR melt peak graphs for the analysis of primers to detect the *acdS* gene in pseudomonads only: qPCR was performed with three primer sets that were designed to amplify the *acdS* gene specifically in pseudomonads **[a]** primer set F481-F941 **[b]** primer set F691/R941 **[c]** primer set F481-F844. In each reaction two positive controls (*acdS* positive genomic DNA and environmental sample) and two negative controls (*acdS* negative genomic DNA and H_20) were utilised and are indicated by arrows on the graphs.

3.5 Discussion

3.5.1 Overview

Land management dramatically alters soil properties, the soil microbiome and crop growth (Muhammed et al. 2018; Doran and Zeiss 2000). The Highfield experiment at Rothamsted Research has previously highlighted both short- and long-term effects of arable, bare fallow and grassland treatments under permanent or conversion managements on such properties (Hirsch et al. 2009; Hirsch et al. 2017). In this study, the Highfield conversion plots were sampled 10 years after the treatments were initially established in 2008. Soil properties including C, N, moisture content and bacterial *16SrRNA* gene abundance all differed, with permanent grassland and conversion to grassland plots supporting a higher bacterial abundance compared to both arable and bare fallow treatments. Conversely, soil pH and *Pseudomonas* spp. *16SrRNA* gene abundance were found to be similar across the nine land use treatments.

Wheat yield, root dry weight and root GA3 content were all significantly lower in the bare fallow to arable conversion plots compared to permanent arable and grassland to arable treatments. Meanwhile, wheat roots sampled from the permanent arable treatment had significantly higher JA concentrations than both conversion plots, suggesting that land management impacts soil properties that consequently alter plant physiology. The rhizosphere soil of wheat was also sampled from the permanent and conversion arable plots, revealing increases in bacterial abundance in rhizosphere soil compared to bulk soil. Conversely, bacterial abundance in bulk soil and rhizosphere soil of wheat grown in the bare fallow to arable treatment did not differ significantly. Interestingly, *Pseudomonas* spp. abundance was found to be similar across bulk soil and wheat rhizosphere soil associated with each of the treatments.

Lastly, the *acdS* gene encoding for the phytohormone modulating enzyme- ACC deaminase-

differed with soil management, with copy number per g^{-1} of soil more abundant in the grassland and arable treatments. The *acdS* gene was additionally enriched in wheat rhizosphere soil, with significantly higher gene abundance compared to bulk soil across each of the permanent arable and conversion to arable treatments. The highest abundance of the *acdS* gene per g^{-1} rhizosphere soil was associated with the grassland to arable plots, followed by the permanent arable and lastly the bare fallow to arable plots. Despite this, the abundance of the *acdS* gene per ng of DNA displayed increases associated with the permanent arable and bare fallow to arable plots compared to the grassland to arable treatment. This suggests that the abundance of microbes with the *acdS* gene increases in the presence of plants, however the distribution of *acdS* microbes increases within rhizosphere communities grown in soils with degraded properties.

3.5.2 Soil properties

Similar to previous observations during the first four years of conversion at the Highfield experiment, soil properties differed significantly across the 9 treatments (Hirsch et al. 2017). After 10 years since the establishment of conversion plots, soils are still recovering from the degradation caused by permanent bare fallow and arable land uses. Meanwhile, the grassland treatment continues to enhance moisture content, C% and N% of the conversion plots. Despite these qualities beginning to increase to levels found in the permanent grassland plots, the conversions were still significantly lower. This highlights the difficulty in restoring qualities associated with soil health once they have been degraded. The conversion of grassland managed soils to arable and bare fallow has continued to significantly decrease soil C, N and moisture content compared to the permanent grassland treatment. Although soil properties in the conversion plots had lower values, they have not yet reached that of permanent bare fallow and arable soils. These findings agree with studies that have associated plant inputs with increases in soil quality parameters, particularly soil C. Depletion of SOC pools in soils is a

global issue and a principal cause of degradation, particularly in semi-arid Mediterranean regions (Diacono and Montemurro 2010). Identifying short and long-term land use impacts on soil C is important in informing the prevention and recovery of soil degradation in future agricultural managements.

Although only basic soil measurements were investigated in this study, these properties can each have dramatic effects on plant physiology and growth. Soil moisture content can adversely impact plants and microbes when too high (waterlogged) or too low (drought) (Cavagnaro 2016; Yu et al. 2018; Voesenek and Bailey-Serres 2015). Higher soil nutrient content as in grassland managed plots are generally associated with increased crop growth (Lal 2006). Soil pH was additionally measured although it did not differ significantly across the treatments, which remains in line with previous studies of the Highfield experiment (Hirsch et al. 2017). Soil pH is a well-known driving factor for alteration of biological factors including shifts in the soil microbiome and crop growth. The Highfield experiment therefore provides an ideal experimental design to study non-pH related effects of land management (Lauber et al. 2009).

3.5.3 Wheat properties

Initial studies at the Highfield experiment found that wheat yields differed significantly in the first four years of conversions (Hirsch et al. 2017). Grassland to arable conversion plots enhanced yields compared to permanent arable, whilst bare fallow conversions resulted in significantly lower yields (Hirsch et al. 2017). After a further 10 years, yields of wheat in the grassland to arable plots did not significantly differ from continuous wheat, however the bare fallow to arable plots still had significantly lower yields compared to continuous arable and grassland to arable treatments. Furthermore, root dry weight of plants grown in the bare fallow to arable conversion was significantly lower. Together these findings suggest the degradation of soil properties in the bare fallow conversion plots continue to influence wheat

growth. Conversely, properties that increased wheat yields in the first four years of grassland to arable conversion plots appear to be stabilising, with yields on average now similar to the permanent arable treatment. Understanding factors contributing to differences observed in wheat yield and physiology is difficult, especially because some measurements such as soil moisture fluctuate considerably over a season. Soil moisture, C and N were lower in the bare fallow to arable plots and could have potentially contributed to physiological differences. In this study, soil physical parameters such as bulk density were not tested due to time constraints but would have helped to further identity driving factors impacting wheat productivity.

Root phytohormone analysis attempted to better understand differences in wheat physiological responses to the different land managements. JA, ABA and GA3 levels significantly differed in wheat roots grown in the different land managements. JA was significantly higher in the roots of wheat growing in permanent arable plots compared to the bare fallow and grassland conversions. JA is well studied for its role in adapting physiology in response to biotic attack and is often associated with defence against necrotrophic or herbivorous pathogens. Susceptibility to pathogens in intensive monoculture practices is well documented (de Bellaire et al. 2010; Piper 1996; Otani et al. 2019). Its therefore plausible that a higher number of pathogens in the permanent arable plots may have been present when compared to the bare fallow and grassland conversion treatments, which had not cultivated wheat for as long (70 years of permanent arable compared to 10 years of conversion plots). Conversely, crosstalk of phytohormones in response to various environmental stimuli makes the interpretation of these results difficult, since JA like other phytohormones is thought to play a role in many physiological responses at different concentrations. Further analysis to investigate the presence of well-known necrotrophic pathogens in wheat rhizosphere samples would have helped to further unravel these findings.

ABA plays a central role in plant stress responses including examples in both biotic and abiotic

stress, however in plant roots ABA is best studied for its role in adapting to low water availability. Higher ABA levels were associated with the permanent arable and grassland to arable plots compared to the bare fallow to arable plots. When looking at soil C, N and moisture content across the permanent and conversion arable plots, the grassland conversions had significantly higher C and N compared to the arable and bare fallow to arable plots in addition to higher moisture content compared to the bare fallow to arable plots. Since the root dry weights were lower in the bare fallow to arable plots, ABA could be playing a role in these physiological differences, perhaps as a result to lower C, N or moisture content in soil. Low ABA concentrations (in ABA-deficient mutants) have been associated with shorter primary root length, which in part can be due to increases in ethylene (Sharp and LeNoble 2002). Higher levels of ABA have been associated with longer primary root length, by limiting ethylene induced inhibition of root elongation (Sharp and LeNoble 2002). Soil compaction results in shorter, thicker roots through the combined action of increased ethylene emission and lower root ABA levels (Tracy et al. 2012). Although soil physical properties were not measured in this study, the arable and bare fallow treatments have previously been shown to have higher bulk density, which could limit root growth (Neal et al. 2017; Watts et al. 2001). This again could explain the lower root dry weight from the bare fallow to arable conversion plots and warrants investigation in future experiments.

GA3 was significantly higher in wheat roots grown in the bare fallow to arable conversion when compared to the grassland to arable and permanent arable plots. Although GA3 is well known for its positive role in stem elongation, it can play different roles in plant roots. Low GA3 concentrations has been associated with root elongation (Bidadi et al. 2010). This could reflect the lower GA3 levels found in permanent arable and grassland to arable plots, in which significantly higher root dry weights occurred compared to the bare fallow to arable treatment. It's difficult to pinpoint variables that altered root development in wheat grown in the bare fallow to arable plots, however lower C, N and moisture content again likely played

some role. Together the lower levels of ABA and higher levels of GA3 could have played key roles in the physiological response of wheat grown in the degraded bare fallow to arable treatment.

Collectively, several factors associated with the different land managements could affect wheat phytohormone levels and physiology. Further studies with more detailed soil analysis are required to better interpret these results. Although some phytohormone levels differed significantly in wheat roots at the time of sampling (during flowering), the phytohormone profile may have varied considerably throughout the crop growing period. Since various environmental stimuli affect crop physiology, repeated sampling would have provided a more comprehensive view of the phytohormone status of wheat in the different land managements. Additionally, phytohormones may differ spatially within plant tissues therefore sampling of the aerial parts of the plant would have proved advantageous for unravelling differences observed across the different land managements.

3.5.4 Bacterial analysis

Abundance of the *16SrRNA* gene across the distinct land managements at the Highfield experiment revealed differences. The permanent grassland plots supported significantly higher abundance of bacteria, closely followed by both conversions to and from previous grassland compared to permanent arable and bare fallow plots along with bare fallow to arable and arable to bare fallow conversions. This highlights that plant inputs from grassland managements effectively restored properties that encourage bacterial growth. Indeed, the C content associated with the grassland soils was significantly higher which is likely to play a role in increased bacterial abundance, since C is one of the main limiting factors of bacterial abundance in soils (Demoling, Figueroa, and Baath 2007). Although conversions to grassland from previous bare fallow and arable treatments supported a lower abundance of bacteria compared to permanent grassland, no significant differences were found. Together this

indicates the potential of grassland treatments to help degraded soils recover microbial biomass and highlights the resilience of a previously managed grassland soil to resist rapid degradation.

Perhaps unsurprisingly, the permanent bare fallow soil supported the lowest number of bacteria with significantly lower abundance than all other treatments except for the permanent arable, arable to bare fallow and bare fallow to arable plots. Again, this is likely a reflection of the lower C content associated with these soil treatments. The abundance of bacteria is considered by many an indicator of soil health, which appears to be true in this study with the grassland treatments supporting higher microbial abundances.

Pseudomonas spp. 16S rRNA gene abundance was also analysed due to the important role that this species can play in plant-microbe interactions. Interestingly, abundance of this bacterial genus was similar across all treatments. A study by Hirsch *et al* also looked at the Highfield plots used in this study but found contrasting results, in which the culturable pseudomonads in bulk soil were 100-fold less abundant in the bare fallow soil compared to the grassland treatment (Hirsch et al. 2009). One reason for this could be due to the differences in time of year sampled, where changes in weather may have influenced many abiotic and biotic factors that can shape pseudomonad community abundance. Although *Pseudomonas* spp. abundance did not appear to differ across the managements, the analysis of functional qualities may have revealed potential impacts of land management on this agriculturally important species. The *Pseudomonas* genus is large and diverse, with many members found in a variety of environments (Preston 2004). In this study, it appears that the pseudomonads are resilient to changes in soil degradation, which highlights the potential of this bacterial species to be used as a microbial inoculant in contrasting agricultural soil systems.

The rhizosphere soil of field wheat grown in the permanent arable and conversion to arable plots was sampled to better understand plant-soil-microbe dynamics. Bacterial abundance in

the rhizosphere of wheat grown in the permanent arable and grassland to arable plots was significantly higher than in bulk soil. Conversely, bacterial abundance was on average higher in the rhizosphere of wheat grown in the bare fallow to arable plots compared to bulk soil but it was not significantly different. Its interesting that wheat grown in the bare fallow to arable plot attracted lower abundances of bacteria compared to permanent arable and grassland to arable plots, despite bulk soil communities supporting similar bacterial abundance across the three treatments. This could be due to other microbial species such as fungi, archaea or viruses thriving in the rhizosphere of wheat grown in the bare fallow treatment. It could also be indicative of differences in plant selections, particularly since lower root dry weight was associated with wheat grown in the previously bare fallow soil. This suggests that root architecture was constrained, which could have altered plant-microbe interactions. *Pseudomonas* spp. abundance was also investigated in the rhizosphere since it is well known as having traits ideal for rhizosphere competency. Despite this, the abundance of pseudomonads again did not significantly differ across bulk soil or rhizosphere soil associated with the different arable treatments. This supports the culture analysis of pseudomonad abundance across bulk soil and rhizosphere communities found in chapter 2, with no significant difference found across these niches compartments. This highlights that both qPCR and cultivating pseudomonads are comparable methods for estimating pseudomonad abundance. Since the rhizosphere is a highly competitive environment, pseudomonads in this study were likely outcompeted by other organisms for nutrients and space in addition to potential predation by nematodes and insects (Mauchline et al 2017). When looking more closely at the permanent arable and grassland to arable treatments, there is large variation in the abundance within the rhizosphere. This could reflect the stochastic nature of rhizosphere colonisation from bulk soil however there appears to be no clear selection for pseudomonads related to the wheat rhizosphere across these treatments. Investigations into the more

closely associated root compartment of wheat may have revealed differences in pseudomonad abundance, however time did not permit this.

3.5.5 *acdS* analysis

The *acdS* gene is associated with modulation of plant ACC levels and is involved in plant-microbe interactions. The permanent grassland and conversions to grassland supported the highest abundance of the *acdS* gene, whilst bare fallow and arable treatments had lower *acdS* gene abundances. The bulk soil and rhizosphere soil of wheat grown in the permanent arable and conversion to arable plots additionally revealed differences, with rhizosphere soil having significantly higher *acdS* gene abundance compared to bulk soil. This is in agreement with other studies, showing increases of the *acdS* gene in the rhizosphere compared to bulk soil (Bouffaud et al. 2018). When looking at these results per g^{-1} soil, the permanent arable and grassland to arable plots had higher abundances of *acdS* than the bare fallow conversion. At first glance, the *acdS* gene in this study appears more associated with treatments that received plant inputs prior to conversion in 2008, compared to the bare fallow to arable conversion which was previously maintained as plant free. These findings could indicate evolutionary relationships formed over years of inhabiting soils that regularly receive plant inputs. Conversely, it's important to consider the lower amounts of DNA that were found in the bare fallow to arable plots when determining *acdS* distribution. This can be addressed by looking at the *acdS* gene copy number per ng of DNA rather than per g^{-1} of soil. Analysis per ng of DNA found that the permanent arable and bare fallow to arable plots were associated with higher *acdS* abundances in the rhizosphere compared to the rhizosphere of wheat grown in the grassland to arable plots. The arable and bare fallow soils present more stressed environments compared to the grassland, with different soil properties and plant responses. Plant stress can lead to increases of ethylene and its precursor ACC, which can thereby select for more *acdS* bacteria in the rhizosphere of wheat growing in degraded soils.

When looking at plant ACC levels found in the roots of wheat, there were slight differences in average across the treatments, however these were not statistically significant according to standard tests. The grassland to arable treatment was associated with a higher level of root ACC compared to the other plots. Since ACC deaminase bacteria can reduce plant ACC levels, it is plausible that increases in distribution of *acdS* bacteria in the bare fallow to arable and permanent arable plots may have modulated plant ACC levels and resulted in lower root concentrations (Belimov et al. 2009). Since primers used only had ~80% efficiency, the *acdS* communities in the Highfield plots is likely to be higher than the results found in this study. If time had permitted, reverse transcriptase qPCR would have been performed on soil samples taken at different time points to analyse *acdS* mRNA levels at various stages of the wheat growing period. This would have helped to understand if the *acdS* gene was expressed more in soils associated with stress, or soils associated with a long history of plant cultivation at different stages of the growing period.

Pseudomonads are well known for their roles in plant microbe interactions, therefore attempts to design *Pseudomonas* spp. specific primers were made to better understand the distribution of the *acdS* gene in an agriculturally relevant bacterial group. Time constraints limited the optimisation and validation of the primers designed, with 2 primer sets found to be inadequate for PCR due to amplification of non-specific products. Preliminary analysis of the Highfield experimental plots with primer set F441-R941 was carried out, despite levels of amplification for the environmental samples found to be below the Cq threshold. Increases in *acdS* pseudomonad abundance was associated in the permanent arable plots. This is interesting when considering the results from a pot experiment which utilised soil sampled at the beginning of 2018 from the Highfield permanent plots (supplementary chapter S8.2.3). Pseudomonads were cultured from bulk soil and the rhizosphere of wheat grown in permanent arable, grassland or bare fallow soil under glasshouse conditions. An increase in

pseudomonads with the *acdS* gene was additionally found in the rhizosphere of wheat grown in the permanent arable plots. Although root phytohormones were not analysed, several variables were measured including leaf chlorophyll content. Leaf chlorophyll was significantly lower in wheat grown in the permanent arable soil indicating potential responses to stress compared to wheat grown in the grassland and bare fallow soils. Although it's clear that more work is needed to validate the preliminary results with primers F441-R941, it provides a good starting point for further investigation into soil-plant-microbe interactions.

3.5.6 Conclusion

Land management affected soil properties, wheat physiology and microbial community structure. Parameters considered indicators of good soil health were more associated with the grassland managed soils, including increased C, N, moisture content and microbial biomass. These properties were degraded in the arable treatment, with the lowest levels found in the bare fallow plots. Despite these clear differences, conversion of arable and bare fallow soils to grassland highlighted the recovery and resilience potential of plant inputs. 10 years after conversion land use driven changes in soil properties still impacts plant biomass and can shape soil-plant-microbe interactions, with alterations in the microbial *acdS* gene observed. Distribution of the *acdS* gene was found in a higher proportion of the microbial communities associated with the more degraded arable and bare fallow to arable treatments, whilst a higher abundance per g soil was associated with the grassland to arable treatment. Overall this study highlights the impacts of land management on multiple factors including soil properties, wheat physiology and microbial composition, thereby providing a glimpse at the complexity of these dynamics. It's clear the effects of land management can persist for many years after converting to an alternative land use, which could have implications for ecosystem services and the restoration of degraded soils in agriculture.

4.0. The impact of land management on pseudomonad phytohormone gene abundance and community selection in wheat cropping systems.

4.1 Introduction

4.1.1 Land management

Land management can alter the chemical, physical and biological parameters of soil**.** Many studies have found changes in microbial community structure across differing land uses, but how this translates into functional shifts is currently not well known (Allan et al. 2014; Foley et al. 2005; Allan et al. 2015; Cardinale et al. 2012). Studies have so far centred around nutrient cycling with impacts on carbon, nitrogen and phosphorus cycling observed across different land-use intensities (Neal et al. 2017; Paula et al. 2014; Perez-Brandan et al. 2019; Hirsch et al. 2017). However, the impacts of land-use on other soil microbiome functions such as plant growth promoting traits including microbial phytohormones that can alter plant physiology are less studied.

4.1.2 Phytohormone genes and pseudomonads

Phytohormones play an important role in responding to changes in a plant's surrounding environment, including climate and a variety of soil properties. Some microbes produce phytohormones thereby potentially modulating plant physiology, whilst plant derived phytohormones have the potential to modulate signalling in root-associated bacteria. Therefore, microbial phytohormones provide an important target for studying plant-soil-microbe interactions across different environments. Various microbial taxa produce an array of phytohormones, however the beneficial pseudomonads are best characterised for modulating plant auxin, cytokinin and ethylene levels (Dodd et al 2010). Many studies have focused on identifying the biosynthesis pathways of bacterial phytohormones, in addition to understanding the associated plant growth promoting effects using microbial inoculants

(Remans, Spaepen, and Vanderleyden 2006). A relatively underexplored area of research is the distribution of bacterial phytohormone genes across a range of soil and plant environments, including different land-use intensities.

4.1.2.1 Auxin

Soil bacteria commonly produce the phytohormone Indole-3-Acetic Acid (IAA) via multiple pathways. Auxins can have various roles in promoting crop growth however high levels are inhibitory. There is also evidence that IAA acts as a signalling molecule in microorganisms, therefore plant produced IAA may act as a reciprocal signalling molecule in microbe-plant interactions (Malhotra and Sriuastava 2009). As previously discussed in section 1.4, there are 5 different IAA biosynthesis pathways identified in microbes, with three found in pseudomonads. These include the Indole-3-Acetamide (IAM), Indole-3-Acetonitrile (IAN) and Indole-3-Pyruvate (IPA) pathways which are all tryptophan dependant (Figure 4.1). Pseudomonad genes found in the IAM pathway include *iaaM* encoding the enzyme tryptophan 2-monooxygenase, which converts tryptophan to an IAM intermediate and *iaaH* encoding indole-acetamide hydrolase, which hydrolyses IAM to IAA (Spaepen, Vanderleyden, and Remans 2007). This pathway is perhaps the best characterised for plant-microbe interactions including both pathogens and non-pathogenic bacteria. The IPA pathway is similarly one of the better studied IAA biosynthesis pathways in bacteria. The *ipdC* gene found in some pseudomonads encodes for an indole-pyruvate decarboxylase enzyme, which catalyses the key step of IAA biosynthesis in the IPA pathway (Patten and Glick 2002a). Nitrilase enzymes encoded by *nit* genes are found in the IAN pathway, converting IAN to IAA. Nitrile hydratase enzymes encoded by *nthAB* genes have also been found in pseudomonads, which link the IAN and IAM pathways by catalysing the hydration of IAN into IAM, which is then converted to the final product IAA by indole-acetamide hydrolase (Vega-Hernandez, Leon-Barrios, and Perez-Galdona 2002). In bacteria, nitrilase and nitrile hydratase enzymes may have many roles

unrelated to IAA synthesis, with few studies exploring the involvement of these enzymes in IAA biosynthesis (Patek et al. 2009; Patten, Blakney, and Coulson 2013a; Coffey et al. 2010).

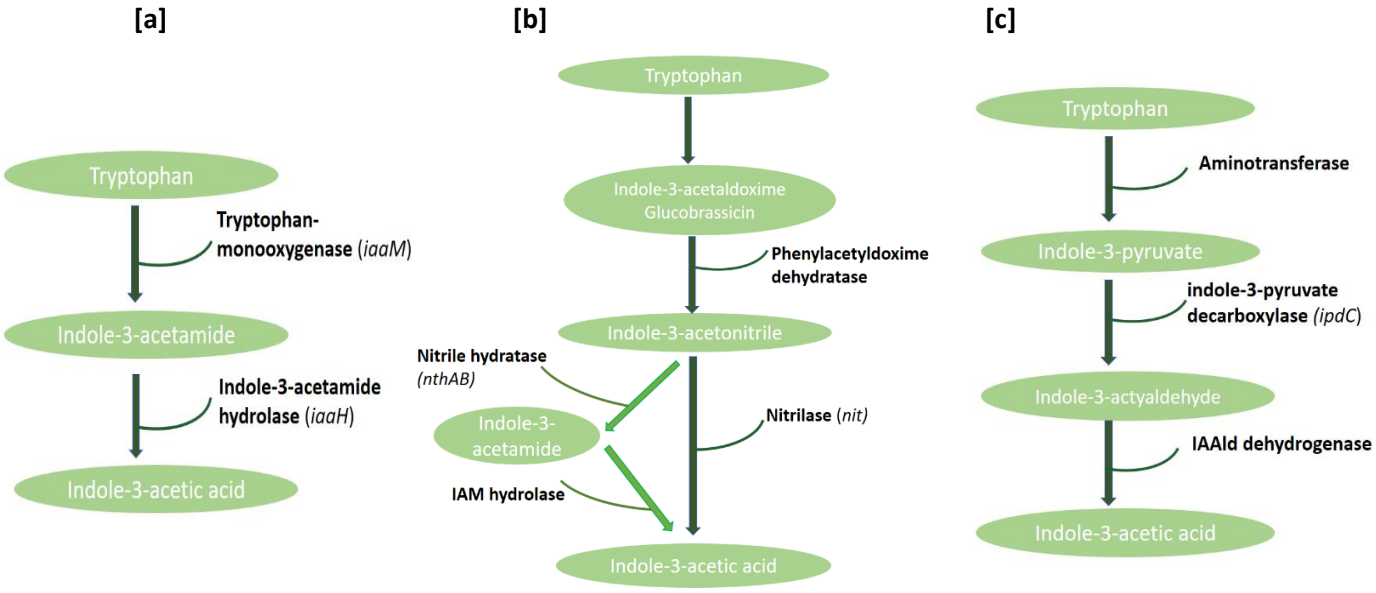


Figure 4.1: Three pathways of IAA biosynthesis found in *Pseudomonas* sp: Various IAA biosynthesis pathways have been identified in *Pseudomonas* species, including **[a]** the Indole-3-Acetamide (IAM) pathway- in the first step tryptophan monooxygenase (*iaaM* gene) converts tryptophan to IAM; in the second step IAM is hydrolysed to IAA by IAM hydrolase (*iaaH* gene) **[b]** The Indole-3-Acetonitrile (IAN) pathway- in the first step tryptophan is converted to indole-3-acetaldoxime by an unknown enzyme; indole-3-acetaldoxime is then converted to IAA by a nitrilase enzyme (*nit* gene). An additional pathway converts indole-3-acetaldoxime into IAM via a nitrile hydralase enzyme (*nthAB* gene), IAM is then hydrolysed to IAA via the final step of the IAM pathway **[c]** The Indole-pyruvate (IPA) pathway- in the first step tryptophan is transaminated to IPA by an aminotransferase. IPA is then decarboxylated to indole-3-acetaldehyde (IAAld) by the enzyme Indole-3-Pyruvate decarboxylase (*ipdC* gene) which is then oxidized to IAA by IAAld dehydrogenase.

4.1.2.2 Cytokinins

Similarly to auxin, cytokinins are best studied for roles in the promotion of crop growth. Natural cytokinins are adenine derivatives, which are classified by the configuration of their N^6-side chain as isoprenoid or aromatic cytokinins; small substitutions in these side chains have pronounced effects on cytokinin activity (Leonard et al. 1969; Schmitz et al. 1972). Isopentenyl transferase (IPTs) are key enzymes in the synthesis of cytokinins *in planta*, with two classes described based on the adenine substrate - these are the adenylate IPTs and tRNA IPTs (Figure 4.2). The best characterised are adenylate IPTs, which can result in the synthesis of isopentenyladenine (iP) and *trans*-zeatin (tZ) cytokinins from the addition of an isoprene side chain to the N^6 position of adenine. Both plant and bacterial cytokinin biosynthesis utilise IPT enzymes to achieve this, however the pathways can differ in both adenine substrate and the side chain donor. In plants the IPT enzyme appears to utilize both adenosine diphosphate (ADP) and adenosine triphosphate (ATP) coupled typically to dimethylallyl pyrophosphate (DMAPP), whereas the bacterial IPT utilizes adenosine monophosphate (AMP) coupled to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBDP) (Lincoln Taiz 2018). In plants this initial step results in iP type cytokinins, however further hydroxylation of the isopentyl side chain can result in tZ type cytokinins when the side-chain substrate is DMAPP. If the side-chain donor is HMBDP, tZ type cytokinins can be formed directly. The *ipt* gene characterised in *Agrobacterium* species is perhaps the best characterised IPT gene in bacteria (Ma and Liu 2009).

The tRNA IPT enzymes are involved in a different biosynthesis pathway, with the most abundant end product typically being *cis*-Zeatin (cZ) (Gajdosova et al. 2011; Schafer et al. 2015). The role of tRNA derived cZ in plants has been debated for some time. This can be attributed to the low abundance levels typically found in plants, leading to the conclusion that tRNAs are not a major source of cytokinin (Klambt 1992). Despite this, some plant species such

as maize and rice contain substantial amounts of cZ, therefore warranting further investigations into their importance and role. Biosynthesis begins with prenylation of an isopentenyl group to an adenine found at position A37 in tRNAs, such as $tRNA^{LEU}$ or $tRNA^{SER}$ (Bjork 1995). DMAPP is commonly cited as the isoprene side-chain utilised in this biosynthesis pathway. An additional step of tRNA degradation is required before the released modified base can be classed as a cytokinin. The *miaA* gene encoding for a tRNA isopentenylpyrophosphate transferase was identified in *P. fluorescens* G-20 and is the only cytokinin biosynthesis gene associated with this bacterial species to date (de Salamone, Hynes, and Nelson 2001).

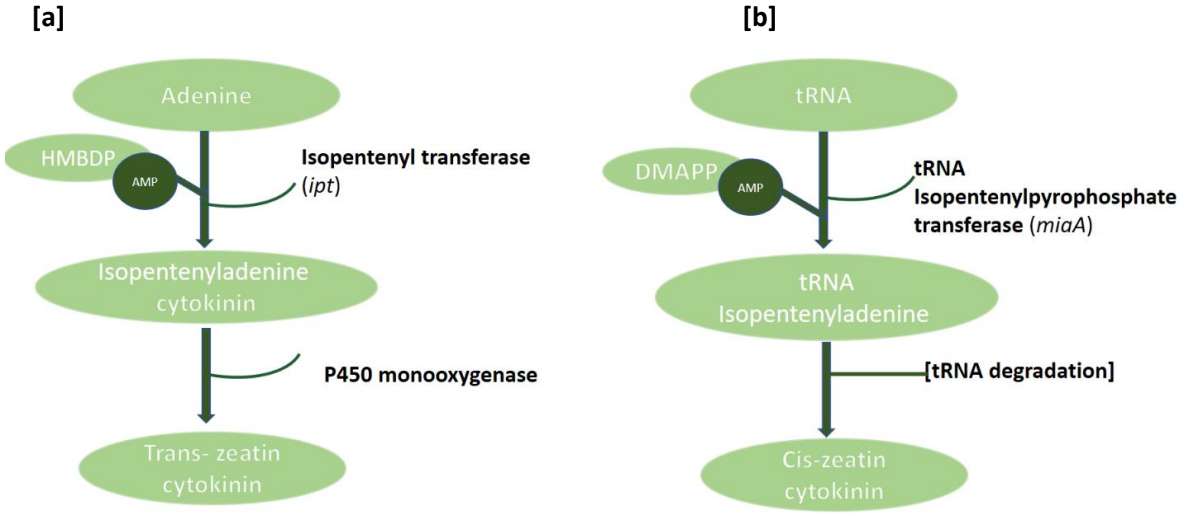


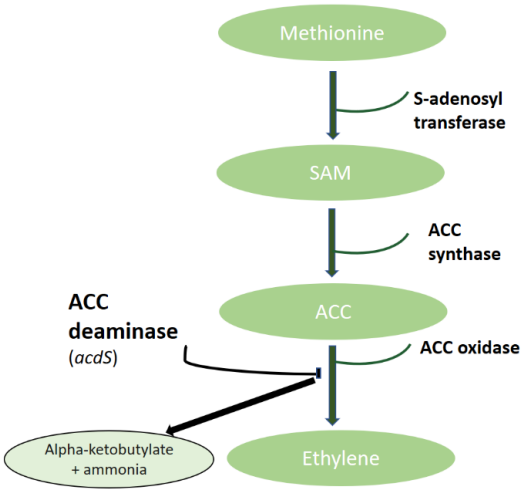
Figure 4.2: Schematic of cytokinin biosynthesis in bacteria: [a] The first step in iP and tZ cytokinin biosynthesis is the addition of the isopentenyl side chain from HMBDP coupled with AMP to the N^6 position of adenine catalysed by an adenylate IPT enzyme (encoded by *ipt* genes in some bacteria). The resulting iP cytokinin can then be converted to a tZ type cytokinin catalysed by a monooxygenase. **[b]** The first step in cZ biosynthesis is the addition of isopentenyl group from DMAPP to an adenine found at position A37 in tRNA, catalysed by an tRNA IPT enzyme (encoded by *miaA* in pseudomonads and other bacteria). The tRNA iP is only considered a cytokinin if the tRNA is then degraded, resulting in a cZ type cytokinin.

4.1.2.3 ACC deaminase

The enzyme amino cyclopropane carboxylate (ACC) deaminase can modulate the phytohormone ethylene, by degrading its immediate precursor ACC (Figure 4.3a). The enzyme catalyses the cleavage of ACC to α-ketobutyrate and ammonia. Ethylene is best studied for its role in stress responses, thus ACC deaminase producing bacteria commonly alleviate symptoms of plant stress by lowering ACC and consequently ethylene. Conversely, ACC provides a C and N source for bacteria able to utilise the substrate, thereby displaying a mutually beneficial relationship between plant and bacteria under stressful environments. ACC deaminase belongs to the tryptophan synthase β-subunit family of pyridoxal 5′-phosphate (PLP)-dependent enzymes (Todorovic and Glick 2008). The homolog D-cysteine desulfhydrase (DCyD) also belongs to this enzyme family, with both sharing the initial substrate catalysis step (Todorovic and Glick 2008). The amino acid residues Lys51, Ser78 and Tyr294 found in ACC deaminase from the model *Pseudomonas* sp. UW4 are essential for ACC deaminase activity, however they are also conserved in the homolog DCyD. This has resulted in an overestimation of true ACC deaminase-containing bacteria in the literature, with some primers yielding non-specific products. Conversely, the Glu295 and Leu322 residues in *Pseudomonas* sp. UW4 are unique to ACC deaminase, enabling better estimations of this enzyme in more recent years (Fujino *et al.*2004; Todorovic and Glick 2008). Most studies have typically focused on identifying the structural gene for ACC deaminase known as *acdS*. The ACC deaminase gene locus also contains the regulatory *acdR* gene encoding a leucine responsive regulatory protein (LRP)-like region upstream of *acdS*. The *acdR* gene has been shown to be essential for optimal functioning of the enzyme (Grichko and Glick 2000b; Li and Glick 2001; Cheng et al. 2008; Prigent-Combaret et al. 2008). LRP, cAMP receptor protein (CRP) and fumarate-nitrate reduction regulator (FNR) binding sites have also been evidenced in the promoter regions of the *acdS* gene and are involved in regulation of the enzyme as shown in (Figure 4.3b) (Prigent-

Combaret et al. 2008). Although mainly studied in bacteria and fungi, it's worth noting that some plants have also been found to have ACC deaminase genes including Arabidopsis (McDonnell et al. 2009).

