Bacterial and fungal diversity effects and the activity of biocontrol agents in the rhizosphere of crop plants.

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Cyflwynir y traethawd hwn er côf am nain gariadus.
Nain a wnaeth yr hyn a allai a'i chariad.

Table of contents.

Thesis abstract.

Bacterial biocontrol agents have previously been identified as alternatives to agrochemicals for the control of crop diseases. The advent of genetic engineering and the identification of the modes of biocontrol that bacteria possess has allowed the potential modification of successful inocula to improve their efficacy in biocontrol. A plant growth promoting rhizobacterium (PGPR) *Pseudomonas fluorescens* SBW25 (WT) was modified to contain the phenazine-1-carboxylic acid biosynthesis pathway. The genetically modified biological control agent (GM-BCA) 23.10 was previously shown to have improved biocontrol activity when compared to WT.

The work described in this thesis assessed the natural microbial community structure and function in the rhizosphere of pea, wheat and sugar beet under field conditions and the potential impact of the release of a GM-BCA may have on these communities, using laboratory based mesocosms. In addition to this, an investigation in to the production and conservation of HCN biosynthesis was undertaken on global collection of biocontrol strains to assess the potential of HCN biosynthesis as another trait for genetic modifications. A detailed field study of the indigenous rhizosphere communities was undertaken using culturable microbiological techniques, carbon source utilization and the development of molecular community profiling approaches on bacteria and fungi. A dominating effect of the plant species was shown and specific enrichment was identified on soil communities with significant effect also seen relating to plant growth stage. These data provided a robust baseline data set for comparisons to mesocosm experiments assessing the potential ecological impact that the release of a GM-BCA may have on the environment. This mesocosm study demonstrated plant growth stage effect dominated shifts in community profile with transient perturbations of indigenous community structure and function identified as a result of the inoculation. The significant change with greatest persistence was the replacement of a large proportion of γ-proteobacteria communities by the inocula.

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Abbreviations.

∝	Alpha
ARDRA	Amplified Ribosomal DNA Restriction Analysis
AWC	Average Well Colour
BBCTAB	Bead beating with hexadecyltrimethylammonium bromide
BCA	Biological Control Agent
bp	base pair
BSA	Bovine Serum Albumin
°C	Degrees Centigrade
CFC	Centrimide, Fucidin, and Cephalosporin.
cfu	Colony forming unit
CLPP	Community Level Physiological Profiling
CTAB	hexadecyltrimethylammonium bromide
DAPG	2,4-diacetylphloroglucinol
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTP	deoxy Nucleoside Tri-Phosphate
EDTA	ethylenediaminetetraacetic acid
FAME	Fatty Acid Methyl Ester
γ	Gamma
g	grams
GM	Genetically Modified
GM-BCA	Genetically Modified- Biological Control Agent
GMM	Genetically Modified Micro-organism
h	hours
HCN	Hydrogen Cyanide
Kb	kilobases
Km	Kanamycin
Kan^R	Kanamycin resistance
LB	Luria-Bertani broth
LBA	Luria-Bertani Agar

LH-PCR	Length Heterogeneity Polymerase Chain Reaction
m	meter
M	Molar
M bp	Mega base pairs
μg	Micrograms
μL	Microlitres
mg	Milligrams
mL	Millilitres
mM	Millimolar
min	minutes
MG-H_20	Molecular grade water
MVSP	Multi Variate Statistical Package
nM	Nanomolar
NJ	Neighbour joining
O/N	Over night
pM	Picomolar
PAUP	Phylogenetic Analysis Using Parsimony
PBS	Phosphate Buffered Saline
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
%	percent
pfu	propagule forming unit
PGPR	Plant Growth Promoting Rhizobacteria
PSA	Pseudomonad Selective Agar
P*tac*	Constitutive promoter
Rpm	Revolution per minute
rRNA	Ribosomal Ribonucleic acid
SBW25	*Pseudomonas fluorescens* SBW25
SDS	Sodium-Doecyl-Sulphate
SOC	Bacterial recovery media
TE	Tris-base with ethylenediaminetetraacetic acid.
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
TSBA	Tryptone Soya Broth Agar

v	Volume
V	Volts
Ver	Version
w	Weight
WT	Wild Type
23.10	*Pseudomonas fluorescens* SBW25::Ptac-*Phz*ABCDEFG-Km

Chapter One: Introduction.

1.1. Introduction.

Over the last decade considerable research effort has been directed towards the study and assessment of biological control agents (BCAs) for use in agriculture. Considerable benefits have been identified in the control of fungal diseases, however, the primary concern is now the wider ecological impact of the introduction of BCAs (51). The fear is that the introduction of BCAs could perturb the natural ecosystem of the inoculated environment. If the perturbation was significant, plant health could be affected, resulting in lower soil fertility, and, in turn, threatening the stability of the entire ecosystem. In all environments the natural balance of nutrients needs to be maintained, if this balance is altered, plant health and indigenous communities may be affected.

The generation of Genetically Modified Micro-organisms (GMMs) with improved biological control ability (9, 14, 18, 47, 52) has heightened the concern of their potential impact on the environment. These Genetically Modified BCAs (GM-BCAs) are designed to have improved efficacy and a wider spectrum of activity against phytopathogens, thus they have the potential for improved ecological fitness. It is possible to investigate the fitness of an organism under natural conditions, however due to the highly diverse nature of the environment it is impossible to predict the behaviour of the inoculum in all situations. Tight legislation is in force to reduce risks and lessen the possibility of unforeseen situations arising as a result of the release of GMMs (51).

1.1.1.Plant growth promoting rhizobacteria.

The soil environment has many physical, chemical and biological factors that affect the growth of a plant. Many of these interactions occur between plants and micro-organisms in the zone of soil (2-3 mm) which is under the direct influence of plant roots, this region is defined as the rhizosphere. Intense microbial activity occurs in the rhizosphere due to the localised high levels of exudates from plant roots, organic matter, and general cell turnover (23). The surface of the root, where many of these interactions occur, is defined as the rhizoplane. However, these two regions are

difficult to separate in terms of microbial ecology (39) and, consequently, they will both be referred to as the rhizosphere.

Bacteria that inhabit the rhizosphere of plants and whose presence there promotes plant growth are known as Plant Growth Promoting Rhizobacteria (PGPR) (49). Numerous species of bacteria are considered to promote plant growth, they include *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Pseudomonas, Rhizobium and Serratia* (2, 5, 20, 21). In addition to these bacteria, some fungi (35) and yeasts (1) have demonstrated plant growth promotion. Plant growth promotion is achieved by many mechanisms, for example, the assimilation of nutrients unavailable to the plant, and their conversion into utilisable substrates (Rhizobia fixing nitrogen for legumes (54), and the provision of phosphorous and other nutrient from mycorrhizal fungi in exchange for fixed carbon from plants (26), and the suppression of disease by bacteria, either by direct resource competition (10, 59) or possessing properties antagonistic to crop diseases (the production of secondary metabolites with antifungal and bacterial properties) (14, 25, 28, 34, 41, 42, 57).

Despite a range of mechanisms involved in plant growth promotion by bacteria, the PGPR mechanisms usually involve disease suppression (27).

The presence of PGPR in the soil has been recognised for over a century (22), whether indirectly in agriculture, by the knowledge that some soils show suppression of fungal crop diseases (59), directly by identification of nitrogen fixation by rhizobia in leguminous plants (54), or by the isolation of bacteria with disease suppressive properties (59). The modern agricultural practice of continuous monoculture has lead to the establishment of disease suppressing rhizobacteria and fungi and, therefore, to the development of soils suppressive to fungal diseases (39). Weller and Cook (59) isolated bacteria, present in soil, that suppress *Gaeumannomyces graminis,* the causal agent of Take-all disease of crops. They identified the bacteria responsible for the decline of Take-all disease, and recognised the potential use of these bacteria for biological control. Research undertaken during the past twenty years has provided environmental microbiologists with a basic knowledge of the role of bacteria in the rhizosphere and an understanding of the mechanisms involved in biocontrol. An appreciation of the mechanisms that regulate the biosynthesis of antimicrobial

compounds involved in disease suppression is an important step towards increasing the reliability of the biocontrol agent. This would improve the potential of bacteria as biological control agents (BCAs) against crop diseases, as an alternative to fungicides (58). Several biocontrol products have been produced commercially to protect crops from fungal diseases. These biocontrol strains include "NoGall", an *Agrobacterium* to control Crown-gall disease of fruit, "Dagger G", a *Pseudomonas fluorescens* to control Damping-off disease in cotton, and others like "Trichodex" (*Trichoderma hazianum)*, "AQ^{10}" (*Ampelomyces quisqualis*) and "BlightBan A506" (*Pseudomonas fluorescens*). Details of other commercial BCAs are available at http://www.agrobiologicals.com. The use of bacteria as BCAs has not been as reliable as the chemical alternatives (58). The underlying problem has been the lack of knowledge of the mechanisms of biocontrol and plant growth promotion. In recent years, directed research into this area of microbial ecology has been undertaken due the reliance on fungicides being unpopular (58). Grounds for this research has been strengthened by the increasing occurrence of fungicide resistance from repeated use (19, 61), concern about fungicide residues on food, and the issue of safety of some fungicides (61). The use of a mixture of bacteria and fungicides has been suggested as one solution to reduce the environmental impact (38).

1.1.2. Fluorescent pseudomonads.

Gram-negative bacteria are the most common colonisers of the rhizosphere, dominated by the fluorescent pseudomonads when enumerated using culturable techniques (27). Fluorescent pseudomonads are a group of PGPR that are ubiquitous soil bacteria. They have been identified in a variety of habitats including soils, plant surfaces, fresh water, sediment, sea water, hospitals, burn victims and biofilms. They have received the most attention in biocontrol research due to their properties of being rapid and aggressive colonisers of the rhizosphere, and they are among the most widely studied bacteria with crop disease suppression abilities. The mode of action of these BCAs is multifactorial, and thus a variety of mechanisms are identifiable as important in disease suppression. These include direct resource competition, induced resistance, siderophore production, niche habitation, and, perhaps the most important, the production of secondary metabolites. Several secondary metabolites, produced by pseudomonads and linked with disease control, have been described. These include

phenazines (34), phloroglucinols (14, 25), pyoluteorin (28), pyrrolnitrin (42), and hydrogen cyanide (HCN) (57). The improved ability of survival and persistence in the environment of BCAs has been attributed to many of these secondary metabolites; mutants deficient in these traits have reduced persistence in the rhizosphere and biocontrol abilities (14, 29, 34, 57). An essential gene for the production of several secondary metabolites, *Pseudomonas fluorescens* CHA0 global regulator (*gacA*), was knocked out. This mutant had significantly reduced ability to control Black Root-Rot in tobacco (29). The production of these secondary metabolites is not limited to the control of plant pathogenic fungi, they have an impact on other indigenous micro-organisms, including bacteria (25), protozoa (46) and fungi (17).