[a]



[b]

Figure 4.3: Schematic of the role of ACC deaminase in reduction of ethylene levels and its biosynthetic gene locus: [a] Ethylene biosynthesis begins with methionine produced by the Yang cycle, this is converted to S'adenosyl-l-methionine (SAM) catalysed by S-adenosyl transferase. SAM is then converted to amino cyclopropane carboxylate (ACC) by ACC synthase, and consequentially converted to ethylene via ACC oxidase. Bacterial ACC deaminase can interfere with the ethylene biosynthesis pathway through degradation of ACC, thereby reducing the amount of ACC available for conversion to ethylene. **[b]** A schematic of the gene locus for ACC deaminase in *P. putida* UW4, showing the structural gene *acdS* downstream of the regulatory gene *acdR* along with Leucine responsive protein (LRP), fumarate nitrate reductase protein (FNR) and cAMP receptor protein (CRP) binding sites.

4.2 General Aim

4.2.1 Overall aim

To assess cultivable pseudomonad community abundance and diversity within bulk soil, rhizosphere and the root compartment of wheat grown in pot experiments utilizing soil from the 3 distinct land managements (arable, grassland and bare fallow) at the Highfield experiment, Rothamsted Research. The rhizoplane and endosphere of wheat was additionally focused on to understand if selection of pseudomonads increases within closer proximity to the root. As little is known about how microbial community structure translates into microbiome functioning, a further aim is to study the distribution of phytohormone genes within the pseudomonad communities. Gaining a better understanding of the distribution of pseudomonad community structure in addition to phytohormone genes across different land managements and niche compartments, may offer insights into farming practices that encourage beneficial plant-microbe interactions.

4.2.2 Specific objectives

In this chapter I aim to:

1. Assess the abundance of culturable *Pseudomonas* spp. isolated from bulk soil, the rhizosphere and the root compartment of wheat grown in pot experiments utilising soil from 3 land managements and create a culture collection of *Pseudomonas* spp.
2. Sequence the *gyrB* gene of isolates in the culture collection and identify isolates by sequence comparison of the *gyrB* gene using BLAST.
3. Assess community phylogeny of the isolates associated with bulk soil, rhizosphere and the roots of wheat grown in soil from 3 land managements.
4. Assess the distribution of the phytohormone genes *iaaM, miaA* and *acdS* in pseudomonad communities associated with bulk soil, the rhizosphere and the roots of wheat grown in soil from 3 land managements.

4.3 Methods

4.3.1 Pot experiments

4.3.1.1 Soil sampling and crop genotypes

Soil was collected from a depth of 20-30 cm for each of the continuous plots of grassland, arable and bare fallow managements at the Highfield experiment. The soils for each treatment were sieved (2 mm gauge), homogenised separately and subsequently used in pot experiments with the hexaploid spring wheat *Triticum aestivum* cv Cadenza. At the time of sampling, arable plots had been under continuous arable with winter wheat (most recently *T. aestivum* cv Hereward-seed coated with an insecticide/fungicide treatment of Redigo/Deter, Bayer CropScience) and receiving ammonium nitrate fertilisation to provide approximately 220 kg-N ha^{-1} y, with an additional 250 kg-K ha^{-1} and 65 kg-P ha^{-1} every three years for 68 years. Only the continuous management plots were sampled in this experiment. The corresponding plot numbers are 10, 17, 26, 30 (permanent grassland), 14, 20, 24, 33 (permanent arable) and 3, 4, 8 (permanent bare fallow)- please see figure 1.6 (chapter 1).

4.3.1.2 Cultivation of crops

Seeds were surface sterilized (75% ethanol wash for 30 seconds followed by 3% sodium hypochlorite, for 10 min and rinsed thoroughly with sterile water) and pre-germinated on filter paper soaked in sterile distilled water in petri dishes. After 36 hours, the pre-germinated seeds were sown into pots containing Highfield soil from the 3 different managements, with five replicates per treatment.

4.3.1.3 Sampling of the rhizosphere and root (rhizoplane + endosphere)

Rhizosphere soil was sampled at the wheat early flowering stage (61 zadoks) by breaking away the bulk soil to reveal soil closely adhering to the root system, which was removed from the

roots by gently dislodging into a sterile tube. To assess pseudomonad communities more closely associated to wheat, the rhizoplane and endosphere were sampled as one and referred to from this point onwards as the root compartment. Once the rhizosphere soil had been collected, unwashed roots were cut and ground using a mortar and pestle.

4.3.2 Bacterial isolation

4.3.2.1 Soil dilutions and isolation of *Pseudomonas* spp.

Homogenised rhizosphere soil and root (1 g) was taken for each sample in addition to bulk soil and diluted separately in 10 mL of sterilised distilled water followed by vortex mixing for 10 minutes to ensure bacterial cells were dislodged from soil particles or the root and in suspension. Serial dilutions were carried out to a dilution factor of 10^{-5} for bulk soil and rhizosphere samples and 10^{-7} for root samples. The suspensions were then spread onto *Pseudomonas* Selective agar (PSA, Oxoid) supplemented with Cetrimide, Fucidin and Cephalosporin (CFC) to select against fungus and other bacterial species. The plates were incubated at 28^{o}C for 24-48 hours. Random selection using the online random number generator (https://www.random.org) was used to select isolates from each treatment for subsequent creation of a *Pseudomonas* culture collection. Six isolates from each replicate (30 total per treatment) were randomly selected for further phylogenetic analysis.

	Bulk	**Rhizosphere**	**Root**	Total
Bare fallow	30	30	30	90
Arable	30	30	30	90
Grassland	30	30	30	90

Table 3.1: Culture collection of wheat grown in different land managements

A culture collection totalling 270 isolates, consisted of 30 isolates per niche compartment for each soil management utilised.

4.3.3 DNA analysis

4.3.3.1 DNA release

MicroLYSIS-PLUS (Microzone) was used for releasing DNA from the isolates per manufacturer's protocol.

4.3.3.2 PCR of the housekeeping *gyrB* gene

Each PCR reaction mixture was 24 µL in total and consisted of 10 x BioLine reaction buffer (2.5 µL), BioLine dNTP mix 25 mM each (0.5 µL), Bioline $MgCl_2$, 50 mM (0.75 µL), forward and reverse primers (both at 0.1 uM), BioLine DNA polymerase (0.25 µL), microLYSIS-PLUS DNA extract as a template (1 µL) and 18 µl of nuclease-free water. The PCR conditions were as below with PCR products (5 µL) examined on a 1.5% (w/v) agarose gel and gene products purified using the MinElute PCR purification kit (Qiagen) according to the manufacturer's instructions.

Degenerate primers and PCR programs for amplification of *gyrB* were as follows:

Forward (UP1): CAYGCNGGNAARTTYGA (Yamamoto and Harayama 1995)

Reverse (UP2r): CCRTCNACRTCNGCRTCNGTCAT (Yamamoto and Harayama 1995)

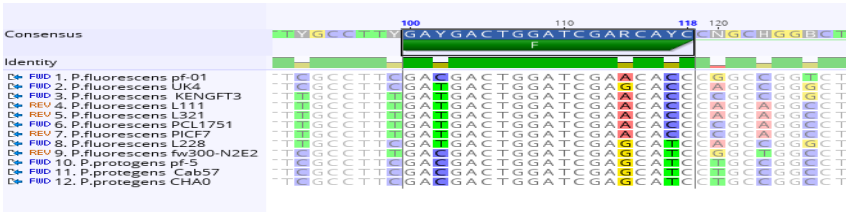
94°C for 5 mins followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 mins and a final elongation of 72°C for 5 mins.

5.3.3.3 Phytohormone primer design

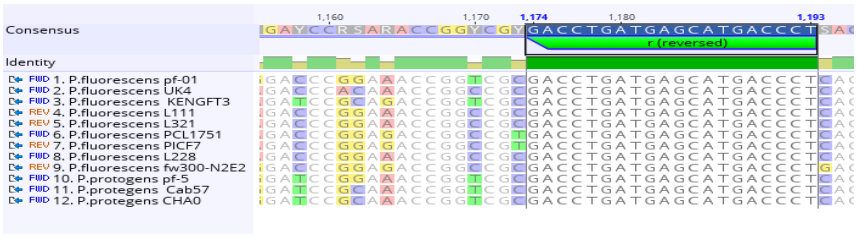
Primers for the ACC deaminase gene *acdS* were already available in the literature by Li *et al* and tested on beneficial pseudomonad strains, however primers for the *iaaM* gene and *miaA* gene in beneficial pseudomonads have not been as widely studied and were therefore designed for this experiment (Li et al. 2015). Reference sequences for *iaaM* and *miaA* genes found in *P. fluorescens* were obtained from the NCBI database and imported into the program Geneious. The sequences were multi-aligned utilising the MUSCLE alignment tool. Segments of

DNA sequence appearing to be conserved across all reference sequences were identified as potential primers. Candidate primers with the most appropriate physical properties such as melting temperature, GC content, base degeneracy number in addition to low dimerization values were chosen. Primer synthesis was carried out by MWG Eurofins.

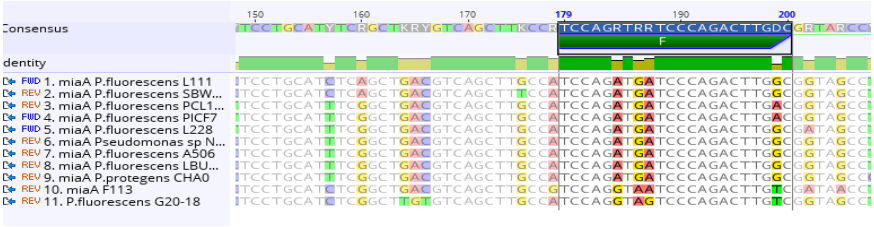
[a] *iaaM* forward primer



[b] *iaaM* reverse primer



[c] *miaA* forward primer



[d] *miaA* reverse primer

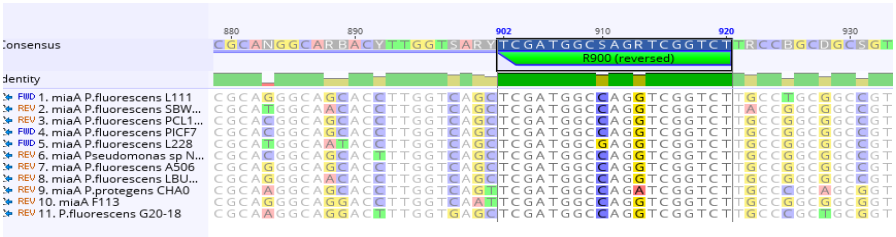


Figure 4.4: Phytohormone gene primer design: Primers were designed based on the aligned target gene sequences in *P. fluorescens* strains obtained from NCBI in the program Geneious. **[a]** Forward and **[b]** reverse primers for the target gene *iaaM* involved in auxin biosynthesis were designed based on conserved regions of DNA. Additionally, **[c]** forward and **[d]** reverse

primers for the target gene *miaA* involved in cytokinin biosynthesis were designed based on conserved regions of DNA.

4.3.3.4 Phytohormone genes PCR

The *acdS*, *miaA* and *iaaM* gene fragments were amplified via Polymerase Chain Reaction (PCR) utilising Microlysis-PLUS released DNA for all 270 isolates. Each PCR reaction mixture was 25 µL in total and consisted of 10 x BioLine reaction buffer (2.5 µL), BioLine dNTP mix 25 mM each (0.5 µL), Bioline $MgCl_2$, 50 mM (0.75 µL), forward and reverse primers (both at 0.1 µM), BioLine DNA polymerase (0.25 µL), microLYSIS-PLUS DNA extract as a template (1 µL) and 18 µL of nuclease-free water. The PCR products (5 µL) were examined on a 1.5% (w/v) agarose gel. The following primers and PCR programs were used:

Degenerate primers and PCR programs for amplification of *acdS* were as follows:

Forward (acdSf3): ATCGGCGGCATCCAGWSNAAYCANAC (26bp) (Li et al. 2015).

Reverse (acdSr3) GTGCATCGACTTGCCCTCRTANACNGGRT (23bp) (Li et al. 2015).

94°C for 2mins followed by 35 cycles of 94°C for 45s, 60°C for 30s and 72°C for 1min and a final elongation of 72°C for 5mins.

Degenerate primers and PCR programs for amplification of *miaA* were as follows:

Forward (miaAF-179): TCCAGRTRRTCCCAGACTTGDC (22bp)

Reverse (miaR-920): AGACCGAYCTSGCCATCGA (19bp)

94°C for 2mins followed by 25 cycles of 94°C for 45s, 60°C for 30s and 72°C for 1 min followed by a final elongation of 72°C for 5mins

Degenerate primers and PCR programs for amplification of *iaaM* were as follows:

Forward- (IAA-AOF-079): GAYGACTGGATCGARCAYC (19bp)

Reverse- (IAA-AOR-1172): >AGGGTCATGCTCATCAGGTC (20bp)

94°C for 2mins followed by 25 cycles of 94°C for 45s, 58°C for 30s and 72°C for 1 min 10s followed by a final elongation of 72°C for 5 mins.

4.3.3.5 DNA quantification and sequence analysis

DNA concentration and purity was analysed using the NanoDrop spectrophotometer ND-1500 (Labtech) following the manufacturer's protocol. Forward and reverse Sanger sequencing was carried out on the *gyrB* PCR product by MWG Eurofins, samples were prepared according to their instructions. Forward and reverse sequences were edited and aligned in the program Geneious. Consensus sequences were then multi-aligned using the software MUSCLE and subsequently trimmed to result in a sequence length of 879bp for each of the 270 sequences. Before phylogenetic construction, the J model test (2.1.10) was used to determine the best model fit for the alignment. A maximum likelihood tree was then constructed utilising a transitional model (TIM 012032) with 1000 bootstraps using the software PhyML (Guindon et al. 2010). The *gyrB* gene sequences of *Pseudomonas aeruginosa* PAO1 and *Pseudomonas fluorescens* F113 were retrieved from the NCBI website and included in the phylogenetic analysis for reference. Sequences were subsequently exported into iTol (https://itol.embl.de/) for viewing and visual amendments. The NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare sequences from individual isolates to the NCBI database.

4.3.4 Statistical analysis

Five replicates were used for each treatment (n=5). When determining CFU, each sample had three technical replicates which were averaged to help minimise human error. A one-way ANOVA was performed when comparing mean CFU across the different treatments. Where data did not follow a normal distribution, the results were LOG10 transformed before statistical analysis. Any significant results indicated were followed by the post hoc Tukey analysis, to identify which treatments differed significantly. All statistical analysis was performed in the software R studio.

4.4 Results

4.4.1 *Pseudomonas* spp. abundance

The mean Colony Forming Units (CFU) of *Pseudomonas* spp. isolated from 1 g of bulk soil, rhizosphere soil or roots associated with wheat, was determined and shown in Figure 4.5. All *Pseudomonas* CFU counts were similar across bulk soil and rhizosphere communities ranging from 10^5-10^6 mean CFU g^{-1}. Although some differences were observed, a one-way ANOVA found no significant differences across bulk soil and rhizosphere niche compartments associated with any of the land managements. The abundance of pseudomonads associated with the roots of wheat was considerably higher, with mean counts ranging from 10^7-10^8 CFU g^{-1} across all three land managements. The abundances were significantly different when comparing the root compartment with both bulk soil and the rhizosphere of wheat grown in each of the land managements (p<0.001). Additionally, differences were observed between the pseudomonad communities associated with wheat roots. The grassland soil supported a higher CFU g^{-1} compared to that of wheat roots grown in the arable soil (p=0.025 according to Tukey post hoc) and bare fallow soils.

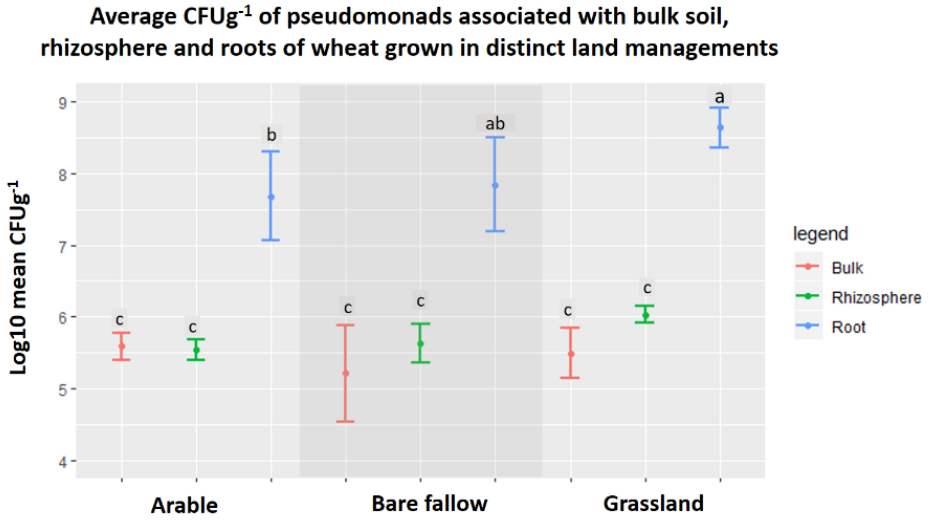


Figure 4.5: The abundance of *Pseudomonas* spp. isolated from bulk soil, the rhizosphere and roots of wheat grown in soils from three different land managements. The mean CFU g^{-1}

± standard deviation of *Pseudomonas* spp. isolated from bulk soil, the rhizosphere and the roots of wheat grown in soils from arable, grassland and bare fallow managed soils. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

4.4.2 Identification of *Pseudomonas* spp. isolates

4.4.2.1 Gel electrophoresis identification of gene fragments

The *gyrB* gene with an expected size of ~1280bp was successfully amplified from each of the selected 270 isolates via PCR, as indicated in figure 4.6.

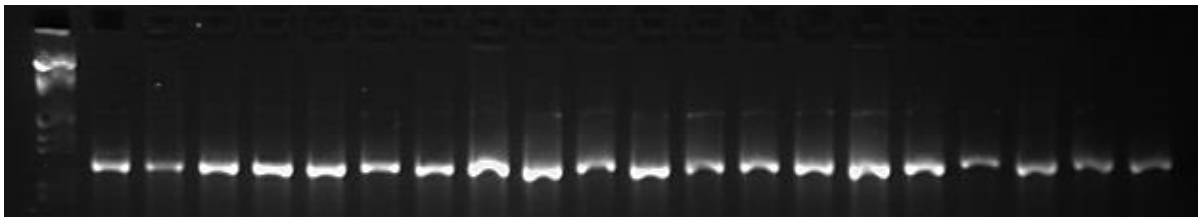


Figure 4.6: *gyrB* DNA fragments amplified from *Pseudomonas* spp. isolates: Gel electrophoresis of PCR products amplified with degenerate gyrB primers (UP-1 and UP-2r). Lane L, DNA marker (lambda DNA-Digested with HindIII); lanes 1 to 20, amplified fragments of the *gyrB* gene from 20 *Pseudomonas* spp. isolates.

4.4.2.2 *Pseudomonas* spp. phylogeny

PCR amplification and sequencing of the *gyrB* gene resulted in 267 forward and reverse sequences that could be aligned together successfully. Phylogenetic analysis of the *gyrB* gene was carried out, with a tree constructed and colour coded according to land management, as shown in figure 4.7 below. Upon visualisation, the pseudomonad communities associated with grassland and arable managed soils appear more diverse when compared to communities associated with the bare fallow soil. Indeed, there are two clades which are dominated solely by pseudomonads isolated from the bare fallow treatment. Despite this, there are clades in which grassland or arable isolates are more abundant compared to isolates from other

treatments. Additionally, phylogenetic trees were built to compare the different niche compartments of bulk soil, rhizosphere soil and the roots of wheat grown in soil from the 3 land managements- as shown in figure 4.8 a, b and c. The resulting phylogenies appear to offer similar information to the phylogenetic tree in figure 4.8.

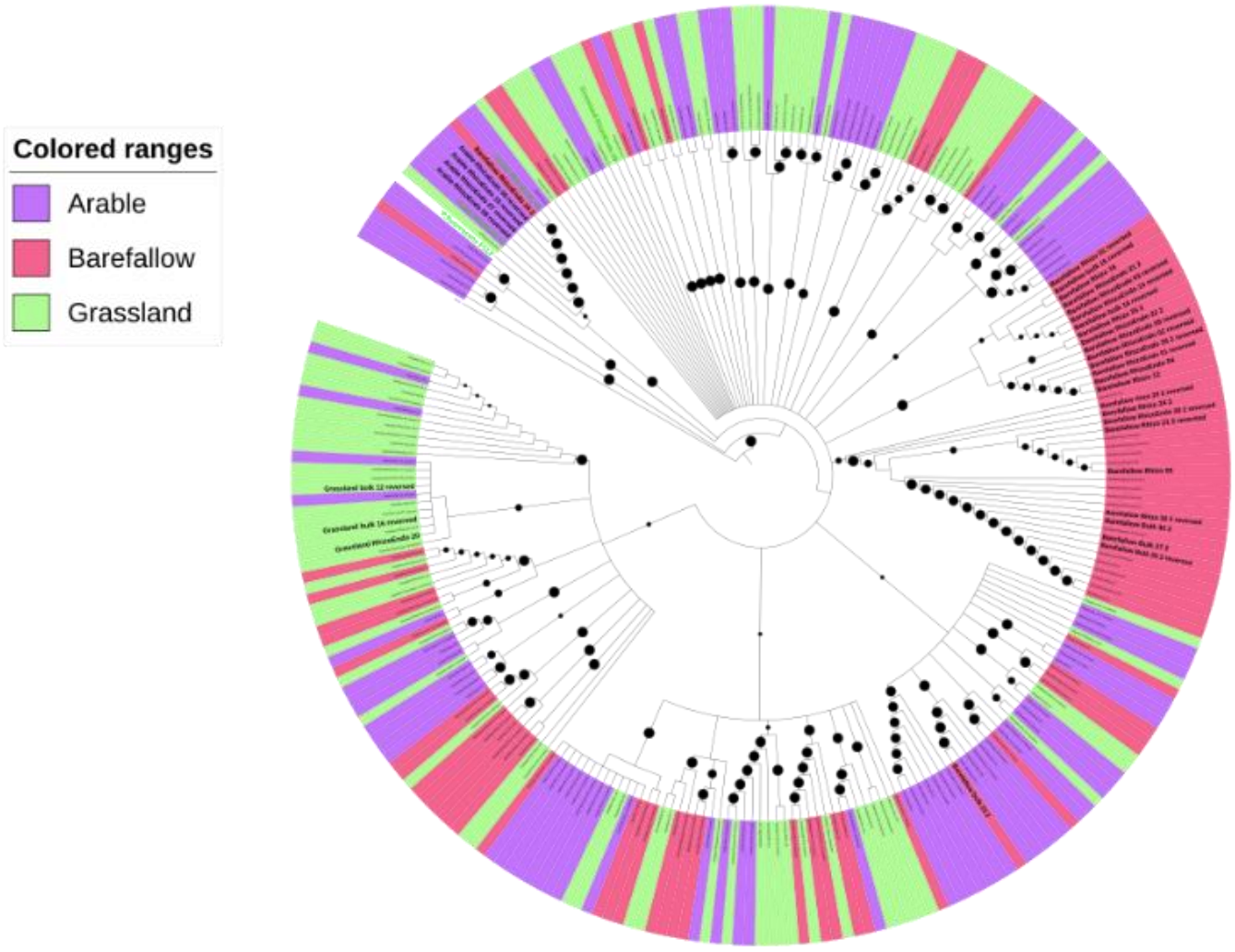
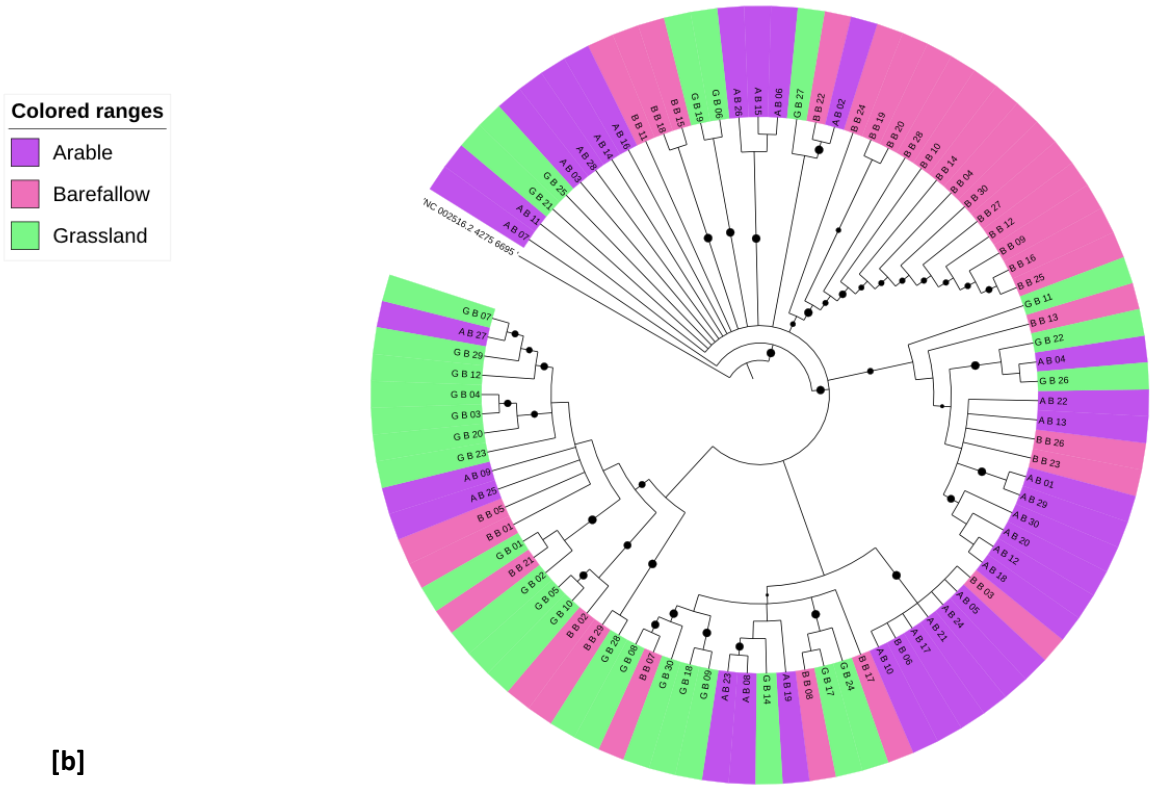
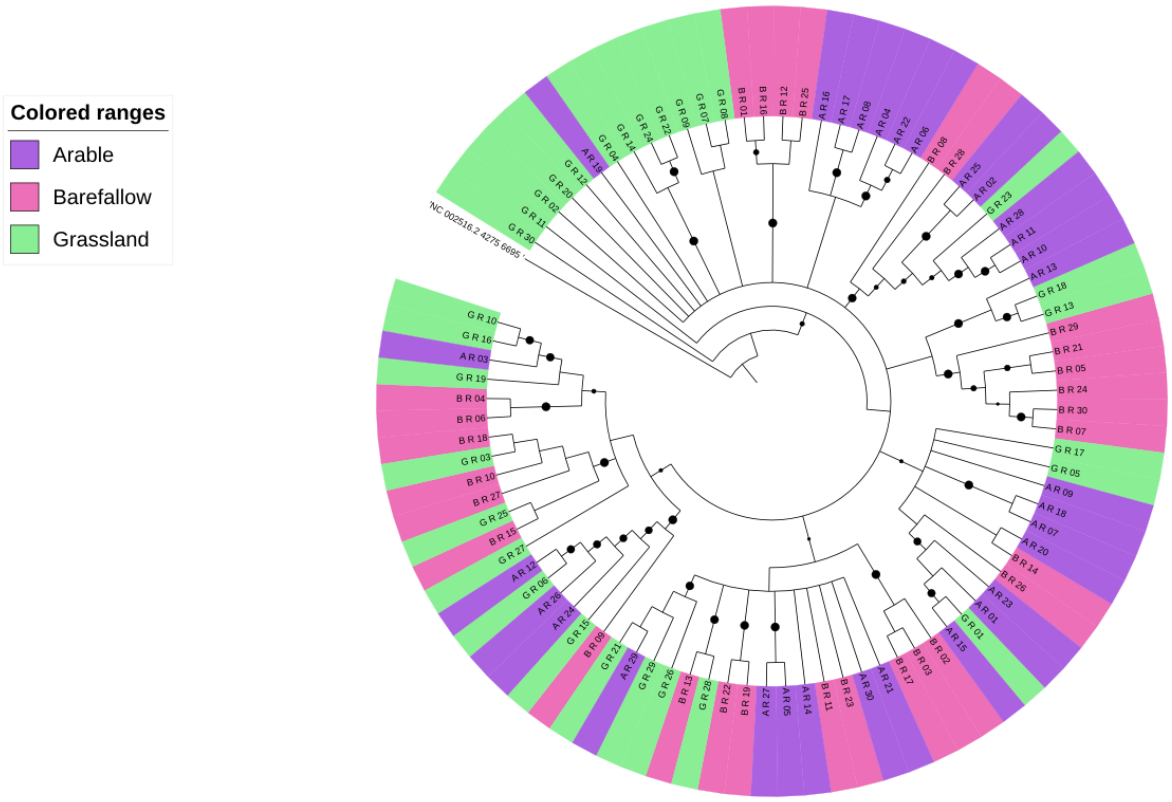


Figure 4.7: Maximum likelihood tree based on *gyrB* nucleotide sequences from *Pseudomonas* strains associated with wheat grown in 3 different land managements: The 276 isolates from bulk soil, rhizosphere soil and roots of wheat grown in three distinct land management regimes (bare fallow, arable and grassland) were phylogenetically analysed utilising an ML tree with a transitional model. Percentage bootstrap values higher than 70% of 1000 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to land management.

[a]


Colored ranges
Arable
Barefallow
Grassland
NC_002516.2:4275:6695

[b]


Colored ranges
Arable
Barefallow
Grassland
NC_002516.2:4275:6695

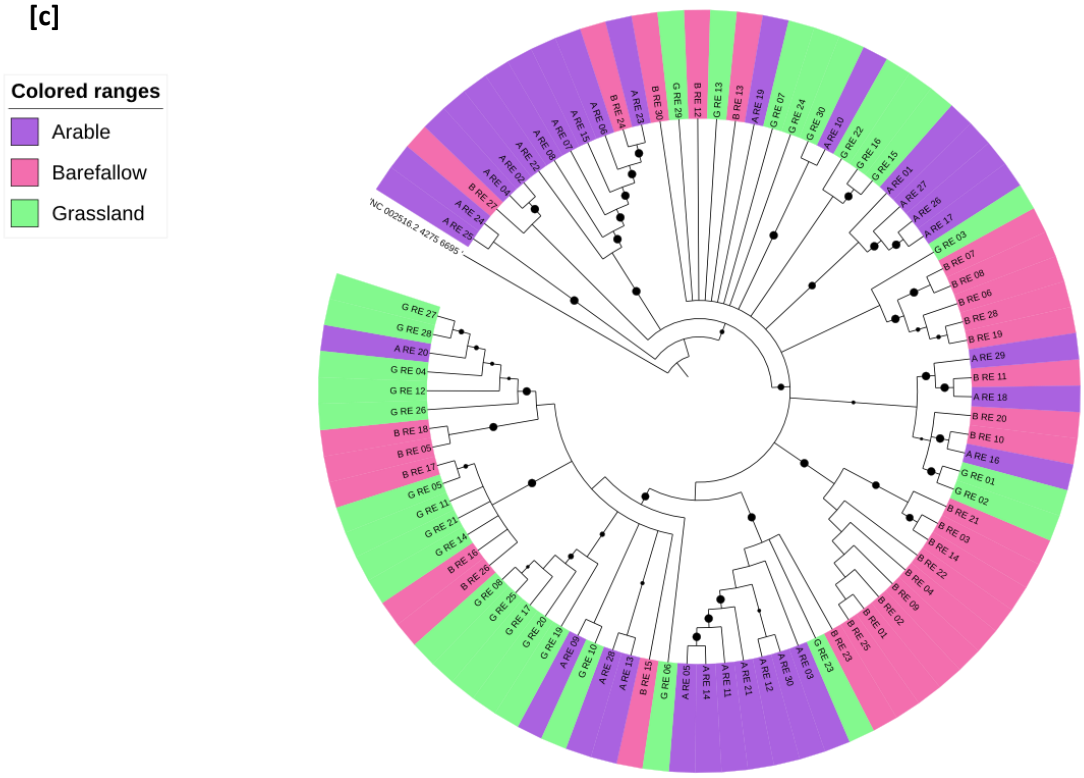
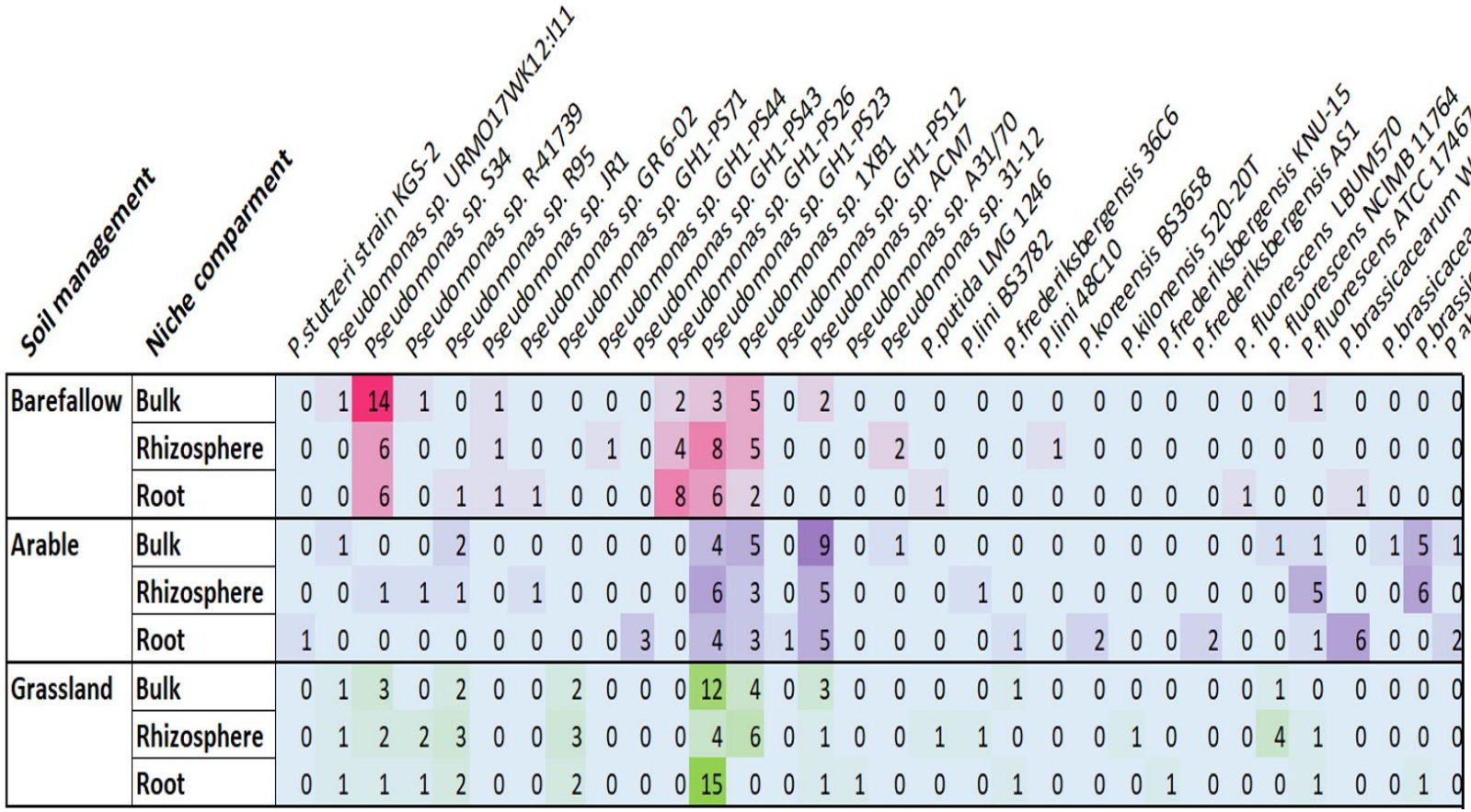


Figure 4.8: Maximum likelihood tree based on *gyrB* nucleotide sequences from *Pseudomonas* strains associated with wheat grown in three different land managements: Isolates from **[a]** bulk soil **[b]** rhizosphere and **[c]** roots of wheat grown in three distinct land management regimes (bare fallow, arable and grassland) were phylogenetically analysed utilising an ML tree with a transitional model. Percentage bootstrap values higher than 70% of 1000 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to land management.