The rhizosphere is a complicated environment with both biotic and abiotic factors affecting the ability of organisms to colonise and survive. Introduced organisms to this environment have to compete against indigenous micro-organisms and the environmental conditions to which they are subjected to, if to survive. The physical conditions to which they are subjected to include: variations in temperature and the percentage of moisture in the soil. These perturbations place large physical and osmotic stresses on bacteria. Additionally, the ability of the bacterium to survive also depends on the mineral composition (survival in one soil type is not indicative of the ability to persist in another) and the percentage of oxygen in the soil (some agricultural soils can have periods or microsites that are anaerobic, thus affecting the survival of obligate aerobes).

Biotic factors affect the survival of introduced organisms. These biotic factors include resource competition, niche competition, predation (10, 59), the bacteria's physiological state on inoculation to the soil (growing the bacteria up in different media can affect survival (15), the timing of release (50), and inoculation method (3, 6). Due to the complexity of the conditions for bacterial survival, the isolation of a bacterium from the environment to which release is planned is the logical approach for the identification of a successful bacterial candidate for biocontrol.

1.1.3. Biological control agents and assessment for general release.

Research has been undertaken to study the possible use of BCAs in agriculture and the mechanisms conferring biological control ability. The genetic identification of biosynthesis operons of secondary metabolite production in biocontrol strains provides the potential utilisation of these genes into further BCAs strains for the potential to increase survival and fitness and perhaps improve the efficacy of biocontrol of a strain (14). Considerable benefits have been identified with use of these organisms (52, 53), however the primary concern is the wider ecological impact of the release of such organisms on the soil ecosystems (53). This concern has been heightened, in recent years, with the increased amount of research into the use of GM-BCAs that have improved efficacy of biocontrol and potentially a wider spectrum of activity against phytopathogens. However, as previously stated this poses an increased risk to the environment. Increased environmental fitness and survival could potentially resulting in a wider habitat range with potentially undesirable spread, greater competition between microbes and antagonism towards essential indigenous species. These factors has resulted in tight legislation for use of GMMs to limit risks and unforeseen circumstances (51).

1.1.4. What is a Genetically Modified Micro-organism (GMM)?

A GMM has been defined as a micro-organism that has been modified by a non-naturally occurring mechanism to contain or modify genes that are not native to the organism. This definition and the legislation that clearly defines what a GMM is set out by the European Union (EU) Directive 90/219/EC. The following are classed as genetic modifications according to Directive 90/219EC: -

i) Recombinant DNA techniques using vector systems:- Work involving recombinant DNA is defined as the formation of new combinations of genetic material by the insertion of nucleic acid molecules, produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host

organism in which they do not naturally occur, but in which they are capable of continued propagation.

ii) Techniques involving the direct introduction into a micro-organism of heritable material prepared outside the micro-organism including micro-injection, macro-injection and micro-encapsulation.

iii) Cell fusion or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

1.1.5. Genetic modification of bacteria to improve their biocontrol ability.

Bacteria with known biological control ability have been genetically modified to improve the efficacy of disease suppression (9, 14, 18, 47, 52). Mechanisms used to improve biocontrol abilities have included the insertion of novel secondary metabolites with antifungal properties (14, 18, 52) and the up regulation of secondary metabolites (9, 47) and sigma factors (47).

To improve the efficacy of the BCA *Pseudomonas fluorescens* SBW25, the Phenazine-1-carboxylic acid (PCA) biosynthetic pathway was introduced as a single chromosomal insertion on a mini disarmed transposon (52). The phenazine biosynthesis pathway has been well characterised, expression of the pathway results in the production of the secondary metabolite PCA whose antibiotic characteristics are effective against a number of plant pathogens. The majority of known phenazines demonstrate broad spectrum antagonistic activity against various species of bacteria, fungi, some higher plants and animals (55). This is a result of the transformation of phenazine compounds by oxidation-reduction reactions, to toxic superoxide radicals in the target cells harmful to the organism (34). Phenazine-1-carboxylic acid controls fungal diseases such as damping off (*Pythium ultimum*) (52), and Take-all (*Gaeuntannomyces graminis* var. *tritici*) (34), and contributes towards the ecological competence of the bacterium in the soil (34). This GM-BCA (23:10) created by Timms-Wilson *et al.* (52) significantly improved its survival and persistence in the rhizosphere and surrounding bulk soil with greatly improved disease control (52), in related studies, *Pseudomonas putida* WCS358r was also modified to contain the PCA

biosynthesis operon from *Pseudomonas fluorescens* 2-79 (18), resulting in a similar effect on biocontrol.

The operon of 2,4,diacetylphloroglucinol (phl) was isolated from *Pseudomonas fluorescens* F113 as an important gene in the conformation of biocontrol of *Pythium ultimum* (14). The operon was transferred into 8 different pseudomonad strains to confer biocontrol ability. Only one strain produced detectable levels of phl, the strain M114, and by the incorporation of this novel element it had a significantly enhanced ability to suppress *Pythium ultimum* in sugar beet (14).

Bacteria have been modified in the regulation of their secondary metabolites to improve biocontrol ability (9, 47). The *rpoD* gene encodes the housekeeping sigma factor σ^{70} in *Pseudomonas fluorescens* CHA0. Attempts to inactivate this gene in order to identify the role it plays in secondary metabolite production were unsuccessful, indicating that it performs an essential role in the bacterium as a global regulator. The incorporation of *rpoD* onto a plasmid with transformation into CHA0 resulted in the increased expression of *rpoD* and an increase in antibiotic production from the bacterium, improving disease suppression of *Pythium ultimum* in cucumbers (47). Delany *et al* (9) introduced a second copy of the 2,4,diacetylphloroglucinol synthesis operon on a plasmid to the biocontrol strain F113. This improved the control of Damping-off disease to equivalent levels as a standard fungicide treatment.