4.4.2.3 BLAST identification

The consensus sequences generated for each of the isolates were cross referenced against the NCBI BLAST database to obtain a species identification. Overall 36 different strains were identified with the majority at ≥ 97% homology and all belonging to the *Pseudomonas* genus as shown in figure 4.9. There appears to be a higher abundance of isolates identified closely to

Pseudomonas spp. GH1-PS23 and *Pseudomonas* spp. 1XB1, which were found in bulk soil and the rhizosphere of wheat across all 3 soil managements. Isolates closely identified to each of these species were also present in the roots of wheat grown in arable and bare fallow soils but not the roots associated with grassland soil. Isolates with close homology to *Pseudomonas* spp. GH1-PS26 was strongly associated with the bare fallow soil treatment, with this species increasing in abundance in the rhizosphere and root compared to bulk soil. *Pseudomonas* sp. S34 was also associated closely with isolates in the bare fallow treatment, with a higher abundance found in bulk soil when compared to the rhizosphere and roots of wheat. This species was also identified in the arable and grassland treatments, albeit at a lower abundance. The arable treatment supported a higher abundance of isolates identified as *Pseudomonas* sp. ACM7 in each of the niche compartments, this species was not identified in the bare fallow or grassland treatments. *Pseudomonas* sp. GH1-PS71 was identified in grassland treatments across all 3 niche compartments, although at a relatively low abundance and was not identified in the arable or bare fallow treatments. The grassland treatment appeared to support the highest level of diversity, closely followed by the arable treatment and the lowest level of diversity associated with the bare fallow soil. Additionally, the diversity of pseudomonads across the different niche compartments of each soil treatment fluctuates. The highest diversity for each of the treatments was associated with either the root (bare fallow and arable) or the rhizosphere (grassland).



Soil management	Niche comparment	P.stutzeri strain KGS-2	Pseudomonas sp. URMO17WK12:I11	Pseudomonas sp. S34	Pseudomonas sp. R-41739	Pseudomonas sp. R95	Pseudomonas sp. JR1	Pseudomonas sp. GR6-02	Pseudomonas sp. GH1-PS71	Pseudomonas sp. GH1-PS44	Pseudomonas sp. GH1-PS43	Pseudomonas sp. GH1-PS26	Pseudomonas sp. GH1-PS23	Pseudomonas sp. 1XB1	Pseudomonas sp. GH1-PS12	Pseudomonas sp. ACM7	Pseudomonas sp. A31/70	Pseudomonas sp. 31-12	P.putida LMG 1246	P.lini BS3782	P.frederiksbergensis 36C6	P.lini 48C10	P.koreensis BS3658	P.kilonensis 520-20T	P.frederiksbergensis KNU-15	P.frederiksbergensis AS1	P. fluorescens LBUM570	P. fluorescens NCIMB 11764	P.fluorescens ATCC 17467	P.brassicacearum Wood1	P.brassicacearum 48H11	P.brassicacearum 38D7	P.ahietaniphila ATCC 700689T
Barefallow	Bulk	0	1	14	1	0	1	0	0	0	0	2	3	5	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	Rhizosphere	0	0	6	0	0	1	0	0	1	0	4	8	5	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Root	0	0	6	0	1	1	1	0	0	0	8	6	2	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Arable	Bulk	0	1	0	0	2	0	0	0	0	0	0	4	5	0	9	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	5	1
	Rhizosphere	0	0	1	1	1	0	1	0	0	0	0	6	3	0	5	0	0	0	1	0	0	0	0	0	0	0	0	5	0	0	6	0
	Root	1	0	0	0	0	0	0	0	0	3	0	4	3	1	5	0	0	0	0	1	0	2	0	0	2	0	0	1	6	0	0	2
Grassland	Bulk	0	1	3	0	2	0	0	2	0	0	0	12	4	0	3	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
	Rhizosphere	0	1	2	2	3	0	0	3	0	0	0	4	6	0	1	0	0	1	1	0	0	0	1	0	0	0	4	1	0	0	0	0
	Root	0	1	1	1	2	0	0	2	0	0	0	15	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0

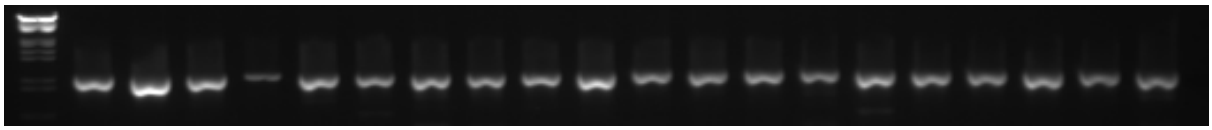
Figure 4.9: Identification of isolates based on *gyrB* gene sequences and compared against the NCBI BLAST database. A BLAST hit table showing strains isolated from bulk soil, the rhizosphere and roots of wheat grown in three distinct land management regimes (bare fallow, arable and grassland). Pseudomonad isolate number is represented in a heatmap to visualise differences associated with each treatment.

4.4.3 Phytohormone gene distribution

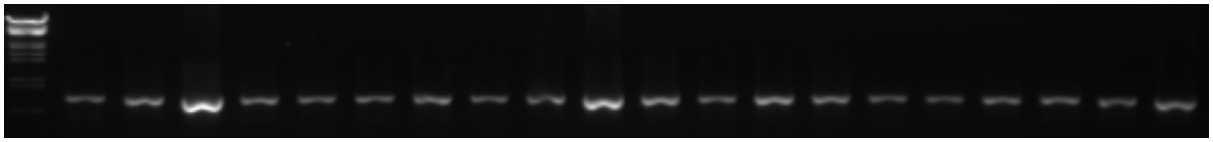
4.4.3.1 Gel electrophoresis identification of gene fragments

Isolates were screened for the presence of the phytohormone genes for auxin- *iaaM,* cytokinin-*miaA* and ACC deamianse- *acdS*. The *iaaM* (~1050bp) and *miaA* (~750bp) genes were successfully amplified in each of the selected 270 isolates via PCR, as indicated in figure 4.10a and b. The *acdS* gene with an expected size of ~680bp as shown in figure 4.10c, was only found in 36 isolates out of 270 isolates thereby representing 13% of the total collection.

[a]



[b]



[c]

Figure 4.10. Phytohormone gene DNA fragments amplified from *Pseudomonas* spp.: Gel electrophoresis of PCR products amplified with [a] *iaaM* primers (IAA-AOF-079 and IAA-AOF-1172) [b] *miaA* primers (miaAF-179 and miaAR-920) [c] *acdS* primers (acdsF3 and acdsR3). Lane L: DNA marker (lambda DNA-Digested with HindIII), lanes 1 to 20: amplified fragments from 20 *Pseudomonas* spp. isolates.

4.4.3.2 The *acdS* gene in *Pseudomonas* spp.

This *acdS* gene appeared to be more abundant in the bare fallow treatment (30 isolates) compared to those isolated from grassland (4 isolates) and arable (6 isolates) as visualised in figure 4.11. Interestingly, the number of isolates with this gene in the bare fallow treatment, increases in number from the bulk soil to the rhizosphere, further increasing in the root compartment.

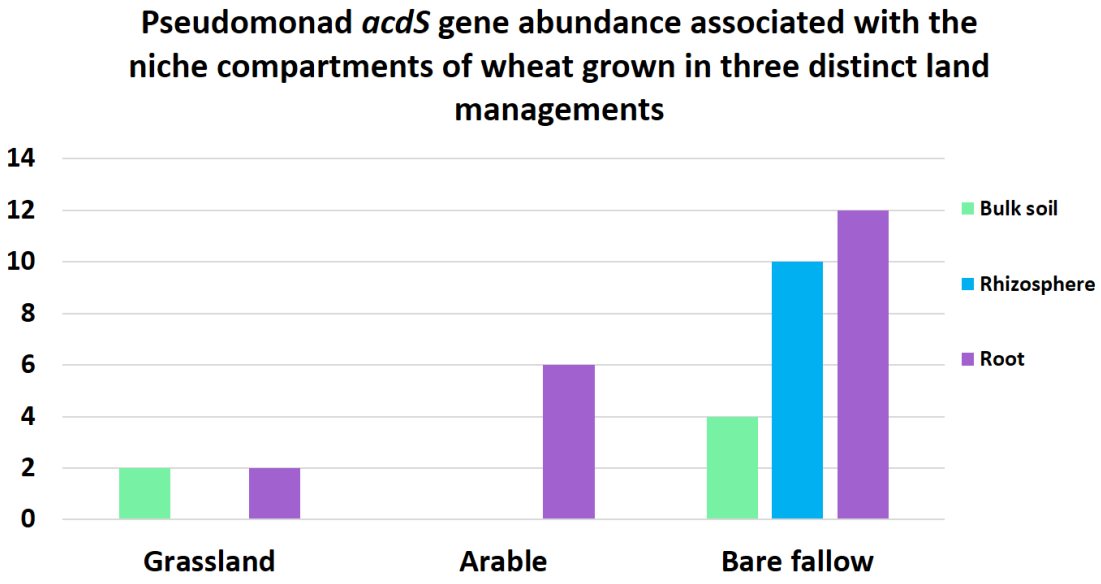


Figure 4.11: The distribution of the *acdS* gene abundance across land managements: A total of 36 isolates were found to have the *acdS* gene via PCR analysis, with abundance of the gene varying across bulk soil, rhizosphere soil and the root compartment of wheat grown in pot experiments utilising a grassland, arable and bare fallow managed soil sampled from the Highfield experiment.

4.4.3.3 The *gyrB* phylogeny of isolates with the *acdS* gene

The *gyrB* gene sequences for isolates identified as having the *acdS* gene were phylogenetically assessed. The phylogenetic tree was constructed and coloured according to land management,

as shown in figure 4.12 below. Upon visualisation, it appears that the ACC deaminase positive pseudomonads associated with bare fallow, grassland and arable managed soils group separately. Interestingly, the *acdS* isolates associated with the bare fallow managed soil appear to be more diverse compared to the grassland and arable isolates. Despite this, the sample size was not equal to properly assess diversity, since the *acdS* positive isolates were 5-fold higher in the bare fallow plots compared to the grassland and arable plots.

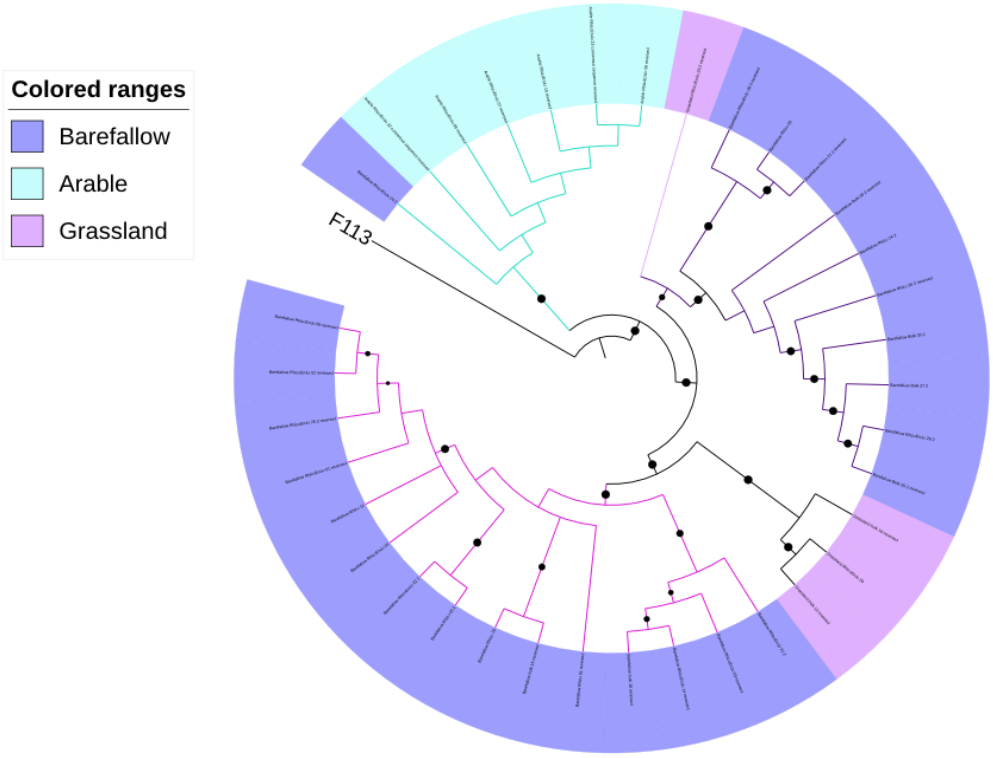


Figure 4.12: Maximum likelihood tree based on *gyrB* nucleotide sequences from *acdS* *Pseudomonas* strains associated with wheat grown in 3 different land managements: The housekeeping *gyrB* gene of 36 isolates identified as having the *acdS* gene from bulk soil, rhizosphere soil and roots of wheat grown in soil from 3 distinct land management regimes (bare fallow, arable and grassland) were phylogenetically analysed utilising an ML tree with a transitional model. Percentage bootstrap values higher than 70% of 1000 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to land management.

4.5 Discussion

4.5.1 Overview

Land management affects soil biodiversity, but how it impacts plant beneficial members such as *Pseudomonas* spp. in both community structure and function is not well understood. In this study, the niche compartments bulk soil, rhizosphere and roots of wheat grown in soil from different land histories, revealed differences in pseudomonad community structure. Both abundance and phylogeny in addition to the *acdS* gene abundance differed across soil treatments and niches. Conversely, the phytohormone genes *iaaM* and *miaA* involved in auxin and cytokinin biosynthesis respectively, were prevalent in the pseudomonad communities analysed. Interestingly, there was a higher abundance of isolates with the *acdS* gene in the bare fallow treatment, which increased in the rhizosphere and root compartments of wheat grown in this soil. Thus, suggesting that soil properties in combination with wheat exudation may have contributed to changes in pseudomonad community structure and functional genes in this study. The pseudomonad communities associated with wheat grown in arable and grassland managements were similar in community structure in addition to *acdS* gene abundance, which differed in the pseudomonad communities associated with the bare fallow management. This could be a result of grassland and arable plots receiving plant inputs compared to the bare fallow soil which had been deprived of plant inputs for >50years.

4.5.2 *Pseudomonas* spp. abundance

Differences were found in the abundance of pseudomonads associated with the niche compartments of wheat grown in soil from the three land management histories. The lowest abundance of pseudomonads were found in the bulk soil of bare fallow, followed by the slightly higher abundances associated with bulk soil of grassland and arable soils. The rhizosphere compartment of wheat grown in the different land histories each had higher pseudomonad abundances compared to bulk soil. Albeit no significant difference was found

between bulk and rhizosphere communities, which agrees with the culturable and molecular analysis used to determine pseudomonad abundance in chapter 2 and 3.

Moving closer to the plant with the root communities, the mean CFU g^{-1} increased significantly by 100-1000 fold compared to the bulk soil and rhizosphere soil compartments. The highest abundance was associated with wheat roots grown in the grassland managed soil, which may result from the higher C content received from root exudation throughout the year, since C availability limits microbial activity (Hobbie and Hobbie 2013). Interestingly, root pseudomonads associated with wheat grown in the arable soil was significantly lower than the grassland treatments, although initially similar across the bulk soil communities. This could be a result of other microbial species increasing in number surrounding the wheat roots. Different selection pressures on root architecture and exudation can alter microbiome composition, favouring other microbial species competing in the rhizosphere and root. Here, the use of metagenetic data would have proved useful in observing the abundance of other microbial species across the niche compartments of wheat grown in the different land use histories.

4.5.3 *Pseudomonas* spp. identification and phylogeny

All bacteria isolated were identified as belonging to the *Pseudomonas* genus from analysis of the *gyrB* gene. A variety of pseudomonad strains were detected via comparison to the NCBI BLAST database, with 36 isolates identified overall that varied across the different managements and niche compartments. This highlights that soil properties driven by land management, in addition to niche compartment of wheat can shape pseudomonad community composition. This was further highlighted by phylogenetic analysis of the *gyrB* gene, which revealed differences in pseudomonad diversity associated with the niche compartments of wheat grown in soils from different land histories. This agrees with several studies that found differences in microbial community structure when comparing land managements. Overall, the grassland treatment showed the highest diversity of

pseudomonads, closely followed by the arable and then bare fallow treatment. This is perhaps not surprising, since the grassland managed soils will have received continuous plant C inputs throughout the year, whilst the arable treatments will have received plant C throughout the growing season. The bare fallow soil differs considerably being devoid of plants for over 50 years prior to use in this study. Not only this, but soil pH has been shown as a dominant driving factor for shifts in microbiome assembly (Xue et al. 2018). Therefore, the differences in pseudomonad diversity could have been driven by factors relating to soil pH, since the grassland and arable plots at the Highfield experiment had a higher pH (pH 6.0 and 5.6 respectively) than the bare fallow plots (pH 5.1). Low pH environments induce changes in plant traits such as concentrations of organic acids, sugars and amino acids in exudates (Liang et al. 2013; Sarasketa et al. 2016). It's possible that such differences in soil properties could have changed *Pseudomonas* community diversity directly, and indirectly through changes driven by plant traits.

4.5.4 Phytohormone related genes in pseudomonad communities.

The phytohormone genes *miaA* and *iaaM* were present in all bacterial isolates tested. Despite evidence that these genes are important for IAA and cytokinin production in bacteria, some studies indicate that the enzymes encoded by these genes also function as signalling molecules in bacteria. IAA participates in gene regulation in some bacterial species (Patten, Blakney, and Coulson 2013b; Spaepen and Vanderleyden 2011a; Spaepen, Vanderleyden, and Remans 2007). Additionally, cytokinins trigger transcriptional changes in bacteria (Samanovic et al. 2018). Although the IAA and cytokinin related genes investigated here appear to be a common trait in pseudomonads, it's not clear whether this is a product of plant-microbe interactions or a trait required for bacterial signalling. Further investigations into the genetic variability and functionality of isolates producing IAA and cytokinins across the different land managements would offer more insights. Conversely, the ACC deaminase gene was not highly

abundant, represented in only 13% of the total pseudomonad culture collection. Since ACC deaminase is not known as a signalling molecule in bacteria, it is more likely that increases in pseudomonads with this gene occurred due to possible plant-microbe interactions.

Studies have found that ACC deaminase bacteria are enriched in rhizosphere communities when compared to bulk soil, indicating ACC as a potential rhizosphere selector (Bouffaud et al. 2018). Interestingly, the ACC deaminase gene was more abundant in pseudomonads from the bare fallow treatment, accounting for 72% of the total *acdS* isolates identified in this study. When looking at the niche compartments, isolates with the *acdS* gene increase from bulk soil to rhizosphere soil, with the highest abundance found in the root compartment. In line with other studies, this suggests rhizosphere selection for pseudomonads with this gene may have occurred in the bare fallow treatment. Since ACC deaminase is associated with helping to relieve stress symptoms through degradation of ACC, it's possible that the bare fallow treatment presented a more stressful environment for wheat than the grassland and arable treatments. Indeed, soil properties differ considerably across the different land histories at the Highfield experiment (Neal et al. 2017). A higher soil bulk density along with lower nutrient status and lower pH can all cause plant stress responses. An optimal pH for wheat growth is between 5.5 to 6.5, therefore the bare fallow treatment may adversely impact crop growth at a pH of 5.1 (Islam 1980; Brito et al. 2018). Lower C and N content in soil can also decrease crop growth by altering physiological responses (Khan et al. 2015). Additionally, a more compacted soil decreases aeration of the roots, which can limit crop growth (Hussain et al. 1999). Each of these factors could potentially stimulate ethylene biosynthesis and root ACC exudation, which could select for ACC deaminase bacteria in the rhizosphere and root compartments.

Studies have proposed that low intensity land uses encourage stronger interactions of plants with the associated soil microbiome based on increases in soil microbial diversity (Estendorfer et al. 2017)**.** In the case of ACC deaminase, higher intensity managements may encourage

stronger interactions, from a less diverse but more functional specific community. Indeed, Mehrabi *et al* found that increased diversity was negatively related to the suppression of take-all disease by *Pseudomonas* spp (Mehrabi et al. 2016). This is further highlighted from a previous study of the Highfield experiment by Neal *et al*, who found that cultivable microbes capable of metabolising P were more numerous in the bare fallow plots and also grew quicker compared to the grassland and arable treatments (Neal et al. 2017). This is interesting as it suggests that lower microbial diversity and biomass does not necessarily translate into reduced functional activity. More studies into the impacts of land management on functional soil-plant-microbe interactions are needed to better understand these dynamics.

4.5.5 Conclusion

Land management affected pseudomonad community abundance and diversity in wheat associated niche compartments. The most diverse communities were associated with the grassland soil followed by the arable treatment and the lowest diversity found in the bare fallow soil. The *iaaM* and *miaA* genes were ubiquitously found in the pseudomonad communities across all treatments however further investigations into the functionality of these traits are required. Only a small proportion of the pseudomonad communities had the *acdS* gene, however its abundance increased in the bare fallow treatment which could be a result of soil-plant-microbe interactions. In agreement with other studies, this highlights that anthropogenic manipulation of land can impact soil microbial communities structurally and influence the abundance of functional genes involved in plant-microbe interactions.

5.0. Screening the genetic and functional potential of *Pseudomonas* spp. to promote wheat growth

5.1 Introduction

5.1.1 Microbial inoculant development

Intensive agriculture has undoubtedly played a significant role in feeding the world's population. Despite this, the global food system faces multiple sustainability issues that must be addressed to produce enough food for future generations. Many avenues are being explored to tackle these issues, with boosted investment into microbial inoculants occurring in recent years (Waltz 2017). Albeit, microbial inoculants have been studied for over a century due to a range of beneficial traits including biofertilisation, bioregulation, biocontrol and bioremediation (Hartmann, Rothballer, and Schmid 2008; Bashan 1998). Chemical fertilizers and pesticides can have harmful effects to the surrounding environment and require utilization of finite resources- as discussed in chapter 1.0. In contrast, microbes are naturally abundant in soils and are desirable targets with potential to improve agricultural sustainability.

One of the attractions of applying viable microbes to agricultural land, is their potential to perceive and respond to environmental stresses with a range of beneficial traits. This could allow for a product that is adaptive rather than having one stand-alone purpose. Despite this potential, microbial inoculants have failed to achieve widespread use in farming systems typically due to inconsistent results (O'Callaghan 2016; Ryan and Graham 2018). This is perhaps not surprising when considering the complex dynamics occurring across the chemical, physical and biological properties of soil. It is inherently difficult to control the establishment, persistence and functional activity of a microbial inoculant applied to agricultural systems, particularly those containing associative symbionts such as *Pseudomonas* spp. Whilst mutualistic symbionts such as *Rhizobium* spp. have achieved more commercial success with

the fixation of N_2 in root nodules offering more predictable results in plants (Checcucci et al. 2017). Root nodules provide a habitat hidden from the species rich rhizosphere, thus reduction in biotic competition enables easier exploitation of this symbiosis. Attempts to manipulate a pre-established microbiome in the rhizosphere is a more difficult task and requires a multitude of ecological considerations including soil parameters and the microbial composition of the rhizosphere.

Additionally, microbial inoculants rely on organisms that can be cultured in the laboratory. This means that inoculant development currently relies on microbes representing less than 1% of the total soil microbiome, albeit some studies have cultivated a much larger proportion of the soil microbiome from utilisation of techniques such as the isolation chip (ichip) (Solden, Lloyd, and Wrighton 2016; Nichols et al. 2010). Although ichip can cultivate microbes *in situ* helping to provide a tangible resource for screening of genomic and some functional traits, the applicability of such microbes for large scale cultivation in a laboratory setting is still a limitation for microbial inoculant research. After isolation and the large-scale cultivation of microbes, these organisms must then survive formulation processes and storage before reaching the end user (Kaminsky et al. 2019). Microorganisms surviving this process are likely to be generations removed from the original isolate, which could have consequences on the desired activity of the product (Takors 2012). For this reason, the purification of active substances from bacteria is of interest, since viability during formulation and storage is not required.

Whether it be for the development of viable inoculants or for the isolation of microbially produced active substances, the progression of culturing techniques would greatly expand the repertoire of organisms available for agricultural purposes. Despite these struggles, the advent of molecular techniques such as genome sequencing and meta-omic studies has provided a surge of new interest in microbial inoculant research (Kaminsky et al. 2019). Many researchers are now looking to the genome as a relatively quick and easy screening process to identify

traits of interest for inoculant development. There is additional interest in genome editing of isolates to enable better survival of organisms during the inoculant development and storage stages, although this technology is currently controversial due to human health fears in addition to the potential spread and negative disruption of ecosystem dynamics.

5.1.2 Genome and functional screening for microbial inoculant development

Whole genome sequencing (WGS) has revolutionised the understanding of microbial genetics from a variety of environments (Patel et al. 2018). Various opportunities exist to investigate the inoculant potential of microorganisms including the presence/absence of genes and gene sequence comparisons at the nucleotide level. This information can help to place a genome into a larger evolutionary and ecological context (Rudman et al. 2018). Research is now reaching an era when sequencing cost and high-throughput data generation are no longer limiting factors (He 2015). As costs for sequencing technologies continue to decrease, microbiological research is set to benefit.

As previously discussed in chapter 1.0, *Pseudomonas* spp. are an ideal target for microbial inoculant development due to their amenability to culture, their ubiquitous presence in the environment and the plethora of PGPR traits associated with many strains. A number of beneficial pseudomonad WGS studies are available in the literature, with particular focus on reference *P. fluorescens* strains F113, SBW25, PF-0-1 and PF-5 (Silby et al. 2009; Paulsen et al. 2005; Redondo-Nieto et al. 2013). Genomic comparisons have revealed large genomic heterogeneity across species, with numerous plant-microbe interaction genes present in the accessory genome (Silby et al. 2009; Redondo-Nieto et al. 2013). Such diversity has led many researchers to conclude that the *P. fluorescens* species actually consists of a species complex rather than representing one singular species. This has been evidenced from phylogenetic studies identifying eight distinct subgroups in the *P. fluorescens* species complex (Garrido-Sanz et al. 2016).

Although the genome can provide a wealth of information regarding genetic potential of isolates, it can only offer clues to functional ability. Genes of interest identified must therefore be functionally verified. There are many techniques available to indicate functional activity such as RT-qPCR however this does not account for posttranslational modifications that can impact protein activity. Qualitative or quantitative plate assays can sometimes be more informative of bacterial function, since they rely on the activity of a functional protein. Many assays have been performed with beneficial bacteria including phosphate solubilisation, siderophore production, ACC deaminase activity and anti-fungal/bacterial competitions assays as discussed in chapter 1.0 (Dutta and Thakur 2017; Hilber-Bodmer et al. 2017) . Additionally, microbes must display a proven ability to promote crop growth within *in vitro* assays or pot experiments before being considered for inoculant application in the field. Despite this, environmental regulatory factors greatly contribute to gene transcription and translation, thereby functional activity observed in the laboratory may not be reflected in the field. Identifying PGPR traits and understanding successful establishment and functional activity within a variety of environments would be of great value towards a more reliable microbial inoculant industry. Screening the genomes of various pseudomonads for beneficial traits is a relatively quick way of finding candidates with multiple genes of interest, which could be utilized in multi-purpose microbial inoculant products.

5.2 Aims

5.2.1 General aim

To screen the genetic and functional potential of pseudomonads identified as having the *acdS* gene to promote crop growth, whilst exploring the ecological context of PGPR genes associated with three distinct land managements (permanent grassland, arable and bare fallow). Identifying and understanding an inoculant which helps alleviate symptoms of stress or promote crop growth, would be of great potential in the early stages of inoculant development.

5.2.2 Specific objectives

In this chapter I aim to:

1. Assess the distribution of several PGPR genes in pseudomonads isolated from different land managements
2. Assess the functional ability of all isolates to produce siderophores, ACC deaminase, solubilise phosphate and produce anti-fungal metabolites.
3. Assess the phylogeny of the *acdS* gene found in pseudomonads
4. Assess the phylogeny of the regulatory *acdR* gene found in pseudomonads
5. Quantify the functional ability of *acdS* isolates to utilise the substrate ACC
6. Assess the ability of salt tolerant *acdS* isolates to promote growth of wheat seedlings under salt stress

5.3 Methods

5.3.1 Culture collection

The culture collection created in chapter 4.0 was utilised in this study with isolates from bulk soil, rhizosphere soil and the root compartment of wheat grown in either bare fallow, grassland or arable soil from the Highfield experiment. See section 4.3 for details on wheat cultivation, soil sampling and pseudomonad isolation. All 36 pseudomonad isolates previously identified as having the *acdS* gene from PCR analysis were chosen to be fully genome sequenced along with 18 negative controls that were not found to have the *acdS* gene- see table 4.1 for isolate information.

Bacterial isolate	Soil	Niche compartment	*acdS* (+/-) PCR screen
GB12	Grassland	Bulk soil	+
GB16	Grassland	Bulk soil	+
GRE20	Grassland	Root	+
GRE29	Grassland	Root	+
GB19	Grassland	Bulk soil	-
GB26	Grassland	Bulks soil	-
GR19	Grassland	Rhizosphere	-
GR26	Grassland	Rhizosphere	-
GRE19	Grassland	Root	-
GRE26	Grassland	Root	-
ARE06	Arable	Root	+
ARE07	Arable	Root	+
ARE08	Arable	Root	+
ARE15	Arable	Root	+
ARE22	Arable	Root	+
ARE23	Arable	Root	+
AB19	Arable	Bulk soil	-
AB26	Arable	Bulk soil	-
AR19	Arable	Rhizosphere	-
AR26	Arable	Rhizosphere	-
ARE19	Arable	Root	-
ARE26	Arable	Root	-
BFB15	Bare fallow	Bulk soil	+
BFB18	Bare fallow	Bulk soil	+
BFB25	Bare fallow	Bulk soil	+
BFB27	Bare fallow	Bulk soil	+
BFB28	Bare fallow	Bulk soil	+
BFB30	Bare fallow	Bulk soil	+
BFR01	Bare fallow	Rhizosphere	+
BFR05	Bare fallow	Rhizosphere	+
BFR12	Bare fallow	Rhizosphere	+
BFR16	Bare fallow	Rhizosphere	+
BFR21	Bare fallow	Rhizosphere	+
BFR24	Bare fallow	Rhizosphere	+
BFR25	Bare fallow	Rhizosphere	+
BFR30	Bare fallow	Root	+
BFRE01	Bare fallow	Root	+
BFRE02	Bare fallow	Root	+
BFRE03	Bare fallow	Root	+
BFRE04	Bare fallow	Root	+
BFRE09	Bare fallow	Root	+
BFRE14	Bare fallow	Root	+
BFRE21	Bare fallow	Root	+
BFRE22	Bare fallow	Root	+
BFRE24	Bare fallow	Root	+
BFRE25	Bare fallow	Root	+
BFRE28	Bare fallow	Root	+
BFRE29	Bare fallow	Root	+
BFB19	Bare fallow	Bulk soil	-
BFB26	Bare fallow	Bulk soil	-
BFR19	Bare fallow	Rhizosphere	-
BFR26	Bare fallow	Rhizosphere	-
BFRE19	Bare fallow	Root	-
BFRE26	Bare fallow	Root	-

Table 4.1 Bacterial culture collection: 54 *Pseudomonas* spp. were isolated from different niche compartments of wheat grown in three land managements and subjected to full

genome sequencing. Of the isolates investigated in this study, 36 were previously identified as having the *acdS* gene from a PCR screen and 18 of the isolates were identified as not having the *acdS* gene.

5.3.2 Genomic DNA extractions

All isolates were grown in LB broth overnight. 1.0mL of overnight culture was used for DNA extraction with the GenElute genomic DNA extraction kit (Sigma-Aldrich) per manufacturer's protocol. This is a relatively easy method to extract DNA by lysing bacterial cells with a chaotropic salt solution, the addition of ethanol binds DNA in the lysate to a silica membrane in microcentrifuge tubes and then eluted using PCR grade H_2O (Biorad). DNA quantity was determined using the Qubit Fluorometric Quantification (Life Technologies) using the manufacturer's instructions. DNA quality was determined using the Nanodrop Microvolume UV spectrophotometer (Thermo Fisher Scientific Inc) by its OD at 260 nm, the 260/280 ratio was used to determine good quality DNA in addition to running the samples on a 1.5% (w/v) agarose gel in 1x Tris-borate-EDTA (TBE) stained with EtBr (0.2 μg ml^{-1}) with 1x TBE as the running buffer. Bands of DNA were viewed under UV light to visually detect any degraded DNA.

5.3.3 Genome sequencing

Genome sequencing for each isolate was performed by Microbes NG with x 30 sequence coverage (https://microbesng.uk/). A total of 30 μL of DNA at a concentration of 50 ng/ μL was sent to Microbes NG, where sequencing libraries for the samples were prepared. Libraries were sequenced on a Illumina HiSeq using a 250 bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15. *De novo* assembly was performed on samples using SPAdes version 3.7 (Nurk, Bankevich et al., 2013) and contigs were annotated using Prokka 1.11 (Seemann *et al* 2014).

5.3.4 Genome analysis

Genome analysis was initially performed by searching for genes within the Prokka annotations received from microbesNG. The genomes were additionally uploaded to the Rapid Annotation using Subsystem Technology (RAST) pipeline for additional annotations (Aziz et al 2008). If genes of interest were not found in the annotated genomes, sequences were download from NCBI and searched for using Basic Local Alignment Search Tool (BLAST) within the RAST pipeline (Aziz et al. 2008). Please see supplementary chapter 8.4 for reference sequences utilised in BLAST searches. A list of genes of interest searched for in this study are shown inTable 4.2.

Function	Target trait	Gene name
Bioregulation	1-aminocyclopropane-1-carboxylate deaminase	*acdS, acdR*
	Indole-3-acetic acid (auxin)	*iaaMH, nit, nthAB, ipdC*
	tRNA isopentenylpyrophosphate transferase (cytokinin)	*miaA*
Anti-fungal	Hydrogen cyanide	*hcnAB*
	2,4-diacetylphloroglucinol	*phlEDBAFGH*
	Phenazine	*phzIRABCDEFGO*
	Pyrrolnitrin	*prnABCD*
	Pyoluteorin	*pltABCDEFGIJKNO*
Siderophore	Achromobactin	*acsABCEDF*
	Pyochelin	*pchR*
	Pyoverdine	*pvdAPMNOEL*
	Ferrous iron transporter	*efeUOB*
Phosphorous metabolism	Alkaline phosphatase	*phoA*
	Phosphate regulation	*phoBR*
	Phosphate transport	*pstS*
	Phosphate DING protein	*dinG*
	Glycerophosphodiester phosphodiesterase	*glpQ*
	Phytase	*phyC*
	Glucose dehydrogenase	*gcd*
Nitrogen cycling	Nitrate reductase	*narGHJI*
	Nitrite reductase	*nirSK*
	Nitrous oxide reductase	*nosZDFYL*
	Nitric oxide reductase	*norBCEDQ*
Abiotic stress tolerance	Periplasmic glucans	*mdoHGD*
	Glycine betaine synthesis	*betABTCI*
	Carbon starvation protein	*cstA*
	Carbon starvation sensing protein	*rspAB*
Biotic stress tolerance	Vancomycin resistance	*vanW*
	Fosfomycin resistance	*fosA*
	Polymyxin resistance	*pmrJLM*
Colonisation	Biofilm	*pgaABCD*
	Flagella structure	*flhAB fliRQ flgDHBC*
	Flagella motor	*motAB filMN*
	Chemotaxis proteins	*cheAVYWV*
	N-acyl-homoserine lactone	*rhlIR lasRI*
	Autoinducer-2	*isrKRABCDFGPQ*
	Type I SS	*lapBCEDP*
	Type II SS	*tadABCZ*
	Type III SS	*yscU yopB icrD*
	Type IV SS (pilus)	*pilABCTQMNOPE*
	Type IV SS (Fimbria)	*pilY1XVWR fimU*
	Type V SS	*tpsBA*
	Type VI SS	*impBCGHJAF*

Table 4.2. Target genes for screening the genome of *Pseudomonas* spp: Various genes of interest were screened for across 54 pseudomonad genomes including PGPR traits involved in

bioregulation, anti-fungal activity, siderophore production, phosphate metabolism, abiotic stress tolerance, biotic stress tolerance and genes related to rhizosphere colonisation.

5.3.5 Phylogenetic analysis

The housekeeping genes *gyrB, rpoB* and *rpoD* were successfully extracted from all 54 genomes and concatenated for phylogenetic analysis. Additionally, the *acdS* and *acdR* gene sequences were extracted from those genomes identified as having the genes and analysed phylogenetically. Sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) alignment tool and a maximum likelihood tree was constructed utilizing a Hasegawa, Kishino and Yano 1985 (HKY85) model with 100 bootstraps in the software PhyML (Guindon et al. 2010).

5.3.6 Functional assays

5.3.6.1 ACC deaminase

Isolates were screened for their ability to use ACC as a sole nitrogen source following the method described in Penrose and Glick (2003) (Penrose and Glick 2003). Bacterial isolates were grown overnight in LB broth at 150 r.p.m at 28°C. Cultures were adjusted to an Optimal Density (OD) of 1.0 at an absorbance of 600nm using a spectrophotometer, before transferring 1mL of culture into 50mL of Dworkin and Foster (DF) minimal medium with ammonium sulfate as a nitrogen (N) source and grown for 24 hours at 150 r.p.m at 28°C.

DF media was prepared as follows (per litre): 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4 \cdot H_2O$, 124.6 µg $ZnSO_4 \cdot 7H_2O$, 78.22 µg $CuSO_4 \cdot 5H_2O$, 10 µg MoO_3, in addition to 2.0 g $(NH_4)_2SO_4$ as a sole N source. Media was adjusted to pH7.2.

After 24 hours cultures were centrifuged at 5000 r.p.m, growth medium was discarded, and cells were washed in DF medium without an N source- this was repeated twice. 1 mL of cells suspended in N free DF medium was transferred to 25 mL of DF + 3.0 mM ACC (Sigma-Aldrich)

as the sole N source. DF + ACC medium was prepared from a 0.5 M stock solution of ACC, which was filter-sterilized through a 0.2 µm membrane, the filtrate was collected and frozen at −20°C for storage. Prior to inoculation, the ACC solution was thawed and a 150 µl aliquot was added to the sterile DF minimal medium in place of $(NH_4)_2SO_4$. Following inoculation, the culture was grown at 150 r.p.m. for 24 hrs at 28°C. Isolates that did not grow in the DF + ACC medium were regarded as not having a functional ACC deaminase enzyme.

5.3.6.1.1 ACC Calibration curve

Quantification of ACC consumption in isolates found positive for ACC deaminase activity was assessed using a colorimetric ninhydrin assay from Li *et al* (2015). 500 mg of ninhydrin and 15 mg of ascorbic acid were dissolved in 60 mL of ethylene glycol and mixed with 60 mL of 1M citrate buffer (pH 6.0) prior to use. Citrate buffer was prepared with 12.04 g sodium citrate dihydrate and 11.34 citric acid in 1 litre of distilled water and adjusted to pH 6.0.

A standard colorimetric calibration curve was performed with varying ACC concentrations. DF medium was prepared with respective ACC working concentrations of 3 mM, 2.5 mM, 2 mM, 1.5 mM, 1 mM, 0.5 mM and 0 mM. 1 mL of each ACC working solution was mixed with 2 mL of ninhydrin reagent in glass test tubes, which were capped, shaken and placed in a boiling water bath (100°C). After 15 min, the tubes were moved into a water bath at room temperature (~20°C) for 2 min. The samples were then shaken for 30s and left to stand at room temperature for 10 min. This resulted in the development of a purple colour as seen in figure 5.1 [a]. The solution was transferred into a cuvette and absorbance was measured at 570 nm by a spectrophotometer to obtain absorbance readings as shown in figure 5.1 [b].

5.3.6.1.2 Screening isolates for ACC consumption

To test the bacterial isolates, cultures grown in DF + ACC medium were centrifuged at 5000 r.p.m for 5mins and a 1 mL supernatant of bacterial culture was added to 2 mL of ninhydrin

reagent in glass test tubes, which were capped, shaken and placed in a boiling water bath (100°C). After 15 min, the tubes were moved into a water bath at room temperature (~20°C) for 2 min. The samples were shaken for 30s and left to stand at room temperature for 10 min before transferring to a cuvette and absorbance measured at 570 nm by a spectrophotometer.

[a] **[b]**

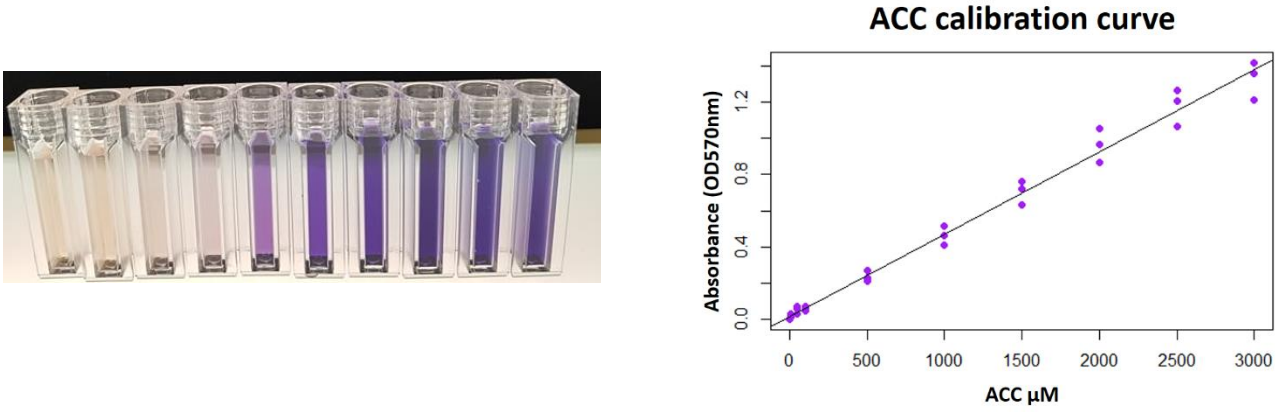


Figure 5.1 Quantification of ACC consumption: Isolates that grew in DF + ACC medium were quantified for ACC consumption at 24hr and 48hr of growth. **[a]** The ninhydrin colorimetric assay results in the development of a purple colour that increases as ACC concentration increases **[b]** A linear model with regression line was plotted using the data obtained from known ACC concentrations.

5.3.6.2 Siderophore production

Bacterial isolates were grown overnight in LB broth at 150 r.p.m at 28°C. Cultures were adjusted to an Optimal Density (OD) of 1.0 at an absorbance of 600nm using a spectrophotometer. 5µL of liquid culture was pipetted onto the centre of a chrome azurol S(CAS) -blue agar plate as developed by Schwyn and Neiland (1987) and grown at 28°C for 5 days (Schwyn and Neilands 1987). The agar plates were prepared as follows: 0.06g of CAS was dissolved in 50mL of ddH_20. In a separate flask 0.0027g of $FeCl_3.6H_20$ was dissolved in 10mL of 10Mm HCL; 9mL of this solution was mixed with 50mL of the CAS solution. Lastly 0.073g of

hexadecyltrimethylammonium bromide (HDTMA) was dissolved in 40 ml of ddH_20 which was then added to the CAS and $FeCl_3$ solution, resulting in a blue colour. The CAS blue solution was autoclaved in addition to 900 mL of 50% LB miller agar at pH 6.8. Once cooled to ~50°C, 100 mL of autoclaved CAS-blue solution was added to 900mL of LB agar, poured into sterile agar plates and left to cool. The CAS/HDTMA complexes tightly with ferric iron to produce a blue coloured agar which changes to orange when a strong iron chelator removes iron from the dye complex (Louden, Haarmann, and Lynne 2011). Isolates producing siderophores therefore develop orange halos around colonies. Plates were photographed after five days incubation and the diameter of colony size (mm^2) was measured and subtracted from the diameter of the orange halo size using the software image J (National Institutes of Health).

5.3.6.3 Phosphate solubilisation

Phosphate solubilisation was analysed using Pikoskaya agar medium (Pikovskaya 1948), which was prepared as follows: 0.5 g yeast extract, 10 g glucose, 5 g calcium tri-phosphate, 0.5 g ammonium sulphate, 0.2 g potassium chloride, 0.1 g magnesium sulphate, 0.0001 g manganese sulphate, 0.0001 g ferrous sulphate and 15 g of agar were dissolved in in 1 litre of ddH_2O, adjusted to pH 7.2 and autoclaved. Moltern agar was poured into sterile Petri dishes at ~50°C and allowed to cool.

Bacterial isolates were grown overnight in LB broth at 150 r.p.m, 28°C. Cultures were adjusted to an OD of 1.0 at an absorbance of 600 nm using a spectrophotometer. 5 µL of liquid culture was pipetted onto the centre of each Pikoskaya agar plate and incubated at 28°C for seven days. Isolates capable of solubilizing phosphate formed a clear halo around colonies from the breakdown of calcium triphosphate present in the agar. The clear halos were measured in diameter using image J and colony size subtracted to determine halo size.

5.3.6.4 Anti-fungal assay

Bacterial isolates were tested *in vitro* for inhibition of the take all fungal pathogen *Gaeumannomyces graminis* var. *tritici*. Potato Dextrose Agar (PDA, Sigma-Aldrich) was prepared according to manufacturer's instructions and adjusted to pH 7.2. A sterile 4 mm cork borer was used to sample a plug of *G.graminis* var*. tritici* grown on PDA, which was transferred to the centre of a fresh PDA plate. 5 µL of bacterial culture was pipetted 2.5 cm distance away from the fungal plug and incubated at 25°C for five days. Plates were photographed and the distance of fungal inhibition caused by the bacterial isolate was measured in image J.

6.3.7 Inoculation of wheat seedling under salt stress

6.3.7.1 Bacterial seed inoculation

Wheat seeds (*Triticum aestivum* cv.cadenza) were surface sterilized (75% ethanol wash for 30 seconds followed by 3% sodium hypochlorite for 10 min) and pre-germinated on filter paper soaked in sterile distilled water in petri dishes for 36 hours. At this stage, seedlings which had coleoptile and radicle emergence were selected for subsequent microbial inoculant application.

Microbial inoculants were prepared by growing isolates with unique *acdR* gene sequences (isolates ARE22, BRE25, GB12, GRE20 and GRE29) along with a negative control without the *acdS* gene (isolate AR20) and heat killed *P.fluorescens* F113 in 1/10 strength LB broth overnight. The bacterial cultures were centrifuged at 5000 r.p.m for 5mins, growth medium was discarded, and cells were washed in 30 mL of phosphate buffered saline (PBS), this was repeated twice to remove nutrients from the growth media. The PBS stock solution was prepared as follows: 8 g NaCL, 2 g KCl, 14.4 g Na_2HPO_4 $2H_2O$, 2.4 g KH_2PO_4 were each dissolved in 1 litre of ddH_2O and autoclaved- this was diluted x10 to achieve a working solution. The bacterial suspensions in PBS were adjusted to achieve 10^5 cells per 1 mL (OD600nm). 3 mL of

bacterial suspension was added to filter paper in petri dishes containing the wheat seedlings and stored in the dark for another 8 hours.

After 8 hours the inoculated seedlings were placed onto agar plates (1.5% agar) with no nutrients as seedlings were previously shown to grow sufficiently from their seed stores. Six seeds were grown in each plate and three true replicates were prepared per treatment. Each plate lid was secured using sealing film (Parafilm, UK) to prevent H_2O loss, the root section was additionally covered by foil to protect bacteria suspensions surrounding roots from the damage of sunlight. Plates were stood upright, and seedlings grown in a glasshouse environment as shown in figure 5.2 [b]. After 96 hours in the glasshouse seedlings were harvested with total average root length and leaf length measured.

[a] **[b]**

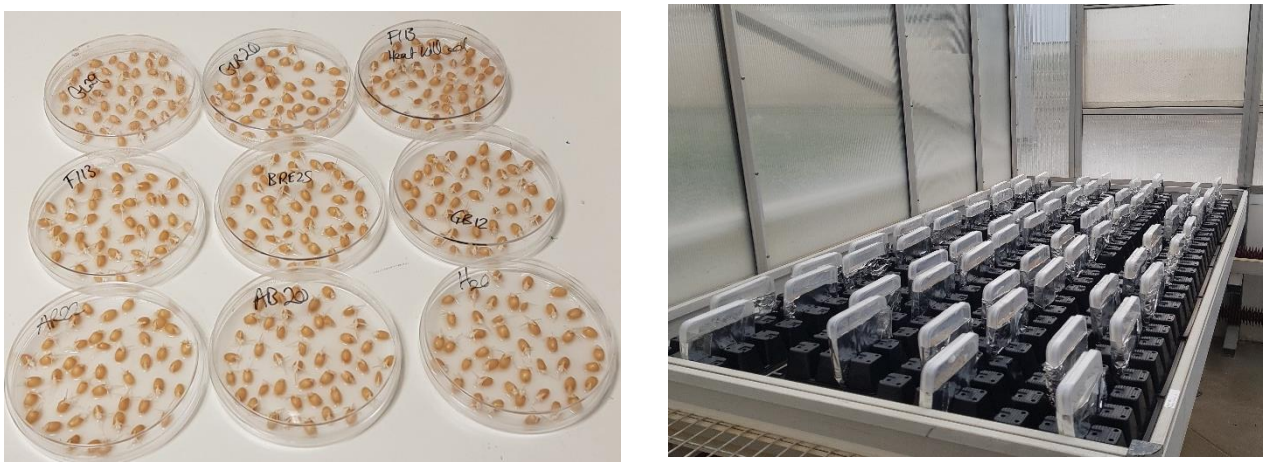


Figure 5.2. Bacterial inoculation of wheat seedlings grown under salt stress: [a] After 36hours of pre-germination in sterile Petri dishes containing sterile H_2O, seedlings with coleoptile and radicle emergence were transferred to sterile Petri dishes containing filter paper soaked in 5 mL of bacterial suspension at 10^5 cells/mL. Three negative controls were used including sterile H_2O, heat killed *P.fluorescens* F113 and an *acdS* negative organisms (AR20).

5.3.7.2 Application of chemical inhibitor and stimulator of ethylene

The application of functional *acdS* isolates is described in the literature to reduced plant ethylene levels to promote crop growth under stressed conditions. To better understand the role of ethylene in the plate assay used for bacterial inoculation, a chemical stimulator 1-aminocyclopropane-1-carboxylate (ACC) and inhibitor 2-aminoethoxyvinyl glycine (AVG) were used. Wheat seeds were pre-germinated as described in section 5.3. After 36 hours germination, seedlings which had coleoptile and radicle emergence were selected for subsequent investigation. A 5 µM and 10 µM filter sterilised solution was prepared for both ACC (Sigma-Aldrich, UK) and AVG (Sigma-Aldrich, UK) and 3 mL of each was added to filter paper in a sterile Petri dish. The pregerminated seeds were transferred to the different chemical treatments and left to soak for 8hours. After 8 hours the inoculated seedlings were placed onto agar plates (1.5% agar). Each plate lid was secured using sealing film (Parafilm, UK) to prevent H_2O loss, the root section was additionally covered by foil as prepared for the bacterial inoculations. After 4 days in the glasshouse seedlings were harvested with total average root length and leaf length measured.

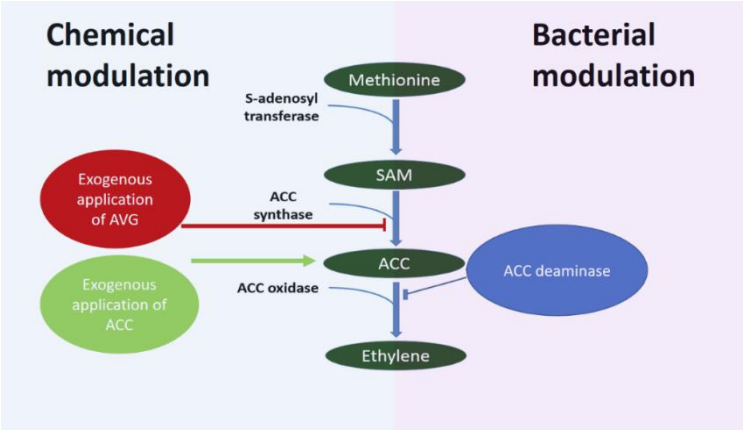


Figure 5.3. A schematic of the ethylene biosynthesis pathway in plants and its modulation by chemical and bacterial applications: Ethylene biosynthesis begins with methionine which is converted to *s*'adenosyl-L-methionine (SAM). SAM is converted to ACC by ACC synthase and

finally to ethylene by ACC oxidase. AVG has been studied for its role in inhibiting ACC synthase activity and thereby reducing ethylene levels, whilst exogenous application of ACC has been shown to increase ethylene levels. The microbial enzyme ACC deaminase has been shown to degrade ACC and thereby reduce plant ethylene levels.

5.3.7 statistical analysis

A one-way ANOVA was performed when comparing mean leaf and root length across the different treatments of bacteria and chemical application *in planta*. Three biological replicates (n= 3) were used for each of the treatments, where one biological replicate had 6 seedlings in each plate. Any significant results indicated were followed by the post hoc Tukey analysis, to identify which treatments differed significantly. All statistical analysis was performed in the software R studio. For gene presence and absence analysis, each strain was given a value of 1 (gene present) or 0 (gene absent). Where no variation was observed in gene presence between all of the isolates, genes were removed prior to conducting the correlation analysis. For correlation analysis of functional activity, each strain was given a value of 0-2, where low to zero activity was scored as 0, moderate activity scored as 1 and high activity scored as 2. Correlation analyses were computed using the Spearman's rank method in Microsoft excel.

5.4 Results

5.4.1 Genome screening

All 54 pseudomonad isolates were successfully full genome sequenced. Genome size varied between 5,957,628 bp and 7,054,452 bp (Table 4.3). A multi-locus sequence phylogeny utilising the housekeeping genes *gyrB, rpoB* and *rpoD* showed similar relationships between isolates as previously seen from analysis of the *gyrB* gene in chapter 4.0. In general, the isolates identified as *acdS* positive in previous screens clustered together according to land management. The 18 *acdS* negative isolates from the three land managements appeared to be more diverse, with no clear groupings according to land management as shown in figure 5.4.

Isolate	Genome size(bp)	GC%	CDS
GL-B-12	6739307	59.6	6113
GL-B-16	7054452	59.4	6452
GL-RE-20	6869057	59.6	6214
GL-RE-29	6374364	59	5801
A-RE-06	6770954	60.9	5964
A-RE-07	6775839	60.9	5961
A-RE-08	6777707	60.9	5958
A-RE-15	6778838	60.9	5954
A-RE-22	6787619	60.9	5973
A-RE-23	6905894	60.7	6101
BF-B-15	6445447	59.6	5785
BF-B-18	6690550	59.4	6009
BF-R-01	6800257	59.5	6182
BF-R-05	6302781	59.8	5650
BF-R-12	6374324	59.6	5792
BF-R-16	6485839	59.6	5816
BF-RE-01	6363420	59.6	5756
BF-RE-02	6357918	59.6	5754
BF-RE-03	6665380	59.5	5972
BF-RE-04	6370867	59.6	5770
BF-RE-09	6368974	59.5	5765
BF-RE-14	6702923	59.5	6026
BF-R-25	6503295	59.6	5836
BF-RE-21	6706620	59.5	6033
BF-RE-22	6520010	59.6	5847
BF-RE-24	6222099	60.9	5794
BF-RE-25	6371407	59.5	5768

Isolate	Genome size(bp)	GC%	CDS
GL-B-19	6524961	58.5	5858
GL-B-26	6541118	59	5900
GL-R-19	6502109	59.5	5854
GL-R-26	6430994	59.9	5777
GL-RE-19	6861571	59.5	6171
GL-RE-26	6432708	59.9	5784
A-B-19	6515109	59.9	5822
A-B-26	6477144	58.9	5800
A-R-19	7022807	59.9	6202
A-R-26	6302608	59.4	5672
A-RE-19	6310608	59	5563
A-RE-26	5937628	60.8	5322
BF-B-19	6598486	59.5	5898
BF-B-25	6511970	59.4	5796
BF-B-26	6741353	58.6	5997
BF-B-27	6253214	59.8	5612
BF-B-28	6247948	59.8	5586
BF-B-30	6299446	59.7	5619
BF-R-19	6858872	59.7	6214
BF-R-21	6263437	59.9	5634
BF-R-24	6623522	59.9	5589
BF-R-26	6572573	58.9	5840
BF-R-30	6356205	59.8	5684
BF-RE-19	6225560	59.9	5588
BF-RE-26	6415110	59.9	5727
BF-RE-28	6589166	59.5	5908
BF-RE-29	6445740	59.7	5769

Table 4.3 Full genome sequencing analysis of 54 *Pseudomonas* sp.: An overview of genome sequencing analysis of 54 *Pseudomonas* sp. isolated from three different land managements

and the niche compartments of bulk soil, rhizosphere soil and wheat roots. Genome size, GC% content and number of coding sequences (CDS) are indicated.

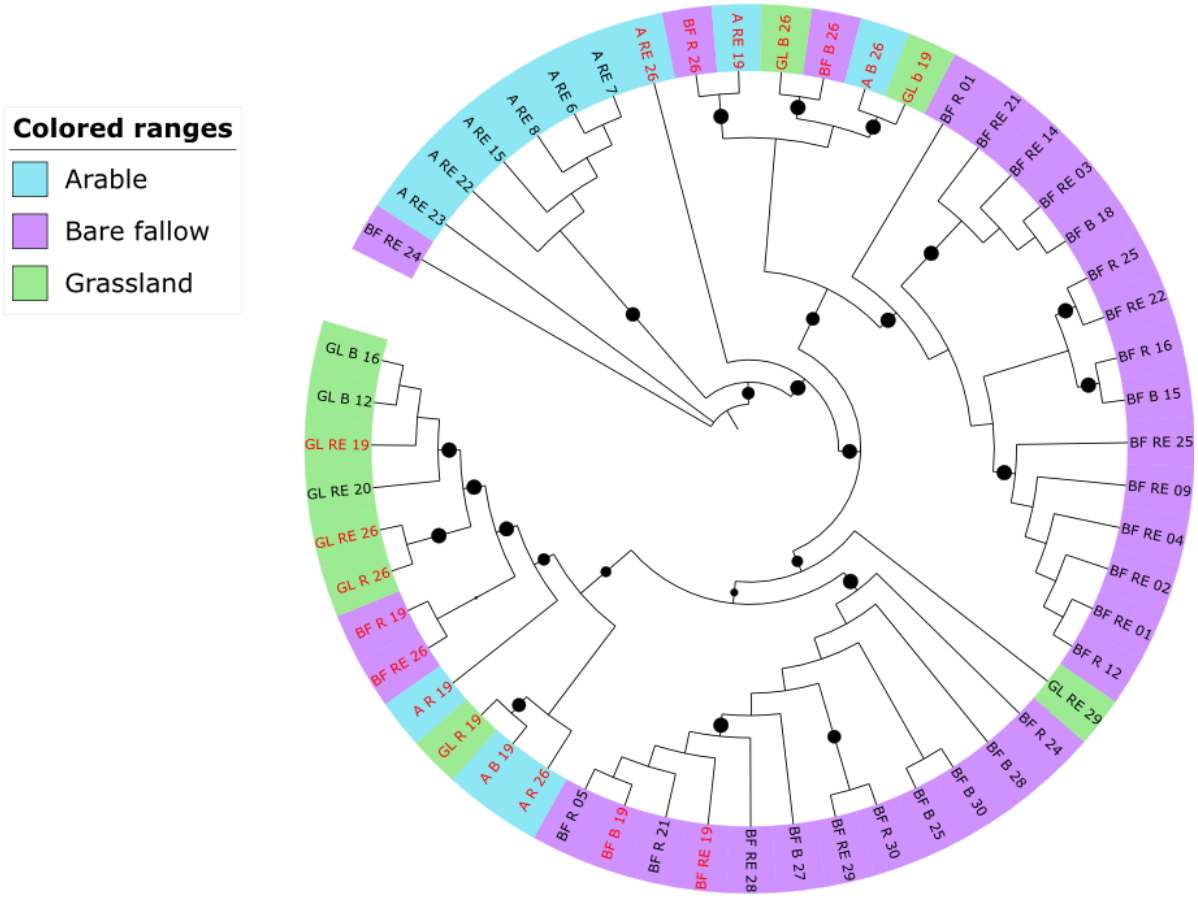


Figure 5.4. Maximum likelihood tree based on concatenation of the *gyrB*, *rpoB* and *rpoD* nucleotide sequences from *Pseudomonas* sp. associated with wheat grown in three different land managements: Isolates from bulk soil, rhizosphere and the roots of wheat grown in three distinct land management regimes (bare fallow, arable and grassland) were phylogenetically analysed utilising an ML tree with a Hasegawa, Kishino and Yano 1985 model and 100 bootstraps in the software PhyML. Percentage bootstrap values higher than 70% of 100 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to land management and isolate names are coloured according to *acdS* gene absence or presence (red: *acdS* negative, black: *acdS* positive).

5.4.2 Screening for phytohormone genes

The genomes were screened for genes known to be of importance in plant-microbe interactions within soils. Firstly, the phytohormone genes previously screened for using a PCR approach in chapter 4.0 were searched for and are summarised in figure 5.5. In agreement with results in the previous chapter, the IAA gene *iaaM* and the cytokinin gene *miaA* were found in each of the *Pseudomonas* genomes. The *acdS* gene was not found in every isolate previously identified by the PCR screen, with 27 out of 36 isolates found to have the ACC deaminase structural gene. All 27 isolates were additionally found to have the regulatory gene *acdR*. Since multiple IAA pathways have been reported in pseudomonads in the literature, nitrilase (*nit)*, nitrile hydratase *(nthAB*), indole pyruvate carboxylase (i*pdC*) and indole-acetamide hydrolase (*iaaH*) were screened for within the genomes. All isolates were shown to have *iaaH* genes, but none were found to have *nit, nthAB* or *ipdC* genes.