The data from these experiments demonstrates that the insertion of novel genes or the up regulation of existing genes has the ability to improve the effectiveness of BCAs by specific genetic modification. However, it should not be assumed that genetic modification of BCAs will always be successful (14). The release of organisms that contain novel genes should be monitored in the environment and ideally the inserted gene would already be present in the indigenous environmental community. This would reduce the potential selective pressure on the inserted trait. The acquisition of a novel genetic element providing a high selective advantage would result in rapid multiplication of the recipient, if the trait were already present in the environment, the selective pressure for multiplication of this trait would be reduced.

1.1.6. Factors that should be addressed when considering the release of micro-organisms into the environment.

The release of organisms into non-indigenous habitats should be carefully considered given the potential environmental impact that they may have. The two main areas for consideration, with particular reference to GMM are:

1) What are the potential risks of the genetically modified trait becoming established in the environment?
2) What impacts, beneficial or detrimental, will the release of these organisms have on the indigenous communities and ecosystem function?

1.1.7. Containment of the GMM trait in the environment.

The release of GMMs containing novel traits increases the potential for establishment of these organisms in the environment by their improved ecological fitness. The improvement of fitness could drive selection of the GMM and increase the potential for the trait entering the horizontal gene pool. Therefore, care has to be taken to limit gene transfer in the organism. There is evidence to suggest that the transfer of genetic material from plants to bacteria can occur in low frequencies (24). This occurred when plants were genetically modified in the chloroplasts in regions where there was homologous sequence to prokaryotes. This underlines the attention detail that should be undertaken to prevent transfer of material between species.

The transfer of genes from bacteria to other organisms is generally limited to the prokaryotic community due to their ability to take up extracellular DNA and their natural ability for genetic transfer. Genetic modification by the insertion of genes into vectors for expression has been universally used for studies in bacteria (48) and is a useful tool for the identification of the traits role in bacteria survival and biocontrol (9, 14). There are several problems associated with the use of plasmids. Bacteria can maintain indigenous plasmids in the absence of selection pressure, however engineered plasmids used for the insertion of novel traits are often lost (43, 56). The

insertion of vectors into a host increases the metabolic load on the organism, which in the absence of a direct advantage reduces its competitive ability (56). The use of plasmids for carrying traits for environmental release contains unacceptable risks for the potential of genetic transfer. Plasmids are naturally mobile elements that transfer between bacteria in the environment (32). They are lost if the increased load to the bacteria is detrimental, or if the plasmid has low stability in the organism (56). The natural transfer of plasmids has been demonstrated by the acquisition of indigenous plasmids to bacteria released into the soil environment (31).

Attempts have been made to design plasmids for use in environmental release that incorporate a suicide mechanism to prevent spread from the site of inoculation. Ramos *et al* (44) designed the first suicide mechanism for the biocontainment of a pollutant degrading bacteria based on a plasmid. The system used the regulatory mechanism of the meta cleavage pathway of the *Pseudomonas putida* TOL plasmid pWWO, the *xyl*S gene, and the *gef* gene in *E.coli* that collapses the cell membrane potential by the production of a pore producing protein. In this system when benzoates are present, the suicide mechanism is repressed. On the complete utilisation of available benozoates, or if the bacteria disperses from the site of inoculation, the suicide mechanism is activated. This plasmid based system was flawed by an unacceptable rate of the suicide vectors failure for bacterial cell death by the leaky expression from the promoter; a more stable chromosomal based system is being developed (44). Chromosomal insertions of genes/operons increases the stability of the modification and reduces the likelihood of transfer (3, 9, 52). Some of the pioneering work for the use of bacteria in field releases was based on the ice nucleation in *pseudomonas syringe* and the competitive exclusion of these bacteria that cause frost damage by mutants deficient this trait (33).

There have been two different micro-organisms released into the environment in the UK. In 1987 a *Rhizobium leguminosarum* was released with a *Tn*5 inserted onto its symbiotic plasmid. This was undertaken to monitor the survival and persistence of the GMM in the environment, and how it survived without the presence of it's symbiotic host (22). There were no adverse affects seen following the release of this organism, with the plasmid maintained in the environment and no evidence of genetic transfer.

The organism was found to persist in the environment in the absence of the host plant (22).

The other micro-organism to be released was *Pseudomonas fluorescens* SBW25EeZY-6KX. This was the first release of a free living, non-symbiotic GMM with a chromosomally inserted gene, and was carried out on sugar beet in Oxford by Bailey *et al*, (3) and by De Leij *et al.* on wheat (7). The aim of these studies were to establish the survival, dissemination and genetic stability of the bacteria to establish whether there were environmental impacts associated with the release of such organisms.

Pseudomonas fluorescens SBW25 was isolated from the phytosphere of sugar beet at the university field station, Wytham, Oxford (3), and it successfully colonises the phyllosphere and rhizosphere of many crop plants including pea, wheat and sugar beet. *P. fluorescens* SBW25 suppresses oospore germination and hyphial extension of *Pythium ultimum* Trow, the causal agent of Damping-off disease (13). However, the mode of action for biocontrol in SBW25 has not been fully determined, as it does not produce any of the typical anti-fungal secondary metabolites previously described (12).

SBW25 was modified by site directed homologous recombination to contain a Kan^R-*xyl*E gene cassette encoding kanamycin resistance and the ability to degrade catechol, and to contain the *lac*ZY gene cassette 1 M bp away from Kan^R-*xyl*E insertion site. This allowed for the monitoring of gene transfer of either element, by reducing the probability of duel transfer due to the large distance between insertion sites. The potential for gene transfer was initially researched in the laboratory with co-inoculations of Wild Type (WT) SBW25 and SBW25EeZY-6KX in media. There was no detected genetic transfer in over 200 generations (3).

In the field release, no genetic transfer was detected from the chromosomally marked bacteria, and therefore any transfer that had occurred was below the levels of detection. These insertions conferred no selective advantage to the bacterium in the environment. If the inserted genetic element encoded a gene increasing the relative fitness of the bacterium there would be an increased selective pressure for this trait. In

the study by Bailey *et al.* (3), genetic material, in the form of plasmids, was transferred to the GMM from the indigenous community (31). This emphasises the frequency and potential for genetic transfer as a natural and common event in the population biology of bacteria as they adapt and respond to their environment.

1.1.8. Ecological impact of the release of GMMs on the environment.

Concern has arisen that GM-BCAs released into the environment would have increased habitat range, competition, and survival between microbes and the antagonism on non-target indigenous species. An increase in the suppression of deleterious micro-organisms is advantageous but an impact on essential plant growth promoting organisms would have severe implications. Plants health in natural soil environments is directly linked to the activity of the microbial associations that they have. This includes the bacterial and fungal pathogens and beneficial involved with a complicated set of interactions. Many of these interactions between plant, bacteria and fungi are tightly linked, indicating a degree of co-evolution. Mycorrhizae are fungal symbionts which demonstrate this by the uptake of minerals for plants in exchange for carbon fixation by plants (26). It is therefore important to address plant-microbe interactions and potential detrimental effects of the introduction of BCAs.

1.1.8.1. Assessing the impact on indigenous bacterial communities.

In this study I have investigated whether the introduction of GMMs has any discernable impact on the indigenous bacterial communities. Studies have primarily focused on the diversity and relative abundance of the components of bacterial communities. In addition studies include assessment of substrate utilisation and metabolic activity. This approach allows detailed studies and facilitates the identification of any shifts in community structure.

There are many techniques available that allow characterisation of community structure. These have been evaluated and tested to provide a practical tool kit to monitor GM-BCAs following release into the rhizosphere. These techniques focus on a number of basic principles, the culturable proportion of micro-organisms present enumerated by plate counts, the amplification of conserved genes with phylogentic

inference in environmental DNA samples by PCR, and the study substrate utilisation assays to give an indication of metabolic potential and activity. The use of PCR in assessing bacterial communities has mainly centred on the amplification of the conserved 16S ribosomal RNA (rRNA) gene. The 16S rRNA gene is genetically conserved in all bacteria, and the gradual molecular divergence of the coding sequence, by insertions, deletions, and mutations, allows phylogenetic inference to be made on the evolution of bacteria and their relationship to each other. There are a number of techniques that have been used in the analysis of the variance that occurs in the 16S rRNA gene sequence and length between bacterial species. These techniques include Denaturing Gradient Gel Electrophoresis (DGGE) (37), length heterogeneity PCR (LH-PCR) (60), and terminal restriction fragment length polymorphism (T-RFLP) (40).

Denaturant Gradient Gel Electrophoresis (DGGE) utilises the property that DNA denatures at different temperatures dependant on GC content (37). A hypervarable region of the 16S rRNA gene is amplified, ~200bp , and run down a polyacrylamide gel with a gradient of denaturant. The PCR primers contain a GC clamp (long string of G and C bases) attached to one end of the product, when the PCR product denatures this clamp holds the two DNA strands together and retards migration in the gel. Therefore a profile of bands dependent on DNA sequence is produced, which effectively creates a "barcode" of 16S rRNA gene fragment diversity based on sequence heterogeneity. Bands can be cut out of the gel and sequenced to identify bacterial species.

Length Heterogeneity PCR (LH-PCR) utilises the same hypervarable 16S rRNA gene region as DGGE but relies on the variability in length of the PCR product (60). The primers for LH-PCR have a fluorescent tag and products are run down a capillary sequencer to size the PCR products, and producing a profile of indicative of the bacterial species present. Another technique is Terminal Restriction Fragment Length Polymorphism (T-RFLP) this is very similar to LH-PCR utilising a longer region of the 16S rRNA gene and digesting the fragment using various restriction enzymes (40). The digested product is run down a capillary sequencer and product sizes are identified. Various species have presence and absence of restriction sites so indications of bacterial community diversity can be implied.

Other techniques to assess the enzyme activity in the soil and the carbon utilisation profiles have been used to monitor the soil communities and detect changes in response to environmental perturbations. The use of Community Level Physiological Profiling (CLPP) has been useful in detecting temporal and spatial changes in the community profiles on plates containing 95 different sole carbon sources. The carbon sources are combined with tetrazolium dye that is reduced by respiring, active bacteria to a purple colour. This can be used to gauge the activity of the bacteria on the sole carbon sources, and identify gain or loss in utilisable carbon substrates in response to environmental perturbations (16).

1.1.8.2. Impact on bacterial communities.

Studies monitoring the diversity of indigenous bacteria and their response to the inoculation of bacterial BCAs have identified perturbations in the indigenous bacterial community profiles. *P. fluorescens* SBW25, and its genetically marked variant SBW25EeZY-6KX, were released in a field experiment to examine the impact and survival of the organisms in the rhizosphere of sugar beet (3) and wheat (7). Monitoring was undertaken to identify the effects of the released organisms on the total bacteria counts, the percentage population of fluorescent pseudomonads, and the population structure in relation to r/K strategists. The introduction of the bacteria caused no significant effects on bacterial numbers present in the rhizosphere. A shift in the population structure was detected with the introduction of the bacteria, and a change was detected in the percentage of r/K strategists that persisted until growth stage 30 (stem elongation). This change occurred in both WT and GM bacteria, but without a consistent trend (7).