Bacterial isolate	ACC deaminase *acdR*	Indole-3-acetic acid *iaaM/H*	Indole-3-acetic acid *nthAB*	Indole-3-acetic acid *nit*	Indole-3-acetic acid *ipdC*	iP transferase *miaA*
GL-B-12	+	+	-	-	-	+
GL-B-16	+	+	-	-	-	+
GL-RE-20	+	+	-	-	-	+
GL-RE-29	+	+	-	-	-	+
A-RE-06	+	+	-	-	-	+
A-RE-07	+	+	-	-	-	+
A-RE-08	+	+	-	-	-	+
A-RE-15	+	+	-	-	-	+
A-RE-22	+	+	-	-	-	+
A-RE-23	+	+	-	-	-	+
BF-B-15	+	+	-	-	-	+
BF-B-18	+	+	-	-	-	+
BF-R-01	+	+	-	-	-	+
BF-R-05	+	+	-	-	-	+
BF-R-12	+	+	-	-	-	+
BF-R-16	+	+	-	-	-	+
BF-RE-01	+	+	-	-	-	+
BF-RE-02	+	+	-	-	-	+
BF-RE-03	+	+	-	-	-	+
BF-RE-04	+	+	-	-	-	+
BF-RE-09	+	+	-	-	-	+
BF-RE-14	+	+	-	-	-	+
BF-R-25	+	+	-	-	-	+
BF-RE-21	+	+	-	-	-	+
BF-RE-22	+	+	-	-	-	+
BF-RE-24	+	+	-	-	-	+
BF-RE-25	+	+	-	-	-	+

***acdS* positive isolates**

Bacterial isolate	ACC deaminase *acdR*	Indole-3-acetic acid *iaaM*	Indole-3-acetic acid *nthAB*	Indole-3-acetic acid *nit*	Indole-3-acetic acid *ipdC*	iP transferase *miaA*
GL-B-19	-	+	-	-	-	+
GL-B-26	-	+	-	-	-	+
GL-R-19	-	+	-	-	-	+
GL-R-26	-	+	-	-	-	+
GL-RE-19	-	+	-	-	-	+
GL-RE-26	-	+	-	-	-	+
A-B-19	-	+	-	-	-	+
A-B-26	-	+	-	-	-	+
A-R-19	-	+	-	-	-	+
A-R-26	-	+	-	-	-	+
A-RE-19	-	+	-	-	-	+
A-RE-26	-	+	-	-	-	+
BF-B-19	-	+	-	-	-	+
BF-B-25	-	+	-	-	-	+
BF-B-26	-	+	-	-	-	+
BF-B-27	-	+	-	-	-	+
BF-B-28	-	+	-	-	-	+
BF-B-30	-	+	-	-	-	+
BF-R-19	-	+	-	-	-	+
BF-R-21	-	+	-	-	-	+
BF-R-24	-	+	-	-	-	+
BF-R-26	-	+	-	-	-	+
BF-R-30	-	+	-	-	-	+
BF-RE-19	-	+	-	-	-	+
BF-RE-26	-	+	-	-	-	+
BF-RE-28	-	+	-	-	-	+
BF-RE-29	-	+	-	-	-	+

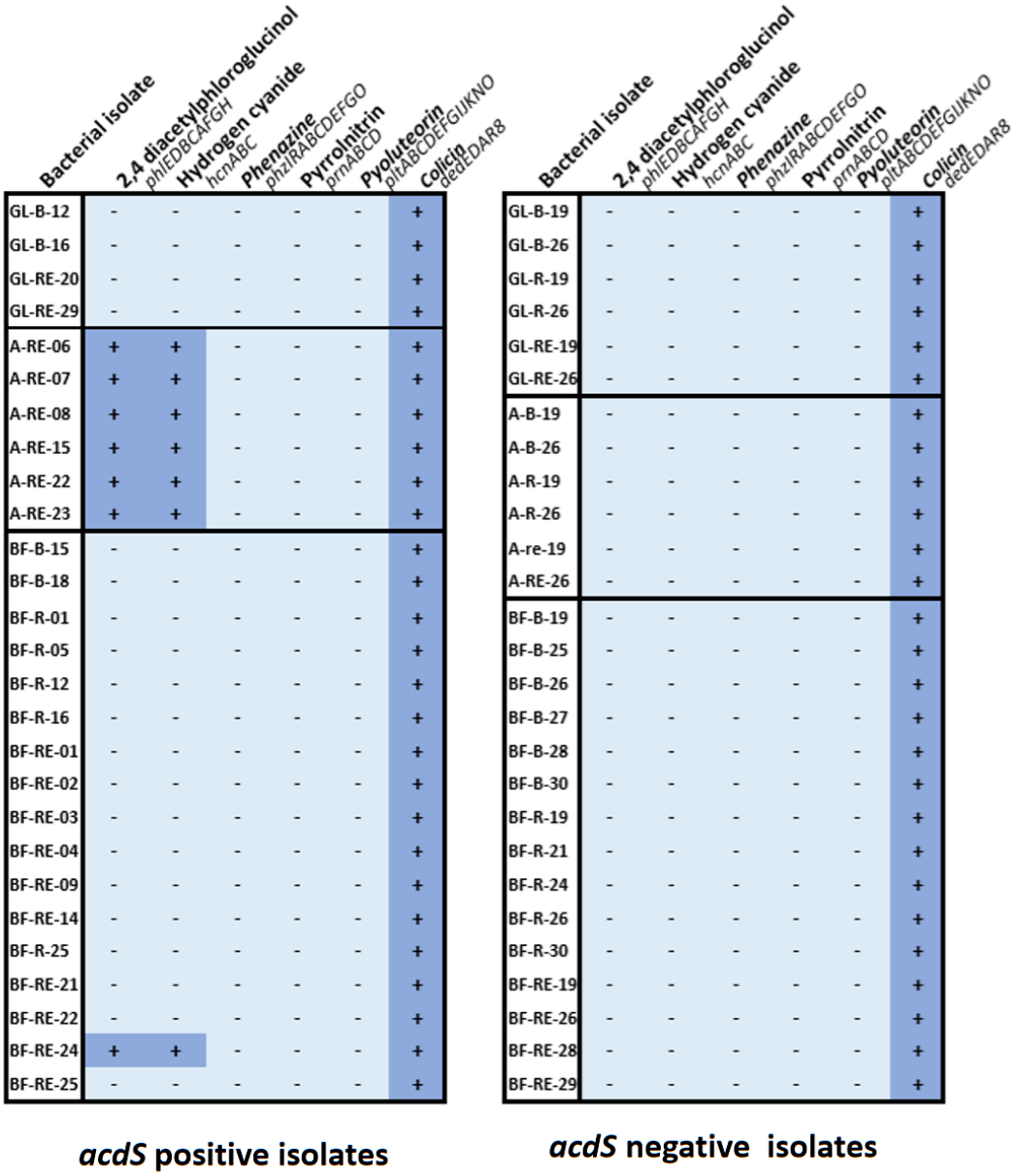
***acdS* negative isolates**

Figure 5.5. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with phytohormone production. Genes involved in the synthesis of ACC

deaminase, auxin and cytokinin in bacteria were screened for in the 54 pseudomonad genomes. Isolates positive for the genes are shown as + and isolates negative for the genes are shown as -.

5.4.3 Screening for known PGPR traits

A screen for anti-fungal genes revealed 7 out of the 54 isolates to have the full gene locus for hydrogen cyanide (*hcnABC*) and 2,4 DAPG (*phlEDBCAFGH*) as shown in figure 5.6. All of these isolates had the *acdS* gene, with six isolated from the root of wheat grown in the arable soil and one isolated from the root of wheat grown in the bare fallow managed soil. Other anti-fungal genes including phenazine (*phz*), pyrrolnitrin (*prn*) and pyoluteorin (*plt*) were not found in the genomes utilised in this study. The bacteriocin colicin (*ded)* was found in each of the genomes suggesting a potential widespread presence of these genes in pseudomonads. Siderophore production was additionally screened for and achromobactin genes (*acs)* were identified in the genomes of A-B-26 and BF-B-26. The siderophore pyoverdine (*pvd*) was also identified in a number of genomes across the different land managements, although those with *acdS* gene isolated from grassland managements were not found to have these genes. Phenazine and pyochelin have also been identified in pseudomonads but biosynthesis genes were not identified in the genomes screened in this study. Ferrous iron uptake genes were identified in a number of genomes, excluding all of those isolated from the bare fallow treatment with no *acdS* gene (figure 5.7).



Bacterial isolate	2,4 diacetylphloroglucinol *phlEDBCAFGH*	Hydrogen cyanide *hcnABC*	Phenazine *phzIRABCDEFGO*	Pyrrolnitrin *prnABCD*	Pyoluteorin *pltABCDEFGIJKNO*	Colicin *dedEDAR8*
GL-B-12	-	-	-	-	-	+
GL-B-16	-	-	-	-	-	+
GL-RE-20	-	-	-	-	-	+
GL-RE-29	-	-	-	-	-	+
A-RE-06	+	+	-	-	-	+
A-RE-07	+	+	-	-	-	+
A-RE-08	+	+	-	-	-	+
A-RE-15	+	+	-	-	-	+
A-RE-22	+	+	-	-	-	+
A-RE-23	+	+	-	-	-	+
BF-B-15	-	-	-	-	-	+
BF-B-18	-	-	-	-	-	+
BF-R-01	-	-	-	-	-	+
BF-R-05	-	-	-	-	-	+
BF-R-12	-	-	-	-	-	+
BF-R-16	-	-	-	-	-	+
BF-RE-01	-	-	-	-	-	+
BF-RE-02	-	-	-	-	-	+
BF-RE-03	-	-	-	-	-	+
BF-RE-04	-	-	-	-	-	+
BF-RE-09	-	-	-	-	-	+
BF-RE-14	-	-	-	-	-	+
BF-R-25	-	-	-	-	-	+
BF-RE-21	-	-	-	-	-	+
BF-RE-22	-	-	-	-	-	+
BF-RE-24	+	+	-	-	-	+
BF-RE-25	-	-	-	-	-	+

Bacterial isolate	2,4 diacetylphloroglucinol *phlEDBCAFGH*	Hydrogen cyanide *hcnABC*	Phenazine *phzIRABCDEFGO*	Pyrrolnitrin *prnABCD*	Pyoluteorin *pltABCDEFGIJKNO*	Colicin *dedEDAR8*
GL-B-19	-	-	-	-	-	+
GL-B-26	-	-	-	-	-	+
GL-R-19	-	-	-	-	-	+
GL-R-26	-	-	-	-	-	+
GL-RE-19	-	-	-	-	-	+
GL-RE-26	-	-	-	-	-	+
A-B-19	-	-	-	-	-	+
A-B-26	-	-	-	-	-	+
A-R-19	-	-	-	-	-	+
A-R-26	-	-	-	-	-	+
A-re-19	-	-	-	-	-	+
A-RE-26	-	-	-	-	-	+
BF-B-19	-	-	-	-	-	+
BF-B-25	-	-	-	-	-	+
BF-B-26	-	-	-	-	-	+
BF-B-27	-	-	-	-	-	+
BF-B-28	-	-	-	-	-	+
BF-B-30	-	-	-	-	-	+
BF-R-19	-	-	-	-	-	+
BF-R-21	-	-	-	-	-	+
BF-R-24	-	-	-	-	-	+
BF-R-26	-	-	-	-	-	+
BF-R-30	-	-	-	-	-	+
BF-RE-19	-	-	-	-	-	+
BF-RE-26	-	-	-	-	-	+
BF-RE-28	-	-	-	-	-	+
BF-RE-29	-	-	-	-	-	+

***acdS* positive isolates**

***acdS* negative isolates**

Figure 5.6. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with antibiotic activity. Genes involved in the synthesis of 2,4 diacylphloroglucinol, hydrogen cyanide, phenazine, pyrrolnitrin, pyoluteorin and colicin were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

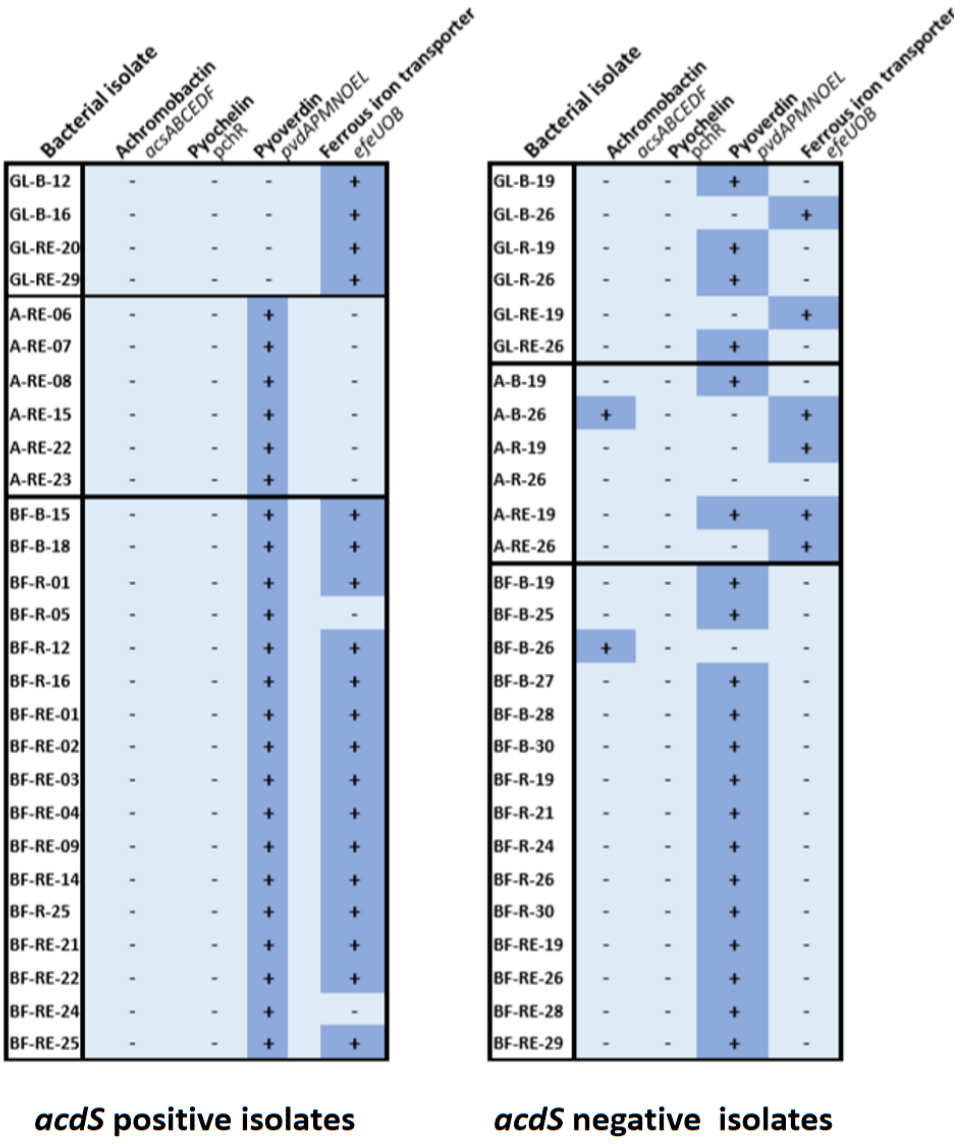


Figure 5.7. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with siderophore production: Genes involved in the synthesis of achromobactin, pyoverdin, pyochelin in addition to an iron transport system were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

5.4.4 Screening for genes involved in nutrient cycling

Various genes involved in phosphate metabolism were identified in the 54 genomes. Alkaline phosphatase (*phoA*) and glycerophosphodiester phosphodiesterase (*glpQ*) in addition to

phoRB genes important for regulation of the *pho* regulon and the *pstS* gene involved in P transport were each identified in all of the genomes. Other genes of interest that were not found in each of the isolates included glucose dehydrogenase (*gcd*) genes involved in the biosynthesis of gluconic acid and found in four isolates. Eight isolates were found to have phytase genes (*phyC*) and phosphate binding DING proteins were additionally found in seven isolates as seen in figure 5.8.

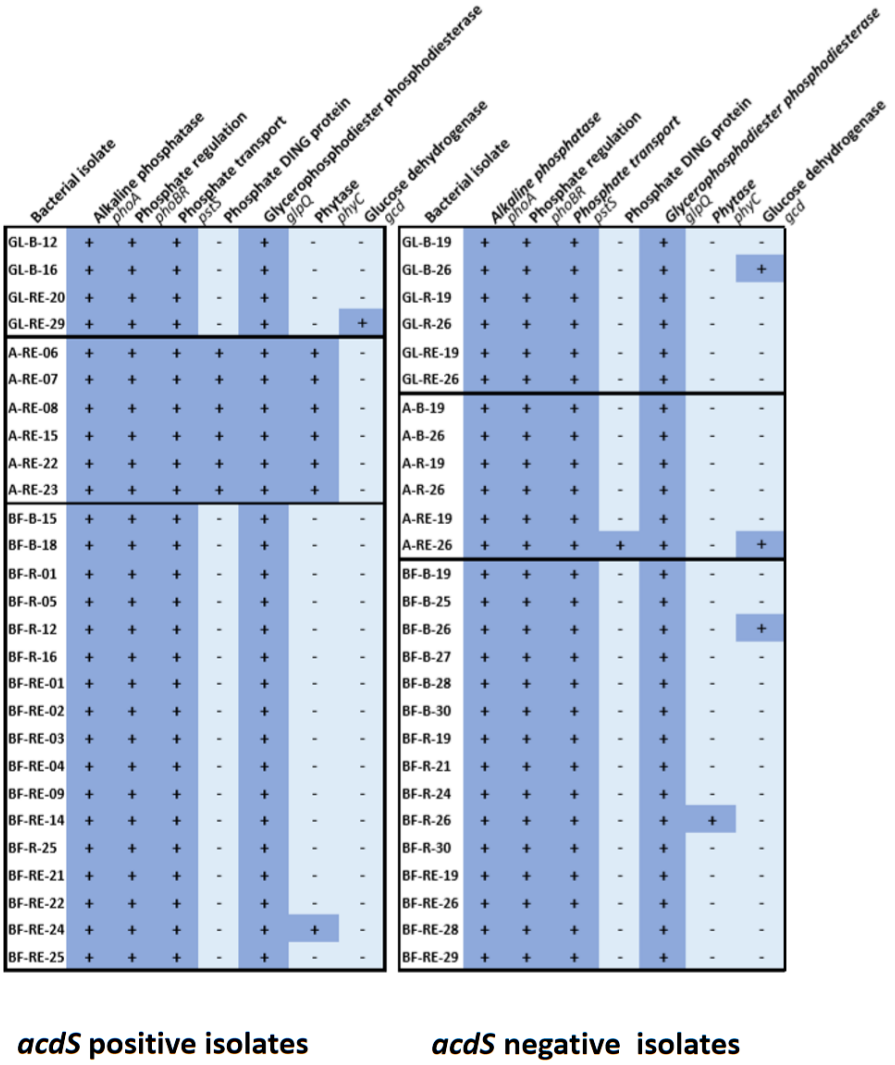


Figure 5.8. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with phosphorous metabolism and transport: Genes involved in the synthesis of alkaline phosphatase, glycerophosphodiester phosphodiesterase, glucose dehydrogenase, phytase, phosphate binding DING proteins in addition to regulation of the *pho* regulon and P transport were screened for in the 54 pseudomonad genomes. Genes identified as either

positive in the isolates (+) or negative (-) are reflected in the table.

Genes involved in dentification were identified mostly in isolates which had the *acdS* gene and isolated from the arable and bare fallow plots as shown in figure 5.9. Albeit some *acdS* negative pseudomonads were found to have such genes. Interestingly, both the *acdS* positive and *acdS* negative isolates from the grassland plots were not found to have any denitrification genes, excluding one isolate (GL-B-26).

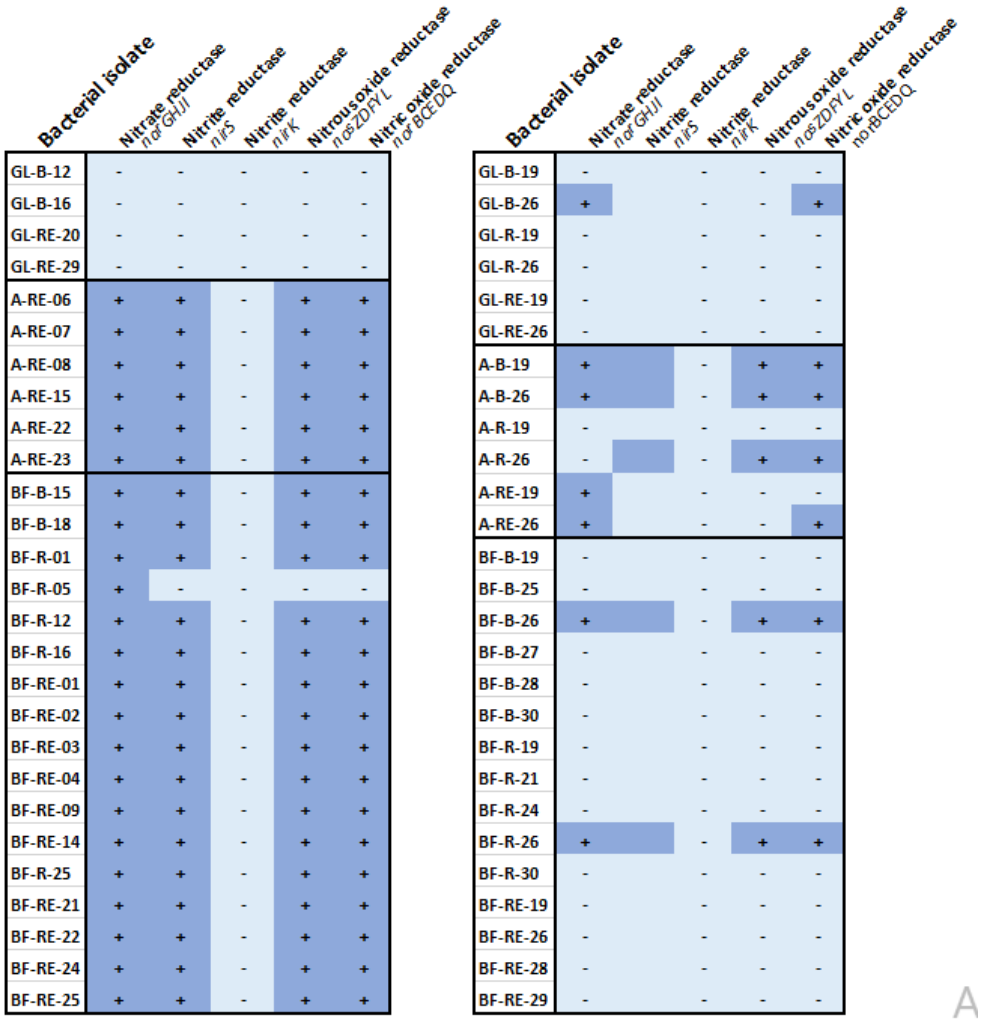


Figure 5.9 . Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with nitrogen cycling: Genes involved in nitrogen cycling were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

5.4.5. Screening for abiotic and biotic stress tolerance

Genes involved in tolerance to osmotic stress were found in each of the isolates including periplasmic glucans (*mdoHGD*) and glycine betaine synthesis (*betABTCI*). Genes involved in carbon starvation (*cstA*) were similarly found in all genomes analysed, however *rspAB* genes involved in the sensing of carbon starvation were only found in six *acdS* positive pseudomonads isolated from the arable treatment and one *acdS* bare fallow isolate. Biotic stress tolerance was also screened for with genes involved in antibiotic resistance. Vancomycin resistance genes (*vanW*) were mainly found in the genomes of *acdS* pseudomonads isolated from the bare fallow treatment, albeit isolates G-B-26 and BF-B-26 without the *acdS* gene were also found to have this gene. Fosfomycin resistance genes (*fosA*) were found in isolates from all land management treatments excluding the *acdS* pseudomonads isolated from the grassland managed soil. Polymyxin resistance genes (*pmrJLM*) were additionally found in isolates with examples found in each land management treatment. A higher abundance of antibiotic resistance genes appeared to be associated with *acdS* positive isolates from the bare fallow treatment (Figure 5.10).

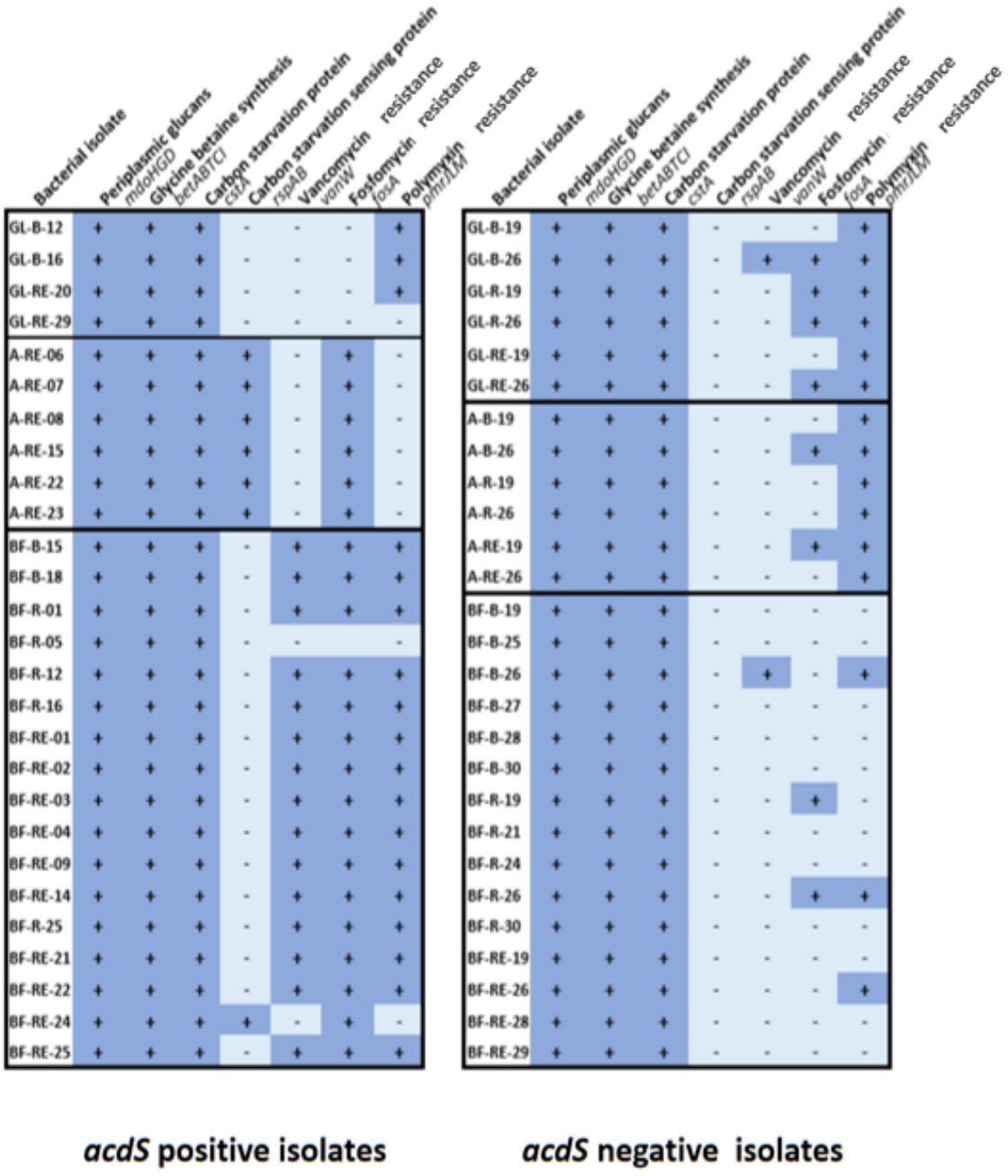


Figure 5.10. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with stress tolerance. Genes involved in the synthesis of periplasmic glucans, glycine betaine, carbon starvation and carbon starvation sensing genes. Biotic resistance genes including vancomycin resistance, fosfomycin resistance and polymyxin resistance genes were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

5.4.6 Screening for rhizosphere colonisation genes

The potential of isolates to successfully colonise the rhizosphere and root compartment of crops was assessed across the 54 genomes. Genes involved in flagellar structure (*flhAV, fliRQ, flgDHBC*) and motility (*motAB, fliMN*) along with key chemotaxis genes (*cheAVYWV*) were found in each of the genomes analysed in this study, suggesting competence in rhizosphere colonisation. Autoinducer 1 (*rhlR lasRI*) and autoinducer 2 (*isrKRABCDFGPQ*) quorum sensing genes were also screened for but were not identified in any of the genomes. Biofilm genes (*pgaABCD*) were identified in ten of the isolates from the arable and bare fallow treatments but not in pseudomonads associated with the grassland treatment as shown in figure 5.11.

Bacterial secretion systems (SS) have additionally been implicated in colonisation and plant-microbe interactions with a variety of genes identified in all 54 genomes analysed. Type IV SS genes involved in pilus assembly (*pilABCTQMNOPE*) and fimbria assembly (*pilY1XVWR*) in addition to type II SS genes involved in secretion of proteins from the bacterial cytoplasm into the extracellular environment (*tadABCZ*) were found in each of the genomes. The type I (*lapBCEDP*), V (*tspBA*) and VI (*impBCGHIAF*) SS genes were found in isolates associated with each of the different land management treatments, albeit were not found in every genome studied. The type III SS genes (*yscU, yopB, icrD*) were not identified in any of the genomes analysed as shown in figure 5.12.

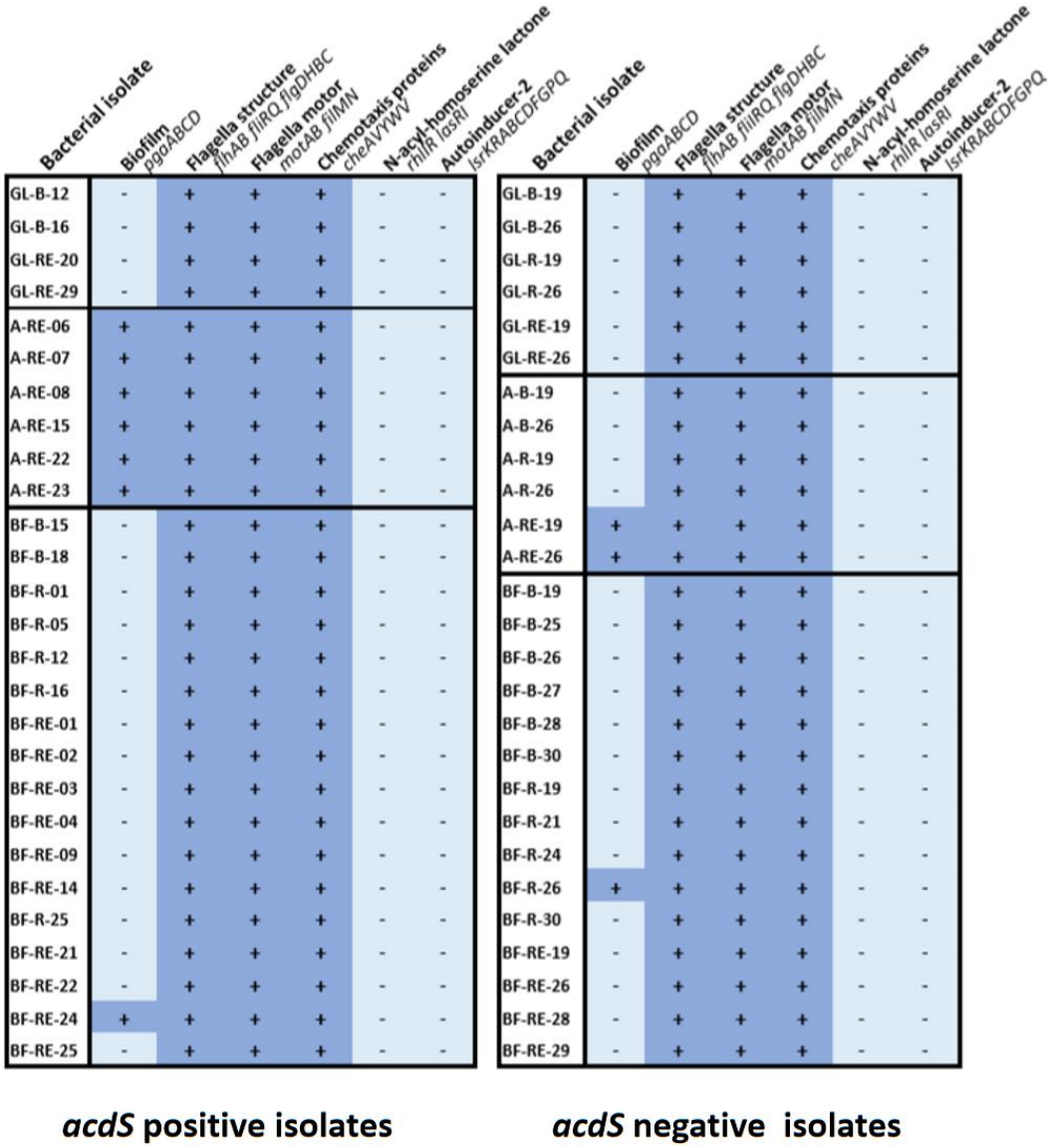


Figure 5.11. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with rhizosphere competence: Genes involved in the biosynthesis of flagellar structure and motility, chemotaxis, quorum sensing and biofilm formation were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

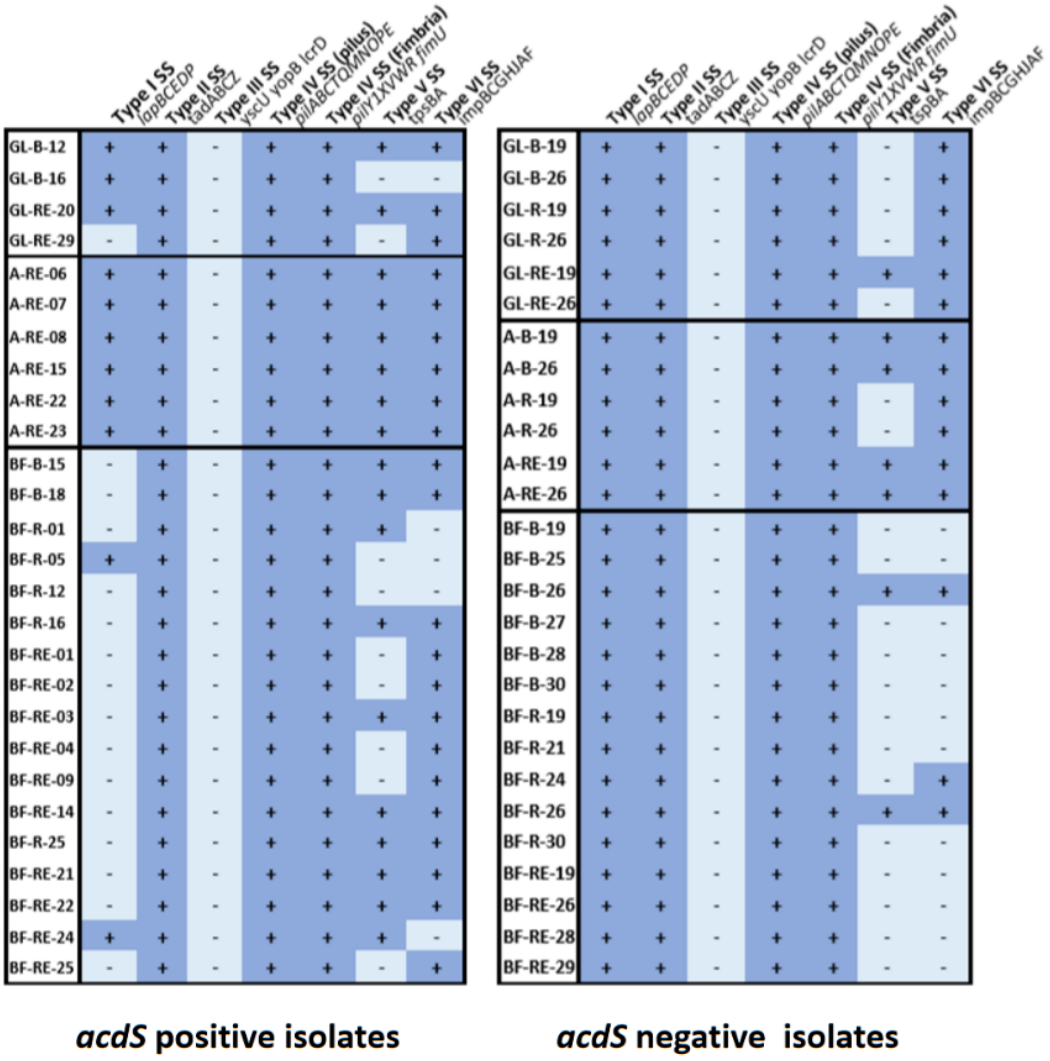


Figure 5.12. Presence/absence table of genes found in 54 *Pseudomonas* spp. genomes associated with secretion systems: Genes involved in the biosynthesis of type I, II, III, IV, V, VI secretion systems were screened for in the 54 pseudomonad genomes. Genes identified as either positive in the isolates (+) or negative (-) are reflected in the table.