A study by Moenne-Loccoz *et al.* (36) concentrated on the introduction of an unmodified BCA *Pseudomonas fluorescens* F113. Data was gathered on bacterial numbers, community structure, ribotype composition of culturable fluorescent pseudomonad communities, and the carbon utilization profiles of the rhizosphere bacteria. The introduction of the bacteria displaced some of the resident pseudomonads, but there was no effect on diversity. There was a change in the carbon utilization profiles caused by the bacterial inoculum, however the importance of this

was not deemed significant as it affected those carbon sources not used by the indigenous rhizosphere communities. It was therefore decided that the perturbations caused by F113 introduction and utilization of these carbon sources was not an environmental risk (36). The impact created by the introduction of this bacterium was anticipated due to the inoculation of a large volume of bacteria, although it was transient.

In these studies, continued persistence of the bacteria in the environment is low, particularly in bulk soil. This decline is probably a result of the high turnover of bacteria that occurs in the plant phytosphere in response to the changing environmental conditions. Theory states that bacteria persist in the environment in low population densities until the environmental conditions are favourable to allow selective growth of a particular species (11). This indicates that a consortium of bacteria inhabit the soil, populating the rhizosphere when conditions suit. There is evidence that bacterial strains can fall below detection levels and then reappear again in subsequent years to colonise plants (3). This however was a single event and very rare with the bacteria not surviving in the absence of visible plant material in bulk soil.

A released GMM has established itself in the natural bacterial community of the soil. *Rhizobium leguminosarum* biovar *vicae* was marked with a transposon, *Tn*5, on its conjugative symbiotic plasmid (22). It has persisted in the soil at the release site since 1987, forming 0.1-1 % of the *Rhizobium leguminosarum* biovar *vicae* population in the soil despite the field site being planted with cereal since 1989. The detection of this bacterium has been possible by the insertion of the *Tn*5 and selection of nodules of introduced legumes. This survival in the environment at low levels without its symbiotic host would appear to agree with the theory of low-level persistence until favourable environmental conditions arise. No environmental problems have been associated with the release of this bacterium (22). This highlights that it should not be assumed that inoculants will not persist in the environment beyond a few years.

1.1.8.3. Impact on indigenous fungal communities.

Limited studies have been undertaken to investigate the effects that releasing BCAs may have on the indigenous fungal communities (4, 18). The primary purpose of such a release is to reduce the effect of disease on plants, thus, by definition, the release of these organisms will cause perturbations in the fungal community. The questions that need to be addressed are what is the impact on the non-pathogenic fungi of crops? and does this have an impact on plant health? Similar techniques as those used with bacterial populations have been undertaken to monitor fungal communities, based on the amplification of the 18S rRNA gene.

In the release of the bacterium SBW25EeZY-6KX in the rhizosphere of field wheat (7), data was gathered on the impact that this release had on the filamentous fungi. It was found that seed inoculation plus foliar application of inoculum resulted in a decline in the fungal density on aerial plant sections, but in general, no significant long-term effects were found on fungal densities during the experiment. A limitation of the data was that the study focused only on culturable filamentous fungi. The plate counts from filamentous fungi can be misleading, as stress conditions induce sporulation, causing the impression of an increase in fungal density.

A more direct study has been undertaken on the effect of the release of BCAs on fungal diversity (30). Wheat seeds were treated with *P.putida* WCS358r and a phenazine producing derivative, WCS358r::phz. Total DNA was isolated from the wheat rhizosphere and 18S amplification was undertaken. Analysis was carried out on Amplified ribosomal DNA restriction analysis profiles, with sequencing to identify fungal species. The introduction of both bacterial strains had caused the suppression of the *Fusarium* population in the rhizosphere until day 40 of the experiment (30). This agrees with initial findings of data based on culturable *Fusarium* (18).

Arbuscular Mycorrhizal (AM) fungi are vital for plant health and nutrient cycling (26). Therefore if BCAs inhibit AM fungal growth there could be a severe impact on plant health and nutrient cycling. A study by Ravnskov *et al.* (45) has indicated that there is no antagonistic effect between BCA *Burckholderia cepacia* and the growth and function of AM fungi. However, this study focused on the direct suppressive

effect on hyphial growth of the bacterium in a root free system, with no study into the fungi-root-BCA interaction. The universal control of fungi present in the plant phytosphere has potentially wider implications. For example if BCAs reduce the entomopathogenic fungi, these fungi especially the zygomycota, cause natural declines in their insect host populations, which include plant pathogens such as aphids (8). The potential removal of these controlling fungi could cause an increase in crop damage by insect pests.

In conclusion there are many techniques to monitor the impact of BCAs on the culturable population densities, indigenous communities structure, and substrate utilisation and metabolic activity in soil, however most studies have focused on the general bacterial communities. More information is required about the fungal communities in the environment, how the introduction of BCAs may alter these communities, and the effect that the bacteria have on plant health. In addition, the impact of GMMs is beginning to be understood, but techniques need to be identified that can rapidly assess the impact of GMM on indigenous species and the potential methods that could be used to reduce the impact of the bacteria.

1.2. Aims.

The aims of this thesis were to address the following questions:

What enrichment effect does the planting of crop species have on the indigenous microbial communities and is this enrichment distinguishable between the three crop species, pea, wheat and sugar beet? This will be addressed using a variety of techniques that compliment each other to assess culturable population densities, carbon utilisation profiles, and the molecular analysis of total bacterial and fungal communities (Chapter 3).