To understand if the ACC deaminase gene was co-selected with other PGPR traits of interest across the three land managements, a Spearman's rank coefficient correlation analysis was preformed to determine any negative or positive correlations (see figure 5.13). Many genes were present/absence in every isolate tested which shortened the gene target list for the

correlation analysis. Interestingly the ACC deaminase gene appeared to be co-selected with different genes according to land management. Anti-fungal metabolite genes along with P and N cycling genes were highly positively correlated the *acdS* gene in the arable treatment. Whilst antibiotic resistant genes along with N cycling genes were highly positively correlated in the bare fallow treatment. Interestingly, these genes were negatively correlated with the *acdS* gene in the grassland treatment whilst alkylphosphonate assimilation was the only gene that appeared to be highly positively correlated with the *acdS* gene in this soil.

	Gene target	Bare fallow	Arable	Grassland
Anti-fungal compounds	2,4 diacetylphloroglucinol (*phl*)	0.17	1.00	0.00
	Hydrogen cyanide (*hcnABCD*)	0.17	1.00	0.00
Siderophores	Pyoverdine(*pvdAPMNOEL*)	0.19	0.71	-0.67
	Achromobactin (*acsABCEDF*)	-0.19	-0.30	0.00
Anti-biotic resistence	Vancomycin resistence (*vanW*)	0.81	0.00	-0.27
	Fosfomycin resistence (*fosA*)	0.81	0.71	-0.67
	Polymyxin resistence (*pmrJLM*)	0.69	-1.00	-0.41
Secretion systems	Type I SS (*lapBCEDP*)	-0.88	0.00	-0.41
	Type V SS (*tpsBA*)	0.47	0.45	0.36
	Type VI SS (*impBCGHJAF*)	0.56	0.00	-0.41
Nutrient cycling	Nitrate reductase (*narGHJI*)	0.88	0.45	-0.27
	Nitrite reductase (*nirS*)	0.94	1.00	0.00
	Nitrite reductase (*nirK*)	-0.27	-0.58	0.00
	Nitrous oxide reductase (*nosZDFYL*)	0.81	0.45	-0.27
	Nitric oxide reductase (*norBCEDQ*)	0.02	0.00	-0.41
	Carbon starvation sensing protein (*rspAB*)	0.17	1.00	0.00
	Phosphate DING protein	0.00	0.85	0.00
	Phosphate outer membrane porin (*opr0*)	-0.02	1.00	0.00
	Alkylphosphonate assimilation (*phn*)	-0.19	-0.30	0.82
	Phytase (*phy*)	-0.02	1.00	0.00
Biofilm	Biofilm (*pgaABCD*)	-0.02	0.71	0.00

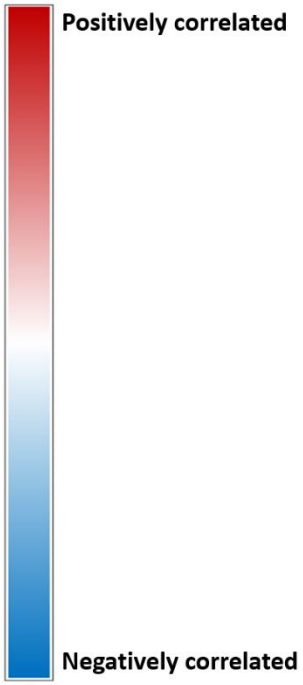


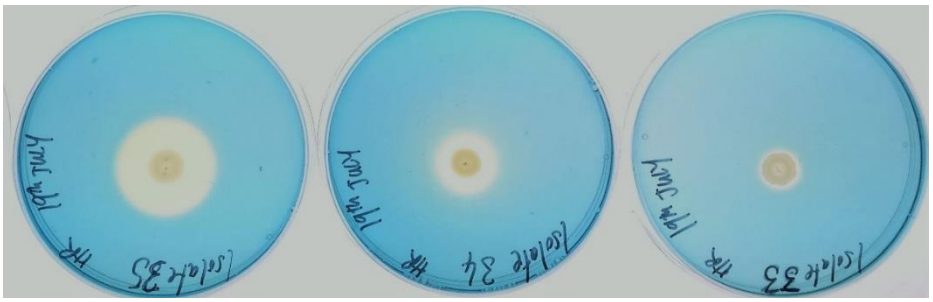
Figure 5.13 Gene presence/absence correlation coefficient analysis: Spearman's rank correlation coefficients between the presence and absence of a selection of PGPR traits. The scale range is from dark blue (highly negatively correlated) to bright red (highly positively correlated), Light colours represent low correlations.

5.4.7 Functional screening for PGPR traits

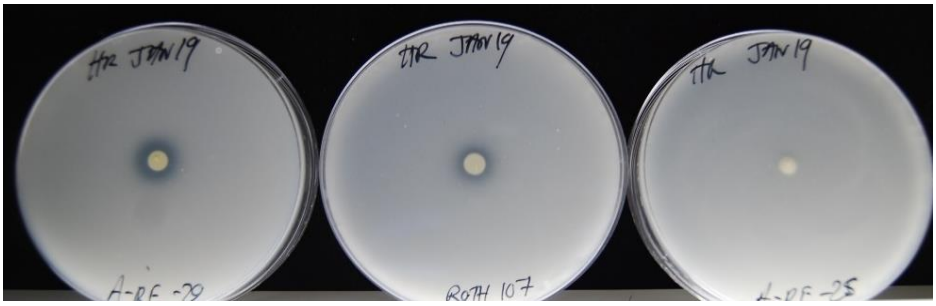
In addition to the genetic potential of 54 *Pseudomonas* sp. to promote crop growth, functional PGPR activity was also screened for utilising high through-put plate assays. Isolates were assessed for their ability to produce siderophores, solubilise phosphate, inhibit fungal growth and to produce ACC deaminase. All isolates displayed siderophore production ranging from low to high activity as seen in figure 5.14 [a]. The ability of pseudomonads to inhibit the growth of pathogenic fungus *G. graminis* was not a widespread trait in the culture collection explored in this study. Seven isolates out of 54 displayed strong anti-fungal activity as shown in figure 5.14 [c]. These isolates all had the *acdS* gene with six isolated from the arable treatment and one isolated from the bare fallow treatment. This finding complements the identification of hydrogen cyanide and 2,4, DAPG genes in the same isolates. Fungistatic activity was also displayed in six of the isolates, in which fungal growth was delayed but not completely inhibited. There was no clear trend in the selection of fungistatic activity across the different land managements.

Phosphate solubilisation was similarly not a wide spread trait found in the culture collection studied however a high proportion of isolates did display low solubilising activity. A-RE-26 was the only isolate found to have strong phosphate solubilisation activity, whilst five isolates were found to have moderate activity. Isolates were additionally assessed for their ability to grow in media containing ACC as the sole N source to indicate ACC deaminase activity. All of the 27 isolates identified as having the *acdS* and *acdR* gene successfully grew in media containing ACC, whilst those identified as not having the *acdS* gene were not found to utilise ACC as a sole N source. Each of these results are summarised in figure 5.15, with low-moderate-high activity of the isolates indicated.

[a]



[b]



[c]

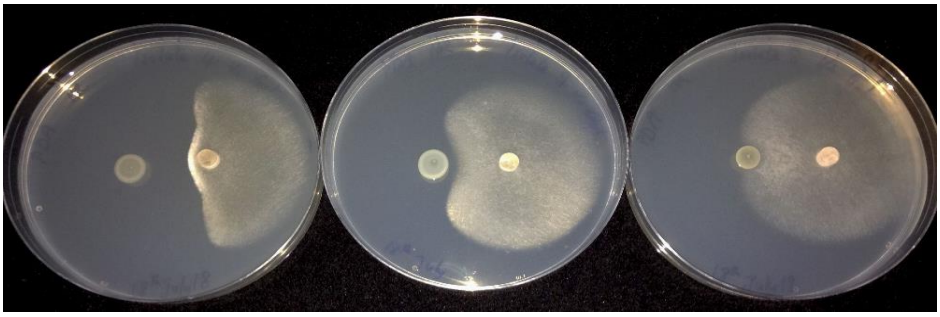


Figure 5.14. Plate assays for the screening of PGPR traits in pseudomonads: Pseudomonads functionally tested for traits related to PGPR activity, this included **[a]** siderophore activity **[b]** phosphate solubilisation **[c]** anti-fungal activity against the fungal pathogen *G. graminis.* Isolates were characterised as having high, moderate or low functional activity as indicated in the photographs from left to right respectively.

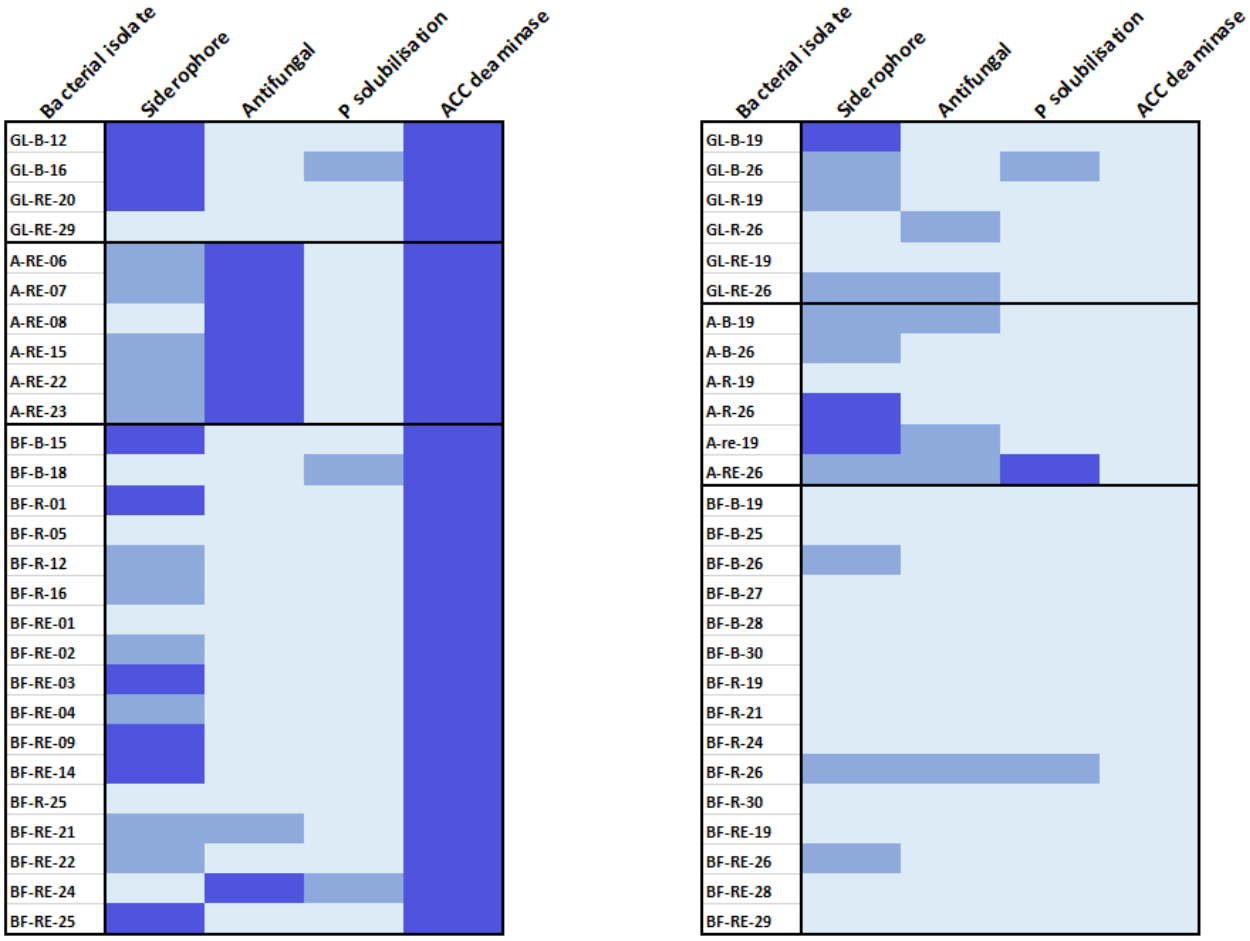


Figure 5.15. Functional activity of 54 *Pseudomonas* spp. to perform PGPR traits: Functional activity involved in siderophore production, phosphate solubilisation, ACC deaminase activity and anti-fungal activity was assessed in the 54 pseudomonad isolates. Activity was characterised as low, moderate or high and is colour coded accordingly (Dark purple: high activity, medium purple: moderate activity and light purple: low to zero activity).

To understand if the ACC deaminase functional activity was co-selected with other PGPR traits of interest across the three land managements, a Spearman's rank coefficient correlation analysis was preformed to determine any negative or positive correlations (figure 5.16). Similarly to the gene presence/absence analysis, anti-fungal functional activity was highly positively correlated with ACC deaminase activity in the arable isolates and moderately

positively correlated in bare fallow isolates. Whilst the anti-fungal activity observed in the grassland isolates was negatively correlated with ACC deaminase activity. Siderophore functional activity was shown to be positively correlated with ACC deaminase activity in the grassland and bare fallow isolates, whilst negatively correlated in the arable isolates. Lastly the functional ability of isolates to solubilise P was found to be positively correlated with ACC deaminase activity in both the bare fallow and grassland isolates, albeit low correlations, whilst negatively correlated with ACC deaminase activity in the arable isolates.

a

	Siderophore	Antifungal	P solubilisation	ACC deaminase
Siderophore	1.00			
Antifungal	-0.06	1.00		
P solubilisation	-0.13	0.68	1.00	
ACC deaminase	0.55	0.13	0.09	1.00

b

	Siderophore	Antifungal	P solubilisation	ACC deaminase
Siderophore	1.00			
Antifungal	-0.12	1.00		
P solubilisation	-0.43	-0.09	1.00	
ACC deaminase	-0.13	0.90	-0.30	1.00

c

	Siderophore	Antifungal	P solubilisation	ACC deaminase
Siderophore	1.00			
Antifungal	-0.36	1.00		
P solubilisation	0.24	-0.25	1.00	
ACC deaminase	0.39	-0.41	0.10	1.00

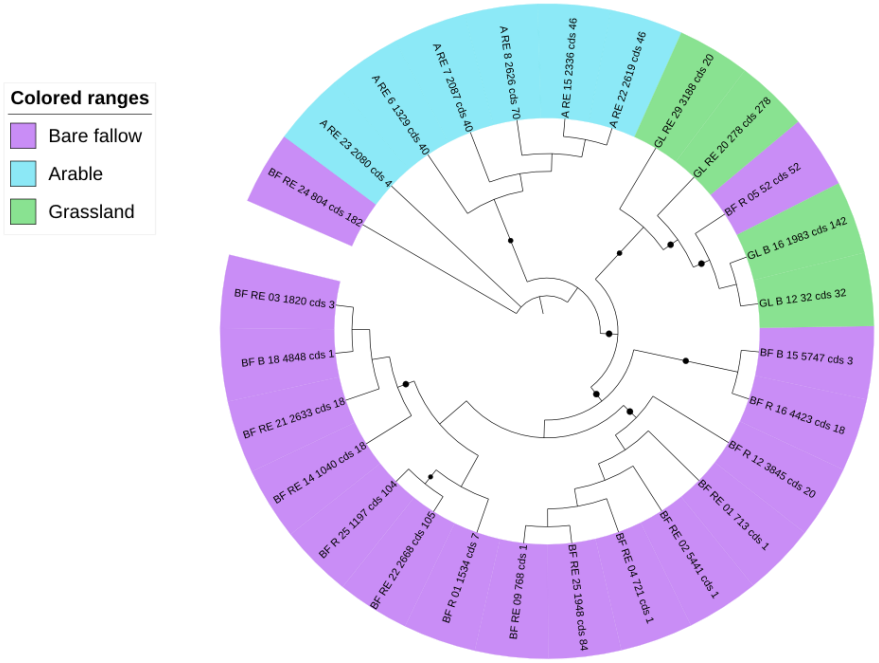
Negatively correlated  **Positively correlated**

5.16 Presence/absence correlation coefficient analysis of functional PGPR traits: Spearman's rank correlation coefficients between the presence and absence of a selection of PGPR functional traits for [a] grassland isolates [b] arable isolates and [c] bare fallow isolates. The scale range is from dark blue (highly negatively correlated) to bright red (highly positively correlated).

5.4.8 ACC deaminase

Pseudomonads isolated from the different land managements resulted in four grassland isolates, six arable isolates and 17 bare fallow isolates with the *acdS* and *acdR* gene. The phylogeny of both of these genes are shown in figure 5.17 [a,b], with distinct clades associated with land management. Out of the 27 gene sequences, twelve *acdS* nucleotide sequences and six *acdR* nucleotide sequences were found to be unique. Sequence alignment analysis identified amino acid residues Lys51, Ser78, Tyr294, Glu295 and Leu322 in each of the 27 *acdS* sequences as shown in figure 5.18. These amino acids have been deemed as essential for ACC deaminase functioning in the reference isolate *Pseudomonas* sp. UW4. Each of the 27 isolates was found to grow in DF media with ACC as the sole nitrogen source, whilst a negative control isolate without *acdS* or *acdR* genes did not grow. Three of the grassland isolates were found to utilise ACC in the growth medium more slowly compared to the arable and majority of bare fallow isolates as shown in figure 5.19. Further to this, the ninhydrin assay measuring ACC consumption found all *acdS* isolates had substantially lower ACC in the growth medium after 48 hours, whilst the negative control had high levels of ACC close to the starting levels of 3 mM.

[a]



[b]

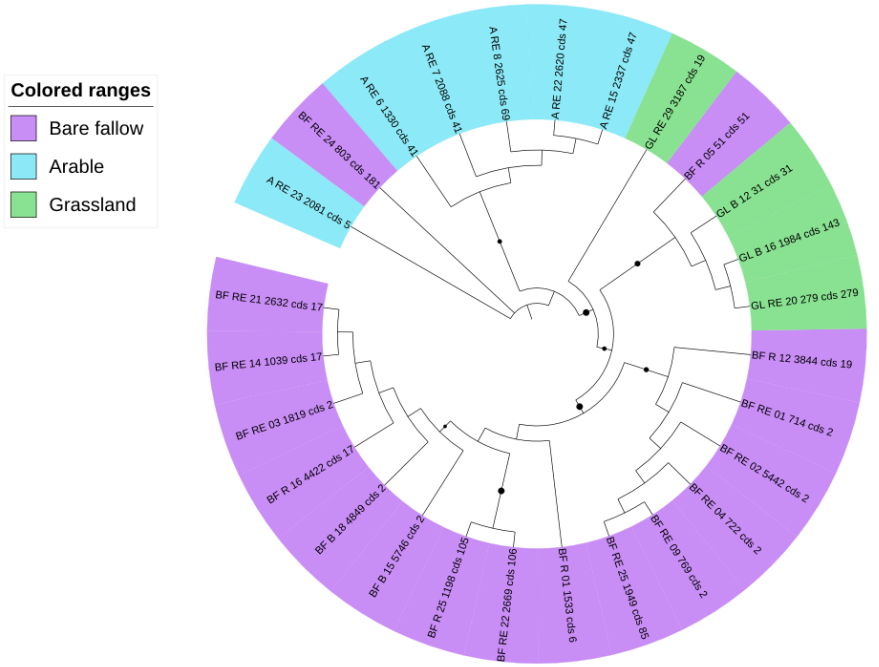


Figure 5.17. Maximum likelihood tree based on concatenation on *acdS* and *acdR* nucleotide sequences from *Pseudomonas* sp. associated with wheat grown in three different land managements: [a] *acdS* and [b] *acdR* nucleotide sequences from bulk soil, rhizosphere and roots isolates of wheat grown in three distinct land management regimes (bare fallow, arable and grassland) were phylogenetically analysed utilising an ML tree with a Hasegawa, Kishino

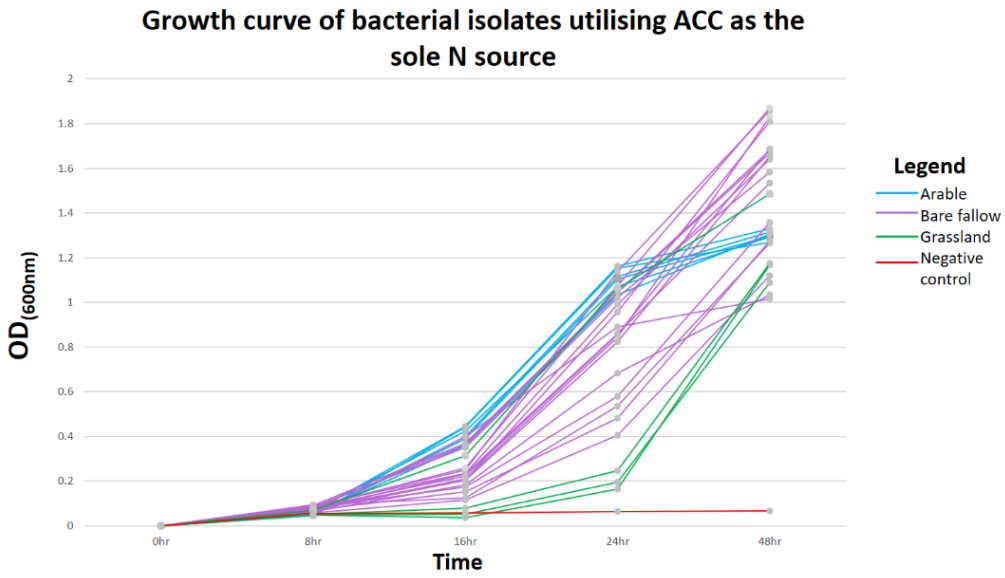
and Yano 1985 model and 100 bootstraps in the software PhyML. Percentage bootstrap values higher than 70% of 100 replicates are indicated by black circles at branching nodes. Individual nodes are colour coded occurring to land management.

[a]

[b]

Figure 5.18. *In silico* analysis of the *acdS* translated amino acid sequences: The amino acids Lys51, Ser78, Tyr294, Glu295 and Leu322 have all been implicated as essential for the functioning of the *acdS* gene in the reference isolate *Pseudomonas* sp. UW4. The alignments shown here with amino acid sequences translated from the *acdS* gene were each found to have all 5 amino acids in the same position as the reference isolate. This indicates that the full genome sequenced isolates found to have the *acdS* gene are potentially functionally important.

[a]



[b]

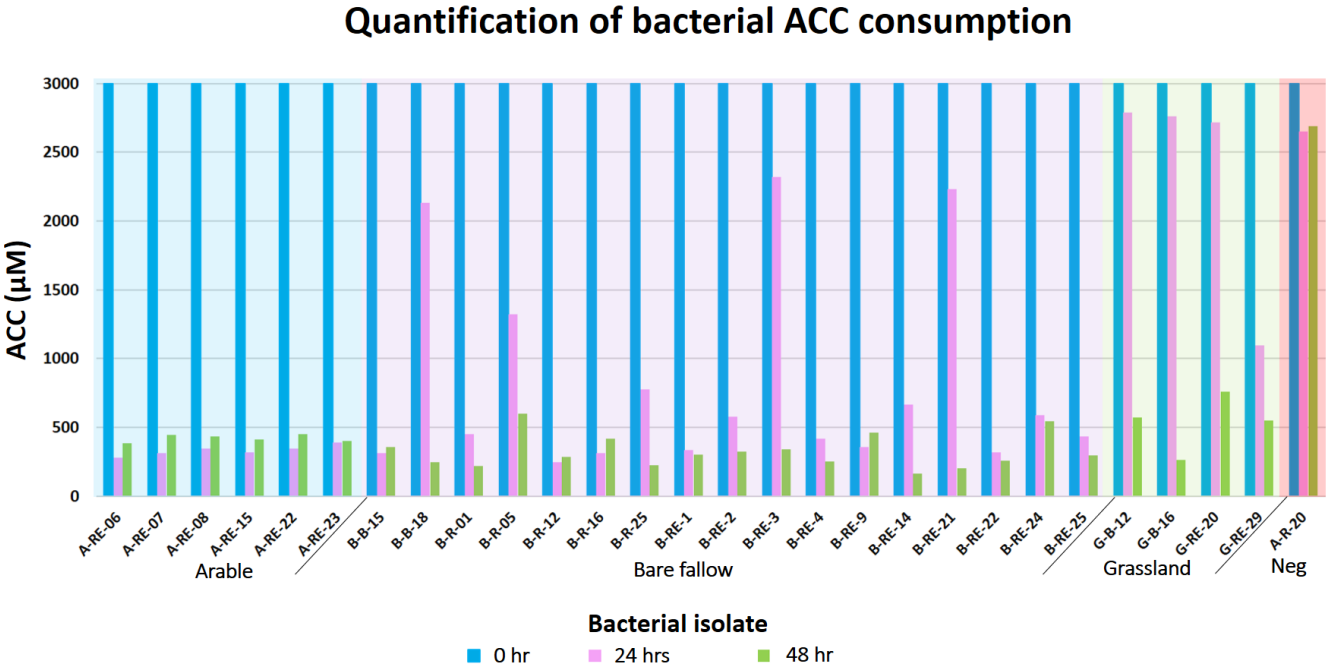


Figure 5.19 Analysis of isolates with the *acdS* gene to grown in media with ACC as the sole nitrogen sou5ce: [a] The optimal density of isolates grown in DF + ACC media was measured at 8hr, 16hr, 24hr and 48hrs after inoculation along with a negative control which did not have the *acdS* gene **[b]** The quantification of ACC consumption was measured using a ninhydrin colorimeter test at 24hrs and 48hrs post inoculation. Differences can be seen in the ability of *acdS* bacteria to utilise ACC as a sole N source across the different land managements.

5.4.9 Plant inoculation assay

5.4.9.1 Bacterial inoculation

Although the pseudomonad isolates had multiple genetic and functional traits that could be further explored for plant growth promotion, salt tolerance and ACC deaminase activity was selected for further study due to simplicity. Salt stress was found to alter crop physiology with no growth observed at 400 μM and 500 μM of NaCl. Seedling growth was significantly reduced at 100 μM NaCl compared to the control of 0 μM and was therefore chosen for further study as seen in figure 5.20.

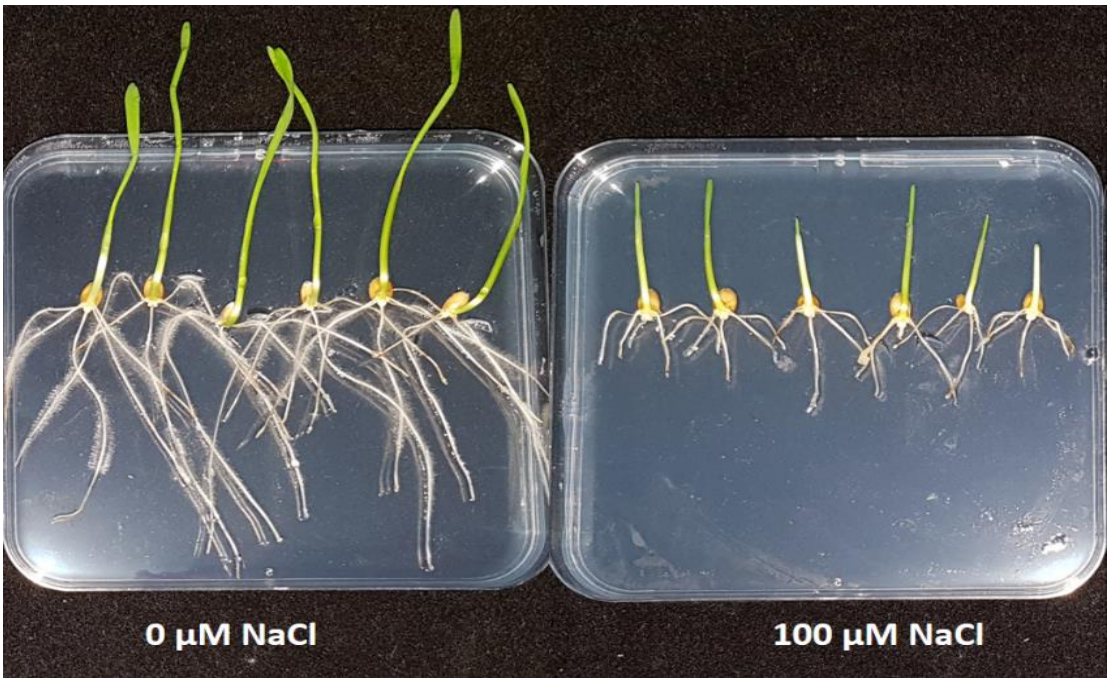


Figure 5.20. Plate assay of wheat seedlings growing on agar under non-salt stressed and salt stressed conditions: Wheat seedlings were grown in plant assays under glasshouse conditions on agar containing 0 μM NaCl or 100 μM NaCl for 5 days in glass house conditions.

Bacterial inoculation resulted in differences observed in root and shoot growth under both non-salt stress and the salt stress conditions (Figure 5.21). It appeared that most of the bacterial applications including the positive control F113 and the negative control of heat-

killed F113 resulted in decreases in growth when compared to the negative control with no bacterial application. Although decreases in average were observed, a Tukey post hoc analysis only found a significant decrease in root length of seedlings inoculated with isolate ARE22, compared to the positive control organism *P. fluorescens* F113 under non-salt stress conditions (p =0.05). All other differences were not found to be significant.

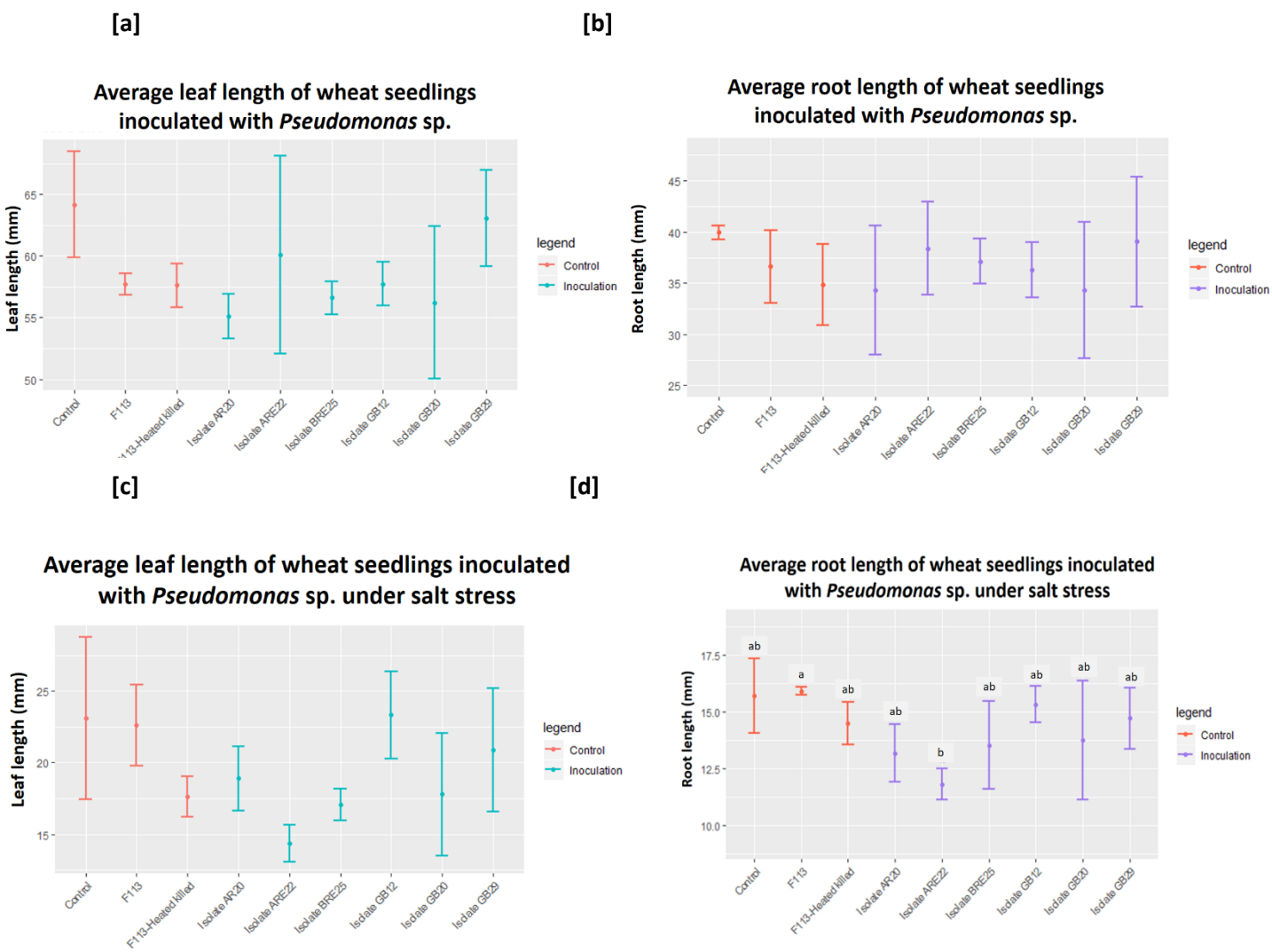


Figure 5.21. Root and leaf measurements of wheat seedlings inoculated with *Pseudomonas sp*. under salt and non-salt stress: Six salt tolerant pseudomonad isolates with the *acdS* gene along with reference strain *P. fluorescens* F113 were each inoculated on wheat seedlings in addition to a negative control of PBS and heat killed *P. fluorescens* F113. The average [a] leaf length under salt stressed conditions [b] root length under salt stress conditions [c] leaf length

under non-stressed conditions and [d] root length under non-stressed conditions were analysed. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

5.4.9.2 Chemical application

The application of the ethylene precursor ACC and the ACC synthase inhibitor AVG revealed significant differences in leaf and root length (Figure 5.22). Under salt stress conditions, AVG and ACC appeared to have antagonist effects, with ACC application at both 5μM and 10μM increasing leaf length whilst AVG at both concentrations reduced leaf length compared to the control. Application of ACC at 10μM increased leaf length significantly compared to the control (p=0.04*), 5μM AVG (p=0.004**) and 10μM AVG (p=0.02*). The average root length under salt stress conditions was found to significantly decrease after AVG application at 10μM compared to control (0.02*) and both ACC applications (p=0.04*, p=0.007** respectively). 5μM application of AVG additionally significantly reduced root length compared to 10μM application of ACC under salt stress (p=0.04*). Whilst ACC application appeared to increase root length slightly compared to the control at 10μM, results were not found to differ significantly.

Under non-salt stressed conditions, ACC application was found to reduce root length whilst AVG application increased leaf length, albeit a significant result was only found between 10μM ACC and 5μM AVG (p=0.04*). Lastly, both ACC and AVG application appeared to reduce root length under non-salt stressed conditions. 10μM AVG reduced root growth significantly compared to the control (p=0.004**), 5μM ACC (p=0.03*) and 10μM ACC (p=0.004**).

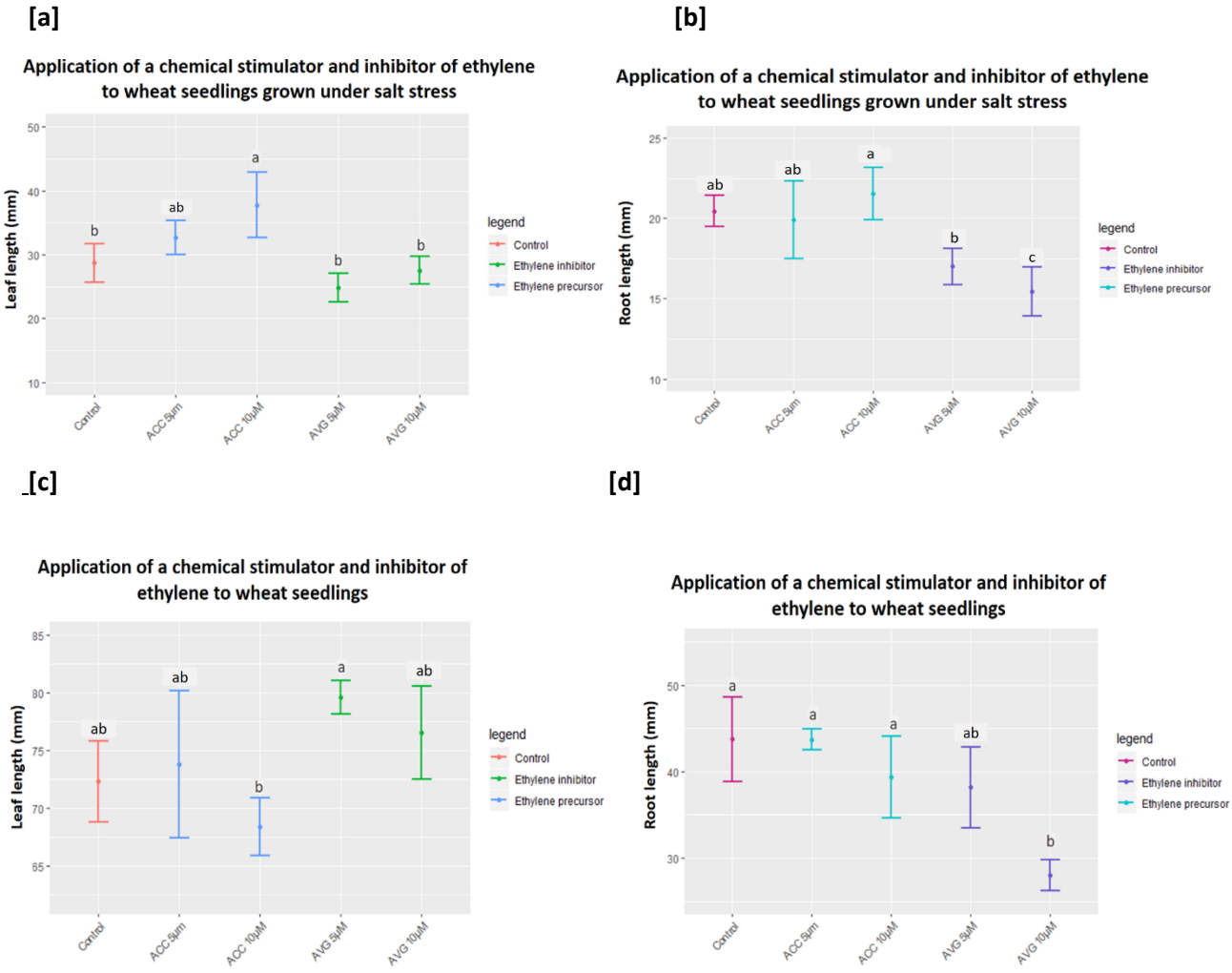


Figure 5.22. Root and leaf measurements of wheat seedlings inoculation with Pseudomonas sp. under salt and non-salt stress: Wheat seedlings grown under salt stress and non-salt stress conditions and were either soaked in the chemical inhibitor (AVG) or stimulator (ACC) of ethylene production. The average [a] leaf length under salt stressed conditions [b] root length under salt stress conditions [c] leaf length under non-stressed conditions and [d] root length under non-stressed conditions were analysed. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

5.5 Discussion

5.5.1 Overview

Microbial inoculants have the potential to play a larger role within agricultural systems in the future, due to the plethora of traits that can benefit crop growth and health (Preston 2004). Genome sequencing technology has recently boosted interest in the utilisation of microbes within farming systems, due to the high throughput analysis of isolates with desirable traits. Despite this, inoculant products have so far failed to gain widespread use, mainly due to a lack in efficacy when applied to the field. Here, 54 *Pseudomonas* sp. isolated from three contrasting land management regimes at the Highfield experiment were successfully full genome sequenced. The genomes were found to have a variety of PGPR related genes and functional activity, which agrees with other studies in the literature (Preston 2004; Silby et al. 2009; Redondo-Nieto et al. 2013).

Many genes involved in PGPR activity were widespread in the pseudomonad genomes including auxin, cytokinin, siderophore, phosphate solubilisation, salt tolerance and various colonisation related genes such as flagellar, fimbriae and chemotaxis genes. On the other hand, genes involved in antifungal activity and ACC deaminase were not ubiquitously found across the 54 genomes studied. Interestingly the seven isolates found to have antifungal genes for hydrogen cyanide and 2,4 DAPG were the only isolates identified as having strong anti-fungal activity against the cereal pathogen *G. graminis.* Other functional screens revealed each of the *Pseudomonas* sp. to have siderophore activity in agreement with the presence of various siderophore genes. The genetic and functional ability for isolates to solubilise phosphate varied, with only one isolate having strong activity.

Further exploration of the ACC deaminase isolates revealed differences in *acdS* and *acdR* genes which appeared to cluster according to land management from phylogenetic analysis. Further to this, the functional activity of the isolates to utilise ACC as the sole N source

revealed differences associated with land management. The arable and most of the bare fallow isolates were quick to respond to ACC as a N source, whilst the grassland isolates except G-RE-29 were much slower to respond. This was further evidenced by quantification of ACC depletion in the growth medium. Isolates with distinct *acdR* gene sequences were selected for inoculation of wheat seedlings *in vitro,* however no clear increase in growth was observed. On the contrary, most isolates appeared to cause a decrease in leaf and root growth highlighting the complexity of understanding plant-microbe interactions. Overall this study helps to highlight the genetic and functional potential of pseudomonads isolated from different land managements, for use within microbial inoculants.

5.5.2 Genome and functional screening

5.5.2.1 Siderophore production

Siderophores are chelating agents that can liberate iron under limited conditions. Iron is an essential element for the growth of microbes and plants, with roles as a catalyst in enzymatic processes, oxygen metabolism and electron transfer (Aguado-Santacruz et al. 2012). Siderophore genes were present in each of the isolates along with functional activity. Pyoverdine genes were most common, whilst achromobactin genes were only found in two isolates from the bulk soil of arable and bare fallow treatments. Siderophore production assessed *in vitro* plate assays displayed a range of low, medium and high activity. No clear correlation was identified between the presence of specific genes and the different levels of function observed. There are numerous gene targets for siderophore production which were not screened for in this study, due to limitations in the annotation software used. BLAST can provide an alternative tool to screen for homologous genes of interest within genomes- however given the high number of PGP genes in the literature time would not permit for this. As sequencing annotations improve in the future, so will the better characterisation of microbes with PGP traits. Siderophores are of interest within microbial inoculants for

rhizosphere competence, giving a competitive advantage in colonisation through iron sequestration (Wandersman and Delepelaire 2004). Conversely, some siderophores can be secreted into the environment and thereby benefit the rhizosphere community by liberating iron, making it accessible to other organisms including plants (Schiessl et al. 2017; West et al. 2006). Siderophore genes and functional activity appeared to be widespread across the pseudomonads studied here, highlighting the potential interest of these strains for microbial inoculant development under iron limited environments and rhizosphere colonisation benefits.

5.5.2.2 Anti-fungal activity

Unlike siderophore production, antifungal genes and functional activity were not as widespread across the culture collection. Antifungal metabolite genes such as pyoluteorin and pyrrolnitrin which have been found in several pseudomonads, were not identified in any of the genomes in this study (Nowak-Thompson et al. 1999; Kirner et al. 1998). Seven isolates were identified as having genes involved in hydrogen cyanide and 2,4 DAPG, which have both been implicated for roles in anti-fungal activity, although their mode of action is not well understood (Islam and von Tiedemann 2011). These isolates were additionally shown to have strong fungicidal activity against the cereal pathogen *G. graminis*. Interestingly the seven isolates all had the *acdS* gene, with six isolated from the arable managed soil and one from the bare fallow managed soil. Indeed, correlation analysis found that antifungal genes and activity were highly positively correlated with the presence of both the *acdS* gene and ACC deaminase functional activity in the arable isolates. As plants exude ACC when under stress, this attracts ACC deaminase producing bacteria to the rhizosphere where they can utilise the ethylene precursor as a nutrient source. A common stress observed in intensive managed soils is an increased abundance of pathogens (Mennerat et al. 2010). It's therefore plausible that wheat grown in the arable managed soil was experiencing stress from a fungal pathogen, providing a

selection pressure for ACC deaminase producing pseudomonads in addition to antifungal activity. This is not an area that has been explored in the literature to date and would require more research. If time had permitted, rhizosphere soil and wheat roots would have been tested for the presence of fungal pathogens across the different treatments. Additionally, seven isolates were found to have fungistatic activity, in which *G.graminis* growth occurred but was reduced compared to the negative control. No correlation could be made between fungistatic activity and the presence of genes potentially responsible for such activity.

5.5.2.3 Phosphorous solubilisation

Phosphorous (P) is essential to living organisms, however the total P available for plant uptake in soils is typically low due to poor solubility and its fixation in soil (Sharma et al. 2013). It's well known that bacteria secrete various enzymes that can release inorganic phosphorous (Pi) under P limited conditions, which can additionally make P more readily available for plant uptake (Glick 1995; Illmer and Schinner 1992). One of the main methods of solubilization studied in bacteria is the production of organic acids, mainly gluconic acid which can solubilize Pi (Adeleke, Nwangburuka, and Oboirien 2017). Glucose dehydrogenase genes involved in the biosynthesis of gluconic acid were identified in four isolates. Whilst alkaline phosphatase and glycerophosphodiester phosphodiesterase genes involved in phosphate metabolism were present in each of the genomes. The *phoRB* genes are important for regulation of the *pho* regulon under phosphate limiting conditions, as is the *pstS* gene involved in Pi transport. Each of these genes were also found in the genomes suggesting that phosphate solubilization potential is common in soil pseudomonads. Indeed, this has been seen across many examples of pseudomonads isolated from a variety of soil environments (Park, Lee, and Son 2009; Peix et al. 2004; Di Simine, Sayer, and Gadd 1998).

Various *Pseudomonas* strains can also degrade organic P compounds, such as phytate and phosphonates. Here only seven isolates were identified as having phytase genes (Cotta et al.

2016). Annotations from the RAST subsystem analysis additionally identified phosphate binding DING proteins in seven isolates (six arable, one bare fallow). Phosphate DING proteins are not well studied in plant-microbe interactions however evidence from human microbiome research suggests a role in virulence though colonisation of intestinal epithelial cells. Upon phosphate limiting conditions DING proteins were localised to extracellular appendages of *P. aeruginosa*, which were involved in adhesion to intestinal epithelial cells (Shah et al. 2014). It is therefore a possibility that these proteins may be involved in plant-microbe interactions, particularly since each of these isolates were sampled from the root (rhizoplane/endosphere), however this is an area that requires more study.

Despite the array of genes identified in genomes that are involved in phosphorous metabolism, isolates were mainly shown to have low solubilising activity from functional plate assays. Five isolates displayed moderate activity and one isolate (ARE26) was found to have high solubilising activity, indicating that these isolates could be of interest for microbial inoculant development in soils with low P availability. No obvious correlation could be made between phosphate solubilising activity and the presence of genes potentially responsible. Again, as annotation software improves over the years so will the characterisation of P metabolism genes in bacteria.

5.5.2.4 Osmotic stress tolerance

Osmotic stress tolerance genes including periplasmic glucan synthesis and glycine betaine synthesis were found in each of the genomes. Periplasmic glucans are involved in osmoregulation in response to osmotic stress (Bontemps-Gallo and Lacroix 2015). Whilst glycine betaine accumulation has been described as an osmo-protectant that balances cellular osmotic pressure under stress (Teixido et al. 2005). There are many examples of pseudomonads in the literature that have displayed salt tolerance and investigated for use within microbial inoculants (Chu et al. 2019; Neifar et al. 2016; Rangarajan et al. 2001). The

interest in salt tolerant bacteria for use within such products is set to increase given the threat of rising sea levels as a consequence of climate change, which increases the risk of salinization of agricultural land (Yeo 1999).

5.5.2.5 Nitrogen cycling

Denitrification involves the reduction of nitrate or nitrite to nitrous oxide or dinitrogen and is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water (Philippot 2007). Pseudomonads are well known denitrifying bacteria present in soils. Here, the majority of bare fallow and arable isolates with the *acdS* gene were highly positively correlated with denitrification genes. Whilst both *acdS* positive and negative grassland isolates were negatively correlated with the presence of denitrification genes. This is perhaps not surprising considering that the arable plots utilised in this study received regular applications of N fertiliser which would have increased soil N availability. Whilst the bare fallow treatment did not receive N applications, it's possible that the higher prevalence of denitrification genes in these isolates compared to grassland isolates was related to the higher bulk density observed in the bare fallow treatment (Gregory et al 2016). A higher bulk density suggests that the bare fallow soil was more compact, which has been associated with an increase in denitrifying bacteria (Pupin et al 2009). This is likely because of reduced pore structure which can lead to more anoxic conditions. In response to oxygen-limiting conditions, denitrifying bacteria produce a set of enzymes to convert nitrite/nitrate to N_2 via nitric oxide and nitrous oxide, helping to generate energy for bacterial survival and growth (Hassan et al 2014)**.**

5.5.2.6 Phytohormone biosynthesis

Similarly to the PCR results found in chapter 4.0, genes involved in auxin biosynthesis (*iaaM)* and cytokinin biosynthesis (*miaA*) were each found in all genomes analysed in this study indicating that the previous PCR screens were accurate. WGS allowed for further screening of

genes involved in other IAA biosynthesis pathways found in pseudomonads- nitrilase, nitrile hydratase and indole pyruvate carboxylase genes were not found in any of the pseudomonads, suggesting that the isolates studied here are not able to produce IAA via the Indole-3-Acetonitrile (IAN) and Indole-3-Pyruvate (IPA) pathways. Indole-acetamide hydrolase *(iaaH*) is required alongside the *iaaM* gene for IAA biosynthesis in the Indole-3-Acetamide (IAM) pathway. Both *iaaM* and *iaaH* were found in each of the genomes, suggesting IAA production via the IAM pathway may occur in each of the isolates. IAA and cytokinin are of interest in microbial inoculants as potentials to alter plant physiology by promoting cell division and elongation, in addition to delaying flowering thereby allowing extended periods of growth (Erturk et al. 2010; Lu et al. 2018). Due to time limitations, the functional ability of isolates to produce auxins and cytokinins was not carried out.

The ACC deaminase PCR screen outlined in chapter 4.0 identified 37 isolates with the *acdS* gene however WGS analysis only identified 26 isolates with this gene. It's likely that PCR inefficiencies led to an overestimation of the *acdS* gene in isolates. Despite this, the abundance of *acdS* isolates in the bare fallow treatment remained highest (17 isolates), followed by arable (six isolates) and grassland (four isolates). The regulatory *acdR* gene was additionally found in each of these isolates, which is essential for the functioning of ACC deaminase. Phylogenetic analysis of the *acdS* and *acdR* gene showed differences in nucleotide sequences, whilst clustering according to land management. This suggests the ACC deaminase genes in this study have been influenced via different evolutionary paths associated with the three different soil managements. Interestingly, the functional ability of the *acdS* isolates to grow in media with ACC as the sole N source differed. Most of the bare fallow and arable isolates grew much quicker over the course of 48 hours compared to the grassland isolates, suggesting these isolates were able to utilise ACC more efficiently. This was reflected by the quantification of ACC consumption utilising a ninhydrin colorimetric analysis. Higher levels of

ACC were found in the media of the slower growing isolates 24 hours after inoculation, whilst all ACC levels were depleted after 48 hours. This could be reflective of the different lifestyles that isolates may have encountered in the different land managements. The grassland managed soil is well known to have qualities associated with soil health (e.g. relatively high SOM, good structure - drainage and aeration), thereby presenting a less stressed environment compared to soil properties in the arable and bare fallow treatment (Hirsch et al. 2009).
Its plausible that isolates colonising crop roots grown under stressed environments, may be quicker to respond to ACC compared to those associated with a less stressed environment. ACC deaminase is a well-known desirable trait for incorporation into inoculants. This study provides insights into the ecological context of *acdS* isolates, with land management impacting gene sequence and functioning. If time had allowed, further genetic analysis of the *acdS* and *acdR* gene would have been studied, in attempt to identify genetic factors responsible for the differences observed in ACC utilisation.

5.5.2.7 Rhizosphere competence

Each of the isolates positive for the above PGPR traits have potential to promote crop growth under different environmental conditions. Although these traits alone are not likely to result in successful inoculants since one of the main issues with inoculant development is the failure of organisms to establish themselves in the diverse rhizosphere. The initial stages of rhizosphere colonisation likely involve motility and chemotaxis genes, which were found in each of the genomes studied. Pilus genes were also identified in each of the genomes which can play roles in adhesion to plant roots. Domination of the rhizosphere is a community effort, in which bacteria of the same species can benefit from fimbriae, biofilm production and quorum sensing genes to help gain establishment and persistence in the root zone. Fimbria genes were found in each of the isolates, but biofilm genes were only found in ten isolates, whilst quorum sensing genes were not detected in any of the genomes. Secretion systems are of importance

in microbe-microbe and plant-microbe interactions, allowing the movement of proteins across the bacterial periplasm and into the surrounding environment. Genes were found in each of the genomes studied for at least one secretion system including type I, type II, type V and type VI. No genes involved in type III secretion systems were found, although these are usually associated with pathogenic microbes. Tolerance to antibiotics is additionally a trait of importance regarding the evasion of attacks from competing organisms. Most isolates were found to have genes involved in antimicrobial resistance with the exception of 12 bare fallow isolates and one grassland isolate. Again, there are numerous antimicrobial resistance genes in the literature that can be screened for, however the annotation software was limited and the number of such genes is likely to be higher in the genomes.

Nutrient acquisition is additionally important for establishment in the rhizosphere, with siderophore production already discussed above. The total genes identified for roles in carbon metabolism were higher on average than in the grassland genomes, followed by arable and then bare fallow isolates as seen in supplementary chapter 8.4. This could be related to the availability of carbon associated with the three land managements, as higher carbon levels have been associated with the grassland treatment, with reductions found in the arable and bare fallow plots. Despite this, the number of genes involved in carbon metabolism did not differ significantly in isolates from the different land managements.

It is clear that the pseudomonads studied here have a wealth of genetic and functional potential for incorporation in inoculants. It's likely that these isolates have many more traits of interest that were not screened for in this study due to limitations in time and annotation quality. As more genes are characterised in the future, genome annotations will improve and allow for the quick identification of many more traits of interest. An additional limitation is the bottleneck in PGP genes screened for, since this is usually based on a list of genes taken from the literature. Its likely that there are many more genes of importance in plant-microbe

interactions, but until they are identified they cannot be quickly screened for in candidate isolates. It is important to remember that despite the identification of genes and functional activity *in vitro*, there are various environmental factors that alter gene and protein regulation. Therefore, if an isolate can perform within a plate assay, it does not necessarily indicate it will perform desirably when applied as an inoculant in the field. For this reason, microbes of interest must be studied in relation to the crop of interest and different soil properties.

5.5.2.8 *In vitro* plant inoculation

Given the diversity found in ACC deaminase gene sequence and function across isolates, it was decided to investigate this PGPR trait further *in planta*. Pseudomonads applied to wheat seedlings under salt stress and non-salt stress conditions revealed differences in seedling growth after 6 days. On average leaf and root length were reduced after bacterial inoculation, albeit these results were not found to be significant against the control, except for a significant decrease in root length after inoculation with A-RE-22. This suggests that application of *Pseudomonas* in this study had a negative impact on seedling growth under salt stress, which contradicts some studies in the literature and highlighting the difficulties of early stage developments in microbial inoculant studies (Pourbabaee et al. 2016; Gupta and Pandey 2019). Interestingly, the negative control of heat killed F113 also appeared to result in decreases in seedling growth, suggesting that a plant-microbe interaction occurred that was not related to a viable trait. It's possible that immune recognition of highly conserved molecular patterns called microbial associated molecular patterns (MAMPs) may have occurred. Flagella is an example of a MAMP that may have been recognised by plant receptors and thereby initiated an immune response, which can result in decreased growth (Liu et al. 2017). Although the application of a single bacterial species is not representative of the microbiome found surrounding roots in the field, its useful to identify and confirm plant-microbe interactions at the preliminary stages of inoculant development.

Another point to raise, is the complex role of ethylene during different developmental stages. Ethylene is generally referred to in the literature as a stress hormone, well known for its role in growth inhibition (Iqbal et al. 2017). Conversely, some studies have found that ethylene levels are important for growth at certain growth stages and stresses (Cao et al. 2007; Lin et al. 2013). Studying the role of ACC deaminase bacteria to reduce ethylene therefore requires more careful consideration of different development stages and plant species. It appeared that increased ethylene from ACC stimulation under salt stressed conditions was beneficial to leaf growth in this study, whilst under non-stressed conditions reductions in leaf growth were found- and vice versa for the ethylene inhibitor AVG. This would suggest that application of ACC deaminase bacteria inhibiting ethylene under salt stress conditions could have negative impacts to seedling growth. AVG was additionally found to significantly decrease root growth in both non-stressed and salt stressed conditions. If time had permitted, the inoculation assay would have been repeated with bacteria sampled from roots for RT-qPCR analysis of the *acdS* gene, in addition to quantification of root ACC content. This would help to unravel if the *acdS* gene was being expressed in this assay and if it reduced ACC levels to result in negative impacts to seedling growth under salt stress. Despite these results, some studies suggest that AVG at concentrations above 5μM can have off target impacts, by increasing root auxin which is a strong inhibitor of root growth (Swarup et al. 2007). It's clear that the complexity of plant physiology in addition to environmental conditions are considerations required for the development of a successful microbe inoculant.

5.5.2.9 Conclusion

The genome analysis of 54 *Pseudomonas* sp. isolated from different niche compartments of wheat grown in soil from 3 land managements, revealed several genetic and functional traits of interest for inoculant development. Interestingly, some PGPR genes appeared to be co-selected with the acdS gene according to land management, which could reflect the different

stresses encountered in the more degraded arable and bare fallow soil compared to the grassland soils. Despite this, organisms selected for genes involved in ACC deaminase activity failed to promote growth of wheat seedlings grown under salt stress and non-salt stress conditions. Overall this highlights the diverse potential of the *Pseudomonas* genus to promote crop growth, whilst providing a glimpse into the difficulties of PGPR inoculation *in planta.*

6.0 General discussion

6.1 General background

Concern surrounds the current global food system and its stability with many socio-economic, political and environmental issues related to agriculture as we know it. From a productivity perspective, farming systems are set to face many pressures including increased supply demand for an expanding population, reduced crop yields as a result of climate change, the loss of agricultural land and limited availability of finite resources. This is compounded by the additional need for a food system that is more environmentally conscious, by encouraging biodiversity surrounding agricultural land rather than reducing it.

The microbiome of soil and plants is an area of interest, promising to promote a more sustainable food system, by harnessing the natural capability of beneficial organisms and thereby reducing the need for chemical applications. Substantial evidence indicates that manipulating the soil microbiome could have real potential in helping to move toward a more sustainable agriculture. Although many advances have been made to help understand microbial inoculant efficacy, products are still prone to failure in the field. This has been associated with the incorporation of organisms without ecological considerations such as the biological, chemical and physical properties of a soil, climate, plant species and developmental stages in addition to rhizosphere competence of strains.

Due the complexity of the microbiome, it is difficult to study PGP traits in depth for microbial inoculant development. *Pseudomonas* spp. are model rhizosphere organisms that are easy to cultivate in the laboratory, ubiquitously found in soil and well known for their ability to perform several PGPR traits. Despite this, the establishment and persistence of these organisms within or associated with the root system of plants requires further study before successful microbial inoculants can be developed. The rise of agriculture has been suggested to negatively impact plant-microbe interactions. Crop domestication and land management

are two examples of anthropogenic actions that have shaped modern agriculture but there are limited studies regarding their impacts on beneficial microbes. This thesis contributes insights into the impacts of crop domestication and land management on beneficial pseudomonad community structure and function in soil and the root associated microbiome of wheat. Additionally, it provides information on pseudomonad isolates that have potential as plant and soil inoculants to improve crop health and yields.

6.2 Pseudomonad community structure in the rhizosphere of domesticated and ancestral wheat

The domestication of wheat has a complex evolutionary history that has resulted in a variety of genetic and phenotypic differences between modern and ancestral species. It has been suggested that modern genotypes of wheat have reduced capabilities to interact with beneficial soil microbes, due to reduced genetic diversity, altered root exudation and altered root architecture compared to their wild relatives (Perez-Jaramillo, Mendes, and Raaijmakers 2016). The study of 17 wheat genotypes ranging from the diploid (AA, BB, DD), tetraploid (AABB) and hexaploidy (AABBDD) genomes were not found to impact pseudomonad community structure in the rhizosphere, with similar results observed in pseudomonad abundance and phylogeny. The study of pseudomonads associated with the rhizosphere of more taxonomically distinct crops including pea, tomato and oil seed rape again identified no clear difference in *Pseudomonas* sp. community structure.

This suggests that crop genotype was not a strong driving factor for changes in pseudomonad community assembly in the rhizosphere. This could possibly have been due to the grassland managed soil utilised, as it has been shown to support high microbial biomass compared to conventional arable systems. This may have made plant induced changes in rhizosphere pseudomonad communities difficult to detect, particularly since soil factors are well known as strong driving factors in microbiome assembly (Xue et al. 2018; Bulgarelli et al. 2012). Despite

this, it's possible that *Pseudomonas* sp. community function varied across the rhizosphere of ancestral and domesticated wheats. Pseudomonads are associated with an array of different traits, with large heterogeneity observed in the accessory genome of strains (Kung, Ozer, and Hauser 2010). Analysis of the genetic and functional capabilities of pseudomonads would have offered further insights into potential impacts of wheat domestication. It's worth noting that there are many other important microbial species that could be altered in the rhizosphere of domesticated and ancestral wheats. Indeed, studies in the literature have identified impacts of crop domestication on the total microbiome associated with rhizosphere soil (Bulgarelli et al. 2015). An additional consideration is the use of pot experiments, which are not always representative of the field environment.

6.3 Land management

Land management is an example of anthropogenic activity in agriculture that can impact the soil microbiome. Vast chemical, physical and biological soil alterations from different land managements can have marked effects on plant physiology and thereby alter plant productivity.

In agreement with previous studies at the Highfield experiment, land management was found to impact soil properties, wheat physiology and microbial community structure. Parameters considered indicators of good soil health were more associated with the grassland managed soils, including increased C, N, moisture content and microbial biomass, whilst these properties were degraded in the arable and bare fallow plots. After ten years since establishment, the conversion of arable and bare fallow soils to grassland successfully increased soil health qualities, thus highlighting the recovery and resilience potential of plant inputs to soil. Conversely, grassland soils converted to bare fallow and arable treatments were shown to have degraded soil properties.

Land use was also found to impact plant physiology, with significant reductions in root dry weight and grain yield found in the bare fallow plots converted to arable. Phytohormones are major signalling components for the adaptation of plant physiology towards their surrounding environment. Thus, soils managed differently may influence phytohormone profiles in crops. In this study, higher levels of gibberellic acid were found in the roots of wheat grown in the bare fallow to arable conversion. This could reflect the lower root dry weights observed in this treatment, since higher levels of gibberellic acid in roots has been associated with reductions in root growth. Jasmonic acid was additionally found to differ significantly, with higher levels found in roots of wheat grown in the continuous arable plots. Jasmonic acid is associated with defence against necrotrophic pathogens and could be indicative of wheat experiencing biotic stress. The association of pathogens in intensive monoculture conditions has been found in many studies (Pankhurst et al. 2003; Krikun et al. 1982).

Root phytohormone changes can alter the soil microbiome. A well-studied trait found in pseudomonads and other microbes is the presence of ACC deaminase, which can modulate plant ethylene levels through degradation of the precursor ACC in the root. Interestingly, the distribution of the *acdS* gene in bacteria was found to be higher in wheat grown in the continuous arable and bare fallow to arable conversion plots compared to grassland to arable conversions. This could be indicative of wheat experiencing stress in both of these treatments as evidenced from reduced root dry weight and yields in the bare fallow conversion plots and the higher levels of jasmonic acid observed in the arable plots. The *acdS* gene was found to increase in frequency in the wheat rhizosphere which is in agreement with other studies in the literature (Bouffaud et al. 2018).

Pseudomonad *16SrRNA* gene abundance was not found to significantly differ across bulk soil or rhizosphere soil associated with the arable and conversion to arable managements. This agrees with previous culture work that found no significant difference in pseudomonad abundance associated with the bulk soil or rhizosphere of domesticated and ancestral wheat.

Since both culture and molecular analysis did not find a significant difference in *Pseudomonas* spp. associated with these niche compartments, it indicates that these results are reliable despite contradictory results reported in the literature (Ali, Charles, and Glick 2014; Hussein and Joo 2018). Several studies of the total microbiome have found that bulk soil and rhizosphere communities differ but are not as distinct compared to rhizosplane and endosphere communities (Edwards et al. 2015; Martínez-Diz et al. 2019). Studies revealed increases in pseudomonad communities and other PGPRs in the niche compartment of roots compared to the bulk soil and rhizosphere (Gottel et al. 2011; Yamamoto et al. 2018). Collectively this research highlights the impacts of land management on multiple factors including soil properties, wheat physiology and microbial composition, providing a glimpse into the complexity of these dynamics.

6.4 The distribution of *Pseudomonas* phytohormone genes across different land management.

Wheat was grown in soil sampled from the three permanent treatments at the Highfield experiment (grassland, bare fallow, arable) and utilised in pot experiments for subsequent sampling of bulk soil, the rhizosphere soil and the root compartment (rhizoplane / endosphere). Land management was shown to affect pseudomonad community abundance and phylogeny in different niche compartments of wheat. The most diverse communities were associated with the grassland soil followed by the arable treatment and the lowest diversity found in the bare fallow soil. Differences in the abundance of pseudomonads associated with bulk soil and rhizosphere were not found to differ significantly, whilst the abundance of pseudomonads associated with the root compartment was significantly higher than both bulk soil and rhizosphere soil across the three land managements. Little is known on how microbial community structure translates into microbiome functioning and there are numerous PGPR targets of interest can be chosen for investigation. Here bacterial phytohormone genes were

studied since they can alter plant physiology through direct modulation of plant phytohormone signalling (Glick 2005; Xu et al. 2015).

Screening for genes of functional relevance revealed all isolates to have auxin genes (*iaaM*) involved in the indole-3-acetamide (IAM) pathway and cytokinin genes (*miaA*). This could be reflective of a common PGPR trait, however these genes have also been implicated in bacterial metabolism. Therefore, further investigation is required to determine the true PGPR potential of these genes. Only a small proportion of pseudomonads were found to have the *acdS* gene, however gene abundance increased in the rhizosphere and root compartments of wheat grown in the bare fallow treatment. This could be indicative of wheat experiencing stress and exuding more ACC into the rhizosphere. The bare fallow soil is well known to have degraded soil properties such as decreased nutrient content and soil compaction, which can provide suboptimal conditions for crop growth (Hirsch et al. 2009). Indeed, ACC deaminase abundance has been associated with stressed environments and the rhizosphere (Timmusk et al. 2011). This suggests the potential role of plant exuded ACC as a rhizosphere attractant for *acdS* bacteria that can utilise the substrate as a nutrient source. Collectively this highlights that anthropogenic manipulation of land can impact soil microbial communities structurally and influence the abundance of functional genes involved in plant-microbe interactions.

6.5 Genetic and functional screening of *Pseudomonas* spp. for plant growth promoting activity

Genome sequencing is a great resource that has revolutionised microbial research with the ability to screen and compare genomes for various traits of interest. Microbial inoculants have recently received increased interest due to the lower cost and easier handling of sequencing technology. Such techniques are helping to gain a deeper knowledge of soil microbes and their genetic potential to perform a range of PGPR traits in addition to successful rhizosphere colonisation. The functional verification of genes is additionally important, by ensuring genes

of interest perform within inoculant applications.

The genome analysis of 54 *Pseudomonas* sp. isolated from different niche compartments of wheat grown in soil from 3 land managements, revealed several genetic and functional traits of interest for inoculant development. A finding that stood out was the potential co-selection of arable isolates with ACC deaminase and strong fungicidal activity in plate assays. This correlated with the presence of hydrogen cyanide and 2,4 DAPG genes. Further research would be required to better assess if this co-selection can occur as an evolutionary pressure from the release of ACC in the rhizosphere, by wheat experiencing biotic stress. Interestingly, the biotic stress related hormone jasmonic acid was previously found in wheat roots grown in the arable treatment, which warrants further exploration into this potential relationship. Phylogenetic analysis of the *acdS* and *acdR* genes suggest that evolutionary pressures exist across the three land managements, as gene nucleotide sequences clustered according to land management. The 27 isolates with the *acdS* gene were functionally assessed, with grassland isolates observed to utilise ACC at a slower rate compared to the majority of bare fallow and arable isolates. This further suggests that different evolutionary pressures exist across the land managements for ACC deaminase function. Since the bare fallow and arable plots are associated with degraded soil properties, it is possible that isolates from these treatments were better adapted to ACC use compared to isolates from the grassland plots in which soil properties are better. ACC deaminase activity was further tested *in planta,* however all isolates failed to promote growth of wheat seedlings under salt stress and non-salt stress conditions. Overall this highlights the diverse potential of the *Pseudomonas* genus to promote crop growth, whilst providing a glimpse into the ecological impacts of different land uses on soil microbial functioning *in vitro*. It additionally highlights the difficulties of PGPR inoculation development.