During the growing season of the three crop species are growth stage dependant effects identifiable and how do they relate in comparison to the effect of the plant species enrichment? (Chapter 3).

Do plants grown in mesocosm conditions with field soil demonstrate the same changes in microbial communities as plants grown under field conditions? (Chapter 3 and 4).

To identify impacts on the indigenous communities as a result of the inoculation of the bacterial biological control agents SBW25 and 23:10., using the assessment of culturable population densities, carbon utilisation profiles, and molecular analysis total bacterial and fungal communities. In addition, to identify if subsections of the bacterial communities analysed by DGGE are influenced by these inocula (Chapter 4).

Hydrogen cyanide biosynthesis has been shown to be significant in biocontrol, how conserved is the expression and synthesis of this trait amongst various known biocontrol and is it potentially a trait that could be incorporated into SBW25 for the improved biocontrol efficacy (Chapter 6).

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Chapter Two: Methodology: Technique optimisation and application for study of soil microbiology.

2.1. Introduction.

A greater understanding of microbial diversity in specialist environments, such as in the soil or rhizosphere, is required in order to assess the resulting impact of extrageneous perturbations on indigenous communities. To begin to address this understanding, studies were undertaken at the University of Oxford field site, Wytham, to model the temporal and spatial structure of bacterial and fungal communities in the rhizosphere in three UK grown crops, pea (*Pisum satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), and sugar beet (*Beta vulgaris* var. *amythyst*). In addition, the potential impact of the inoculation of biocontrol agents (BCAs) and genetically modified biocontrol agents (GM-BCAs) on indigenous communities were measured in mesocosms.

Licensing of new agrochemicals requires that their toxic effects on higher organisms, the residue persistence and their environmental toxicity be measured. No measures of indigenous microbial communities are made to assess any potential detrimental effects on diversity, although research is now being undertaken to address this. As bacterial BCAs for the suppression of fungal phytopathogens are being developed as an ecologically safer alternative to agrochemicals, the impact on the indigenous microbial communities must be addressed to prove they are effective and acceptable. By monitoring the growth of individual crop species, in a simulated agricultural environment, a detailed baseline study can be made of the indigenous microbial community structure during a growing season. This study would also show the effect on soil microbial communities resulting from the planting of a single plant species. These data will be used for a comparison with laboratory experiments where BCAs and GM-BCAs are applied to soil mesocosms to measure the impact and significance of these treatments. The techniques discussed are applicable to the indigenous microbial communities for the analysis of this soil environment, but they are equally transferable to other environments. Many approaches can be undertaken to determine the composition of a microbial community, each providing a different perspective on community structure and function. In this chapter I will introduce the various methods that have been used for analysis of microbial community structure and determination

of function. There are two main methodological approaches to this, molecular, and, culture dependent. Both have their advantages and disadvantages in community analysis.

2.1.1. Sampling from the plant rhizosphere.

Our studies investigate the plant rhizosphere. The rhizosphere is the soil around the root that the plant has direct influence over. This zone was identified as the most critical and actively dynamic region for the study of natural populations (8). Direct interactions occur in this region between the plant and soil microbes, and it is very important for plant health (53) Investigations into the rhizosphere populations is particularly important for the monitoring of responses to the inoculation of rhizosphere colonising BCAs and the influence on the indigenous populations that have important relationships for the plant.

In sampling the rhizosphere it is important that samples are only taken from the rhizosphere and processed in identical and reproducible ways. The rhizosphere in this thesis is defined as the soil adhering to the roots that are < 1 mm away from the rhizoplane. This was achieved by removing plants from the soil and the shaking until all non-tightly adhering soil was removed. This typically left < 1 mm of soil on the rhizoplane. If this does not occur and the co sampling of bulk soil is undertaken, artificial variance within sample time points, and in particular, between samples time points may occur. Sampling of large areas will dilute the rhizosphere effect. It has been identified that the bulk soil environment is very stable, with rhizosphere communities resulting from an enrichment of the soil environment (9, 57). This enrichment results in the selection of bacteria that are specifically adapted for this habitat. As a result of this specific environmental selection, perturbations of conditions could potentially result in greater perturbations in the microbial communities. As ultimately BCAs are also designed to improve plant health we are studying the rhizosphere which is the zone that is directly going to influence plant health.

2.1.2. Indigenous communities.

The indigenous soil communities fall into two groups: the culturable fraction, those that can be grown *in vitro*, and the un-culturable fraction, those that cannot presently be grown *in vitro*. The relative proportions of each fraction vary depending on soil type, history and the local environmental conditions but estimates for culturable bacteria and fungi lie between 1-5% of the total microbial population from the bulk soil (44). The proportions of populations that are culturable from the rhizosphere are significantly higher, with estimates in the region of 70 % (Kowalchuk, personal communication).

2.1.3. Estimations of population densities.

2.1.3.1. Isolation of microbial populations.

Thousands of different selective media types have been developed for the cultivation of microbial populations. These media vary from the most basic, to the highly complex, requiring precise nutrient balances and specialised growth conditions (39). There are numerous media that are commercially available in liquid form and freeze dried powders. For lists of media, and what they isolate, there are guide manuals from many manufactures e.g. Difco Manual 11^{th} ed., Becton Dickenson and co., sparks, Md., 1998; or the less detailed, Oxoid product list (www.oxoid.com), or for the formulation of more customised media, laboratory guides, such as Maniatis *et al.* (29), or the handbook of media for environmental microbiology (1) are a good starting point.