6.6 Future prospects and challenges of the microbiome research in agriculture.

With the advent of next generation sequencing and the development of novel bioinformatic approaches, the capacity to investigate the composition of the soil microbiome and its function has greatly improved. An avalanche of studies now exists in the literature that focuses on microbiome structure across multiple environments, mostly based on a singular gene e.g. 16S rRNA. Whilst these studies are great for observing differences in microbiome assembly, they cannot offer information on how this translates into altered community function. Technologies such as meta-transcriptomics, meta-proteomics and metabolomics can help to unravel factors involved in microbiome assembly, dynamics, and function which is highly desirable in microbial inoculant development (Bakker et al, 2013). Additionally, there is a need for an increase in spatio-temporal studies if the microbiome is to be better understood. Typically, samples are taken of the microbiome that represent one snap-shot in time. Whilst this can yield useful information it's not necessarily representative of the system studied, as microbiome dynamics are non-static. The development of mathematical and computational models of microbial interactions with specific community components, will also be vital for a deeper understanding of the microbiome. Such advances could also reveal different evolutionary forces (mutation, selection, migration, and drift) that shape the functional capabilities of soil microbial communities. This would help to identify if the success of a bacterial lineage is enabled by the host or by other microbial members of the community. Increased accessibility of these technologies will allow better insights in microbial communities however multidisciplinary approaches will also be important, with a more integrative approach to microbial inoculant development required. Considerations of plant genotypes and signalling in the rhizosphere along with environmental factors such as climate and soil type must be studied. This may require microbial inoculants to offer a more tailored approach for application in environments that are vastly distinct. Such an approach may offer a solution to the unpredictability typically observed in commercialised inoculants to date, in which organisms are generally introduced into ecologically inappropriate environments.

An additional area of research that may help to progress inoculant development is the use of genome editing technologies. The establishment and persistence of microbes introduced into the rhizosphere has often been unsuccessful, with transient changes in the microbiome observed. The creation of novel microbes or synthetic communities offers a new but currently underexplored approach to engineering the microbiome. Microbes with functional traits of interest could potentially be engineered for increased fitness and survival during the formulation and storage development of microbes. An additional potential route to manipulate the soil microbiome is through genetic engineering of plants that have altered root morphology or exudation that promote beneficial interactions with soil microbes. Since genome editing technology is considered a controversial topic, it may be many years before this kind of research progresses. The release of genetically engineered microbes into the environment comes with various considerations including safety and ecological concerns. Successful microbial inoculants developed to improve crop growth have the potential to shape microbial communities negatively in the long term, by reducing soil biodiversity. Albeit, many researchers are beginning to look at the application of microbial assemblages that better represent communities in the natural environment.

It has been suggested that the complexity existing in soil systems is unlikely to see successful manipulation of the soil microbiome from inoculant applications, at least until a better understanding of dynamics in the soil microbiome is achieved. Instead, the management of farming systems with techniques that have been shown to improve biodiversity and microbial biomass may offer an alternative route to manipulate the soil microbiome. It's well documented that land management practices such as those incorporating higher organic matter levels can positively alter the soil microbiome compared to intensive systems (Muhammed et al. 2018, Din et al. 2013). Whether land management practices or inoculants are developed, the overarching theme is that the microbiome is unambiguously important in providing ecosystem services in agricultural soils.

6.7 General conclusion

The soil microbiome is a dynamic and complex environment that has been shown to provide many ecosystem services. *Pseudomonas* spp. include agriculturally important strains that have the potential to provide multiple benefits to crop growth in a sustainable way. Despite this, microbial inoculants have typically failed to produce reliable results, which can be attributed to the incorporation of microbes into ecologically unsuitable environments. In this thesis a range of genetic and functional potential was displayed in multiple pseudomonad isolates, which was shown to be influenced by land management. Whilst the study of wheat domestication on pseudomonad rhizosphere communities revealed no clear differences in community structure. This suggests that soil properties may be a strong driving factor for pseudomonad assembly compared to the plant genotypes studied here.

Functional traits were additionally shaped by land use, particularly *acdS* gene abundance which appeared to be more associated with stressed environments in the arable and bare fallow soils. Phylogeny of the *acdS* nucleotide sequence in pseudomonads revealed differences associated with land management. Along with differences found in bacterial ACC utilisation, this suggests that factors associated with land management can drive the evolutionary trajectory of ACC deaminase in pseudomonads. Attempts to manipulate plant physiology through the application of ACC deaminase isolates was not successful and highlights some of the issues relating to microbial inoculant development. Increased investments into microbial inoculant development in the future will help to unravel the intricate dynamics occurring in soil-plant-microbe interactions and allow for better manipulation of the rhizosphere.

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8.0 Appendices

8.1 Supplementary data chapter 2.0

8.1.1 Statistical analysis

[a]

	SUM of squares	Degrees of freedom	Mean squares	F value	Significance
Crop species	1.35E+14	19	7.10E+12	3.594	7.92e-05 ***
Residuals	1.19E+14	60	1.98E+12		

[b]

	SUM of squares	Degrees of freedom	Mean squares	F value	Significance
Crop species	1.341e+11	7	1.915e+10	0.329	0.935
Residuals	1.860e+12	32	5.813e+10		

[c]

	SUM of squares	Degrees of freedom	Mean squares	F value	Significance
Crop species	4.203e+12	4	1.051e+12	4.893	0.01**
Residuals	3.221e+12	15	2.147e+11		

Table 5.1: ANOVA output tables [a] ANOVA output table from the one-way ANOVA analysis of CFU g^{-1} counts recorded in the rhizosphere associated with different crop species **[a]** domesticated and ancestral wheat species **[b]** repeat experiment of domesticated and ancestral wheat species **[c]** taxonomically different crops

8.1.2 Pseudomonad communities associated with taxonomically distinct crops

8.1.2.1 Pseudomonad abundance

As the results obtained for the analysis of pseudomonad community structure across different grass genotypes yielded no clear differences in both abundance or diversity, a second experiment looking at taxonomically distinct crops was carried out in the same grassland managed soil. The mean CFU g^{-1} of *Pseudomonas* spp. isolated from rhizosphere soil associated with each of the genotypes was determined and is shown in Figure S6.1 Significant differences were found in pseudomonad abundance associated with the rhizosphere of oilseed rape (OSR) and wheat with the lowest mean CFU g^{-1} compared to the rhizosphere of tomato with the highest CFU g^{-1} (p <0.009 and p=0.04 respectively).

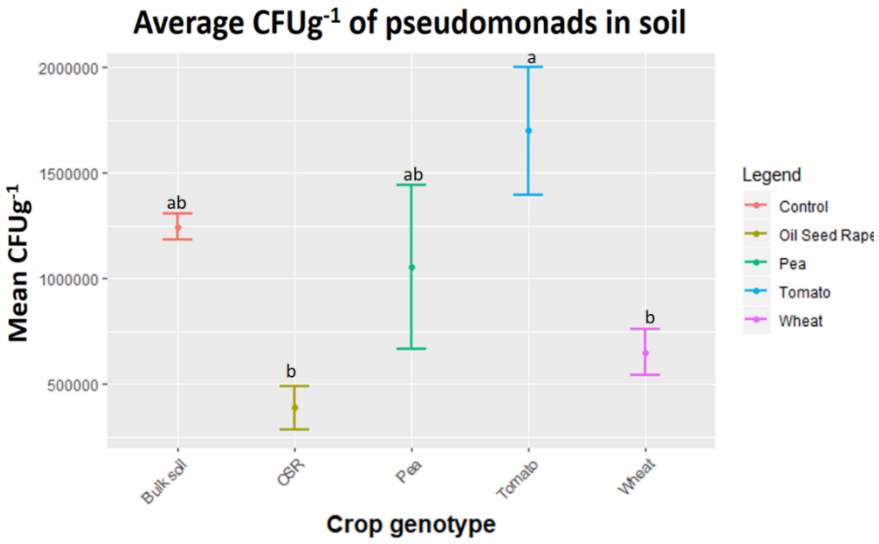


Figure S6.1 The abundance of *Pseudomonas* spp. isolated from rhizosphere soil associated with taxonomically distinct crop genotypes: The mean CFU g^{-1} ± standard deviation of *Pseudomonas* spp. isolated from rhizosphere soil associated with four crops from taxonomically different families, in addition to a bulk soil control. Bars sharing the same letter are not significantly different according to Tukey's HSD post hoc test.

8.1.2.2 *Pseudomonas* community phylogeny

PCR amplification and sequencing of the *gyrB* gene resulted in 50 forward and reverse sequences that could be aligned together successfully (10 isolates per treatment). Phylogenetic analysis of the *gyrB* gene was carried out, with a tree constructed and colour coded by crop genotype and bulk soil, as shown in figure S6.2. As with the previous experiment, there appears to be no grouping of isolates from one or a collection of treatments, indicating that *Pseudomonas* spp. diversity doesn't vary greatly across taxonomically distinct crops, when grown in a grassland managed soil. The consensus sequences were compared against the NCBI BLAST database and a total of 11 different *Pseudomonas* spp. were identified (figure S6.3). As with the previous experiment the strains *Pseudomonas* sp. R-41739 and *P. fluorescens* NCIMB 11764 appear to be more common across each of the crop genotypes in addition to *Pseudomonas* sp. GH1-PS23. On this occasion, *P. frederikbergensis* AS1 appeared to be associated more with bulk soil than the rhizosphere of the different crops.

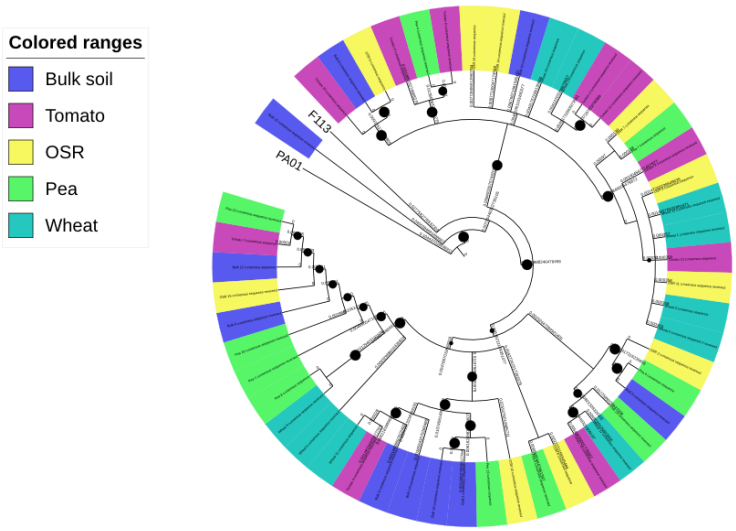
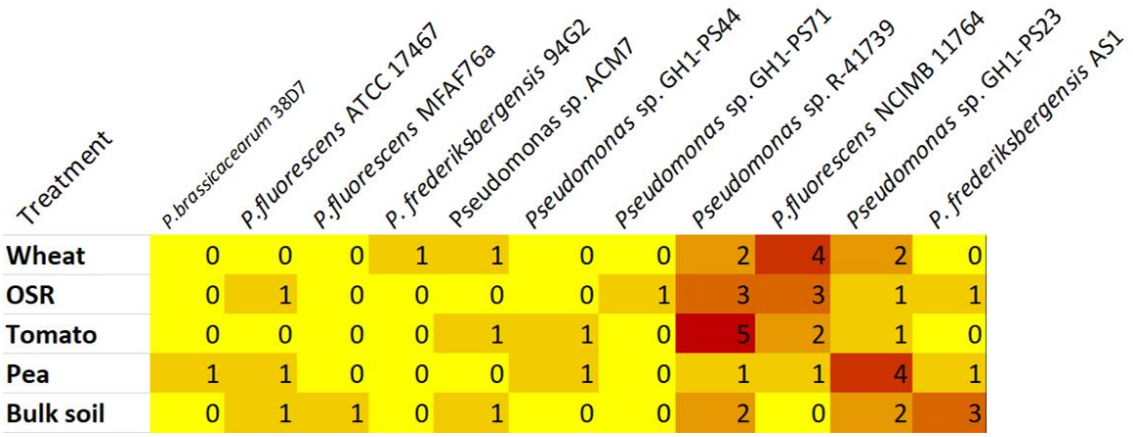


Figure S6.2. Maximum likelihood tree based on *gyrB* nucleotide sequences from *Pseudomonas* strains associated with different crop genotypes: 50 isolates from four crop genotypes and a bulk soil control were phylogenetically analysed utilising an ML tree with a

HKY model. Percentage bootstrap values higher than 70% of 1000 replicates are indicated by black circles at branching nodes.



Treatment	*P.brassicacearum* 38D7	*P.fluorescens* ATCC 17467	*P.fluorescens* MFAF76a	*P. frederiksbergensis* 94G2	*Pseudomonas* sp. ACM7	*Pseudomonas* sp. GH1-PS44	*Pseudomonas* sp. GH1-PS71	*Pseudomonas* sp. R-41739	*P.fluorescens* NCIMB 11764	*Pseudomonas* sp. GH1-PS23	*P. frederiksbergensis* AS1
Wheat	0	0	0	1	1	0	0	2	4	2	0
OSR	0	1	0	0	0	0	1	3	3	1	1
Tomato	0	0	0	0	1	1	0	5	2	1	0
Pea	1	1	0	0	0	1	0	1	1	4	1
Bulk soil	0	1	1	0	1	0	0	2	0	2	3

Figure S6.3: Identification of isolates based on *gyrB* gene sequences and compared against the NCBI BLAST database. Strains isolated from the rhizosphere of different crop species and a bulk soil control were cross referenced against the NCBI BLAST database. Cells which are coloured red represent the highest and those coloured yellow represent the lowest number of *Pseudomonas* species identified.

8.2 Supplementary data chapter 3.0

8.2.1 Statistical analysis

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
% soil moisture content	**Treatment**	8	323.2	40.40	21.71	**1.18e-09*** **
	Residuals	26	48.4	1.86		
Soil pH	**Treatment**	8	3.096	0.3870	1.602	0.173
	Residuals	26	6.279	0.2415		
Soil % C	**Treatment**	8	1.2944	0.16181	32.03	**1.47e-11*** **
	Residuals	26	0.1313	0.00505		
Soil % N	**Treatment**	8	0.27096	0.03387	41.57	**6.97e-13*** **
	Residuals	26	0.02119	0.00081		

Table 5.2 ANOVA output tables: ANOVA output table from a one-way ANOVA analysis of soil properties including soil moisture, soil pH, % carbon (C) and % nitrogen (N) from 9 different land managements

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
Grain weight (t ha^{-1})	**Treatment**	2	17.484	8.742	24.51	<0.001***
	Residuals	8	2.854	0.357		
Root dry weight (g^{-1})	**Treatment**	2	0.9502	0.4751	4.232	0.027 *
	Residuals	4	2.6947	0.1123		

Table 5.3 ANOVA output tables: ANOVA output table from a one-way ANOVA analysis of grain weight and root dry weight of wheat grown in soil from three different land management histories

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
ACC	**Treatment**	2	917295	458648	0.471	0.630
	Residuals	24	23348257	972844		
***Trans*-zeatin**	**Treatment**	2	0.06959	0.03479	3.146	0.061
	Residuals	24	0.26540	0.01106		
Isopentenyladenine	**Treatment**	2	0.0104	0.00519	0.108	0.898
	Residuals	24	1.1509	0.04796		
Gibberellin A1	**Treatment**	2	0.0691	0.03454	0.397	0.677
	Residuals	24	2.0882	0.08701		
Gibberellic acid (A3)	**Treatment**	2	182.5	91.25	7.408	**0.003****
	Residuals	24	295.6	12.32		
Gibberellin A4	**Treatment**	2	0.181	0.09064	0.491	0.618
	Residuals	24	4.431	0.18465		
Indole-3-acetic acid	**Treatment**	2	2718	1359	0.806	0.459
	Residuals	24	40487	1687		
Abscisic acid	**Treatment**	2	697.4	348.7	3.413	**0.050***
	Residuals	24	2452.2	102.2		
Jasmonic acid	**Treatment**	2	27089	13544	13.27	**<0.001*****
	Residuals	24	24492	1021		
Salicylic acid	**Treatment**	2	0.1848	0.09239	2.36	0.166
	Residuals	24	0.9395	0.03914		

Table 5.4 ANOVA output tables: ANOVA output table from the one-way ANOVA analysis of root phytohormones from wheat grown in soil from three different land managements**.**

[a]

Gene target		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
16S rRNA bacterial (bulk soil)	**Treatment**	8	2.979e+09	372421360	12.42	3.88e+07***
	Residuals	26	7.797e+08	29988897		
16S rRNA *Pseudomonas* (bulk soil)	**Treatment**	8	4.830	0.6038	1.539	0.194
	Residuals	26	9.807	0.3923		
16S rRNA bacterial (bulk vs rhizosphere)	**Treatment**	8	2.393	0.4786	1.263	0.333
	Residuals	26	5.306	0.3790		
16S rRNA *Pseudomonas* (bulk vs rhizosphere)	**Treatment**	8	2.032e+09	406331715	13.62	5.87e-05 ***
	Residuals	26	4.178e+08	29841115		

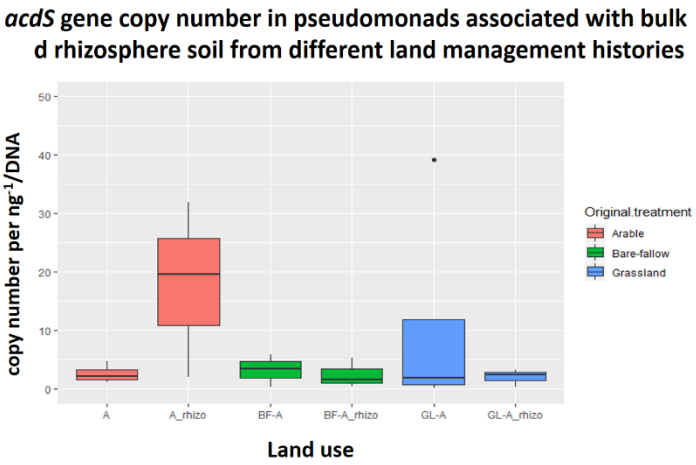
[b]

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
***acdS* bacterial (bulk vs rhizosphere g^{-1} soil)**	**Treatment**	5	2.904e+12	5.808e+11	20.73	5.1e-06 ***
	Residuals	14	3.923e+11	2.802e+10		
***acdS* bacterial (bulk soil vs rhizosphere ng^{-1} DNA)**	**Treatment**	5	593459	118692	14.64	3.91e-05 ***
	Residuals	14	113526	8109		

Table 5.5 ANOVA output tables ANOVA output table from a one-way ANOVA analysis of [a] bacterial and *Pseudomonas* sp. 16S rRNA gene abundance across the bulk soil of 9 different land managements in addition to rhizosphere soil of wheat grown in the permanent arable and conversion to arable plots [b] microbial *acdS* gene copy number associated with bulk soil and rhizosphere soil from permanent arable and conversion to arable plots**.**

8.2.2 Pseudomonad specific *acdS* qPCR analysis at the Highfield experiment

[a]



[b]

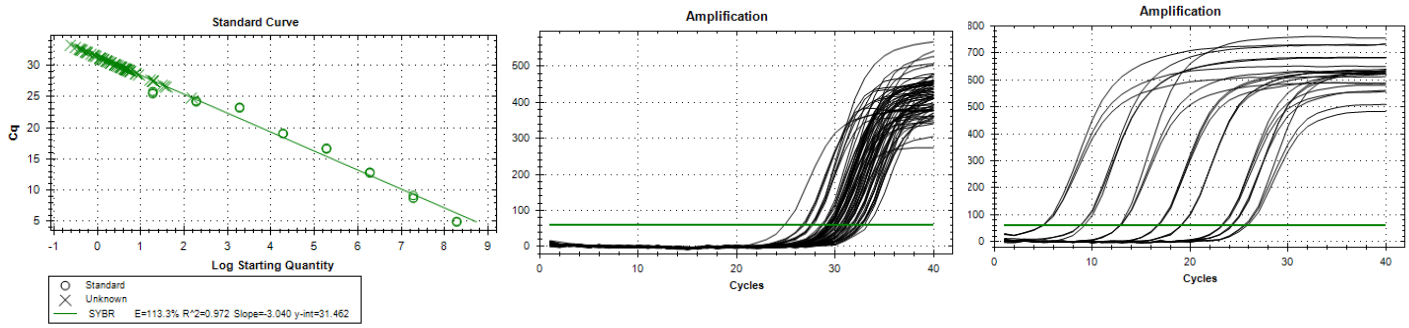
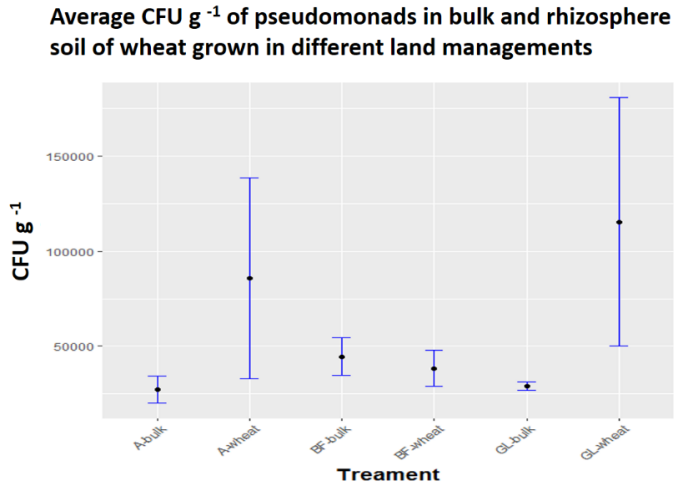


Figure S6.4 Quantitative PCR of the *acdS* gene in pseudomonads across bulk soil and the rhizosphere of wheat grown in different land management histories. Quantitative PCR analysis of the *Pseudomonas* sp. *acdS* gene utilising the primer set F481-F941, in bulk soil and rhizosphere soil sample from different land management's **[a]** pseudomonad *acdS* gene copy number ± standard deviation per ng^{-1} DNA from bulk soil from all 33 plots at the Highfield experiment **[b]** qPCR calibration standard curve in addition to amplification curve of samples and standards.

8.2.3 Cultivable pseudomonads isolated from pot experiments utilising wheat grown in soil from the Highfield experiment

[a]



[b]

[c]

	Bulk	Rhizosphere
Bare fallow	0/6	1/6
Grassland	0/6	0/6
Arable	0/6	3/6

Figure S6.5 Culturable pseudomonas associated with bulk soil and the rhizosphere of wheat grown in pot experiments utilising soil from the permanent land managements at the Highfield experiment [a] The mean CFUg^{-1} of pseudomonads associated with bulk soil and rhizosphere soil of wheat grown in soil sampled from the permanent arable, bare fallow and grassland soil at the Highfield experiment **[b]** Gel electrophoresis of PCR products amplified from DNA released from pure cultures of pseudomonads using the degenerate *acdS* primers (acdSF3/acdSR3), target fragment size is ~600bp **[c]** table summarising the number of pseudomonads found to have the *acdS* gene. Six isolates were tested for each treatment/niche compartment.

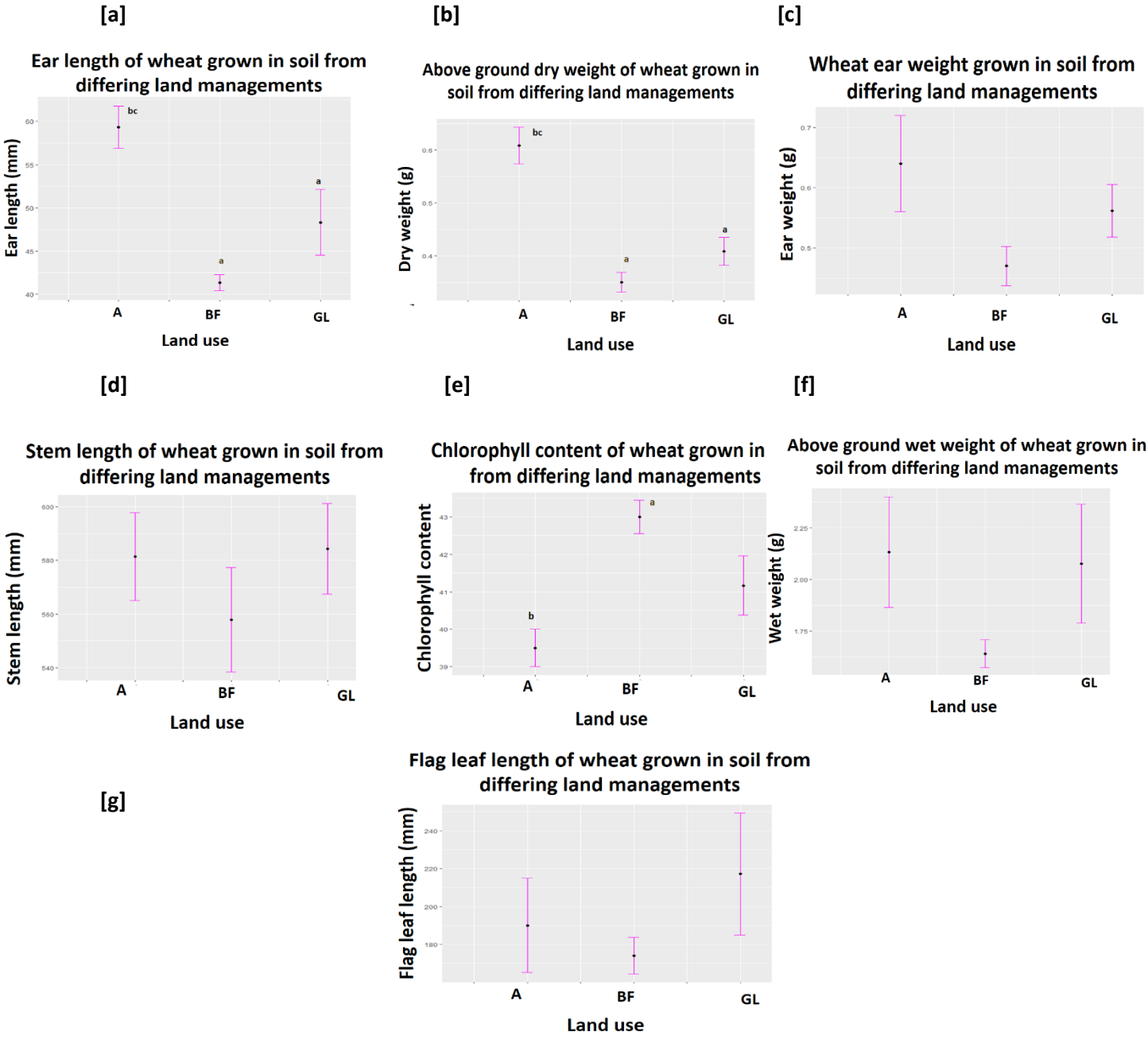


Figure S6.6 Above ground measurements of wheat grown in pot experiments utilising soil from different land managements: Soil sampled from the permanent arable, bare fallow and grassland plots at the Highfield experiment were used to grow wheat in pot experiments under glasshouse conditions. Numerous traits were measured from the wheat grown (n=6) including **[a]** ear length **[b]** above ground dry weight **[c]** ear weight **[d]** stem length **[e]** flag leaf chlorophyll content **[f]** flag leaf length **[g]** above ground wet weight. Treatments found to

differ significantly from each other are represented by the following letter: **a** arable, **b** bare-fallow, **c** grassland.

8.4 Supplementary data chapter 4.0

8.4.1 Statistical analysis chapter 4.0

	SUM of squares	Degrees of freedom	Mean squares	F value	Significance
Treatment	65.52	8	8.190	45.76	<2e-16***
Residuals	6.44	36	0.179		

Table 5.6: ANOVA output tables: ANOVA output table from the one-way ANOVA analysis of LOG10 transformed CFU g^{-1} counts recorded for the niche compartments of wheat grown in soil from 3 different land managements**.**

8.5 Supplementary data chapter 5.0

8.5.1 BLAST analysis for genes of interest

The annotations provided by Prokka and RAST software did not include all genes of interest investigated in this study. Gene nucleotide sequences were therefore downloaded from NCBI database for BLAST analysis of genomes in the RAST pipeline. Below are the squences downloaded:

Phenazine

>AF195615.1:4873-6786 Pseudomonas chlororaphis autoinducer synthase PhzE complete cds

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GGCCGATATCGACTTGCCTGCCACCTCGATCGGCGCGCCTCGCCTGGATGTACTGGCGCTGATCCCCTAC

CGCCAGATTGCCGAACGCGGTTTCGAGGCGGTGGACGATCAGTCGCCGCTGCTGGCGATGAACATCACCG

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>AF195615.1:6789-7625 Pseudomonas chlororaphis autoinducer synthase PhzF

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Phytase: NC_016830.1

>NC_016830.1:3336378-3338303 Pseudomonas fluorescens F113, complete genome

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Alkaline phosphatase

>NC_016830.1:1070213-1071754 Pseudomonas fluorescens F113, complete genome

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GA

Glycerophosphodiester phosphodiesterase

>NC_016830.1:5269135-5270262 Pseudomonas fluorescens F113, complete genome

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Nitrile hydratase (*nthAB)*

>NC_019670.1:3908924-3909523 Pseudomonas sp. UW4, complete genome

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>NC_019670.1:3909566-3910228 Pseudomonas sp. UW4, complete genome

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ACGCAATATTACGAACGCTACGTCATTGCCACGGCCACCTTGCTGGTCGAAACCGGCGTTATTACCCAGG

ACGAACTCGATCAGGCGCTGGGTTCACCCTTCAAATTGGCCAACCCTTCCCACGACAAGGGCCGCCCCGC

CATCACCGGCCGTCCGCCATTTGAAGTTGGCGACCGGGTAGTGGTTCGCGACGAATACGTGGCCGGCCAC

ATACGCATGCCGGCCTATGTACGCGGCAAGGAAGGCATCGTCCGACACCGCACCTCGGAAAAATGGCCGT

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8.5.2 Analysis of carbon genes identified across 54 pseudomonad genomes

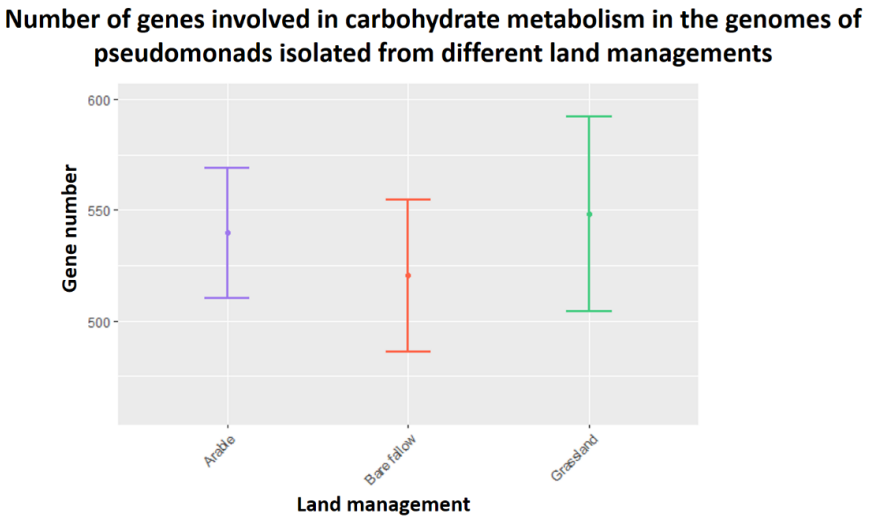

Figure S6.7 Number of genes involved in carbohydrate metabolism identified in the genomes of pseudomonads isolated from three distinct land managements: The total number of genes identified from analysis in the RAST pipeline for genes involved in Carbohydrate metabolism were identified. Averages were compared across the genomes of isolates from the land management histories (arable, bare fallow and grassland).

8.5.3 ANOVA tables

The statistical analysis of differences observed in leaf and root length observed from the application of eight different inoculation treatments in addition to application of chemical inhibitors and precursor of ethylene was carried out, with ANOVA results shown in the tables below.

[a]

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
Bacterial inoculation leaf measurement 0.1M NaCl	**Treatment**	8	245.8	24.58	2.104	0.0891
	Residuals	18	186.9	11.68		
Bacterial inoculation root measurement 0.1M NaCl	**Treatment**	8	42.64	5.330	2.569	0.046*
	Residuals	18	37.24	2.075		
Bacterial inoculation leaf measurement 0M NaCl	**Treatment**	8	236.3	23.64	1.228	0.314
	Residuals	18	293.6	18.35		
Bacterial inoculation root measurement 0M NaCl	**Treatment**	8	103.3	12.91	0.627	0.745
	Residuals	18	370.7	20.59		

[b]

		Degrees of freedom	SUM of squares	Mean squares	F value	Significance
Chemical application leaf measurement 0.1M NaCl	**Treatment**	4	304.2	76.05	7.309	0.00508**
	Residuals	10	104.0	10.41		
Chemical application root measurement 0.1M NaCl	**Treatment**	4	78.01	19.502	7.464	<0.00472***
	Residuals	10	26.13	2.613		
Chemical application leaf measurement 0M NaCl	**Treatment**	4	124.7	53.68	3.499	0.0492*
	Residuals	10	153.4	15.34		
Chemical application root measurement 0M NaCl	**Treatment**	4	493.2	123.30	8.51	0.00294***
	Residuals	10	144.9	14.49		

Table 5.7 ANOVA output tables Output table from a one-way ANOVA analysis of leaf and root lengths of wheat [a] inoculated with pseudomonads and [b] with chemical inhibitor and stimulator of ethylene grown under salt stress and non-stressed conditions