Despite the diverse range of environments that microbes can inhabit and differential conditions required for growth, fundamentally there are some basic requirements for growth. They require a carbon and energy source, a source of nitrogen, electron acceptors and some basic minerals. Depending on the micro-organism, the carbon and energy source can vary from just carbon dioxide and hydrogen, to very complicated media with amino acids, carbohydrates, nitrogen bases, and undefined nutrient

sources such as yeast extract (44). The nitrogen source most commonly used is ammonium, with the electron acceptors typically as oxygen, carbon dioxide, ferric iron, nitrate, and sulphate (44). Many rhizosphere dwelling organisms can grow on these basal media, and even more on nutrient rich varieties, allowing the growth of most culturable bacteria. The use of antibiotics has allowed the growth of selected organisms. Antibiotics, such as cyclohexamide, are used to suppress growth of eukaryotes *i.e.* yeast and fungi, where as aureomycin is used to suppress bacterial growth for the selection of fungi. Thus antibiotics are used for the selection of individual organisms. Resistance to mercury is a plasmid borne trait that has been commonly identified in indigenous microbial communities at the Wytham field site so the addition of mercury chloride will select micro-organisms that have natural resistance. Kanamycin resistance is routinely used as a laboratory selective marker in genetic manipulations (29), or the use of natural antibiotic resistances of specific bacterial groups such as the combination of centrimide, fucidin, and cephalosporin (CFC) for the selection of pseudomonads (Oxoid, UK).

An important consideration in the growth of micro-organisms is the physical conditions required for optimal growth. These include temperature, oxygen concentrations, light, and incubation time. Prokaryotes can survive and multiply in temperatures ranging from -1.2 °C to 113 °C (26). It is logical that those microbes adapted to one temperature would not be successful at another. Oxygen levels are another important factor; organisms range from being aerobic to obligate anaerobes, where oxygen is toxic to them (26). The time of incubation of organisms also needs to be addressed. For example, *E.coli,* whose average doubling time in the laboratory in 20 minutes, would form distinct colonies on an agar plate in 12 hours, whilst a fungal isolate can take 7 days to achieve substantial growth visible by the naked eye on an agar plate. Although the principles of microbe growth are very simple, care has to be taken to explore the possible conditions required for their successful growth. Although alone the technique of culturable population enumeration does not tell you a great deal, coupled with the identification of a number of isolates or in combination with molecular methods it can give a useful insight into microbial populations.

2.1.3.2. Estimation of the total and active bacterial populations using microscopy and flow cytometry.

Techniques have been developed using cytochemical stains for the quantification of active bacterial populations in environmental samples. The use of combinations of stains allow the estimations of the active fraction of bacterial communities in comparison to total population counts (56). These "active bacterial" stains utilise artificial electron acceptors that incorporate into the bacterial cells. Frequently used acceptors are 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2- (*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) (56). Incubation with either of these salts causes the accumulation of formazan crystals in actively respiring bacteria. As the bacteria accumulate these crystals there is increased colour intensity (red) visible by fluorescent microscopy. Conventionally, active population numbers were estimated by microscopy counts and compared to total bacterial cell counts under the microscope using a general DNA stain with a different colour, The most commonly used was the LIVE/DEAD® Baclight™ Bacterial viability kit (Molecular probes, USA), which utilises SYTO® 9 green fluorescent nucleic acid stain which stains bacteria with intact cell membranes and propidium iodide which stains cells with damaged membranes. This would give an estimation of the total population numbers in the environment and proportion that is active.

Microscopy work is time consuming and can be subjective. An alternative method to this has been developed utilising the properties of differential staining as described above. This technique is known as flow cytometry and has allowed the automation of bacterial counts for the estimation of population densities (42). This technique was traditionally used in clinical microbiology, however it has since then been adapted and developed for use with environmental samples. Flow cytometry is carried out on "clean" bacterial cell suspensions. This is achieved by the separation of the bacteria from particulate matter, such as soil, clay, and organics, using a Nycodenz density gradient (30). This produces bacterial cell suspensions suitable for analysis by flow cytometry, thereby allowing the analysis of soil communities for total bacteria counts and activity (56). The samples are split, with one half stained for total counts and the

other for active cells. Bacteria are stained for total counts using a general DNA stain, such as SYBR® Green (Molecular probes, USA), and the active proportion are stained using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), as described above (56). The bacterial suspensions are run in a single cell stream through the flow cytometer and illuminated by a laser (42). Measurements are taken of the level of fluorescence and the light scatter. These measurements then correspond to the number of bacteria that have taken up each dye, and thus relative counts of abundance can be determined (42). The use of flow cytometry has not, to date, been used on soil fungal populations, although techniques are being developed to more accurately identify and count airborne fungal spores (38).

2.1.4. Bacterial identification techniques.

Plating methods simply provide us with total viable bacterial counts, however, once isolated, several techniques can be used to provide details of the proportion bacterial species in a sample. Some of the methods available for this identification are described below.

2.1.4.1. Ribosomal RNA gene amplification and sequencing.

The most widely used technique for bacterial identification is the amplification and sequencing of the conserved small ribosomal subunit (SSU), 16S rRNA gene. The 16S rRNA gene has been used as a phylogenetic marker since the late 1970's for bacterial identification. The 16S rRNA gene is genetically conserved in all bacteria, and the gradual molecular divergence of the coding sequence, by insertions, deletions, and mutations, has allowed a database of over 100,000 sequences to be created (Ribosome Database Project (RDP), http://RDP.cme.msu.edu/html/). Using these changes, phylogenetic inference can be made on the evolution of bacteria and their relationship to each other. The 16S rRNA gene it is not the only gene that can be used for distinguishing between bacterial strains. Other genes identified with a capacity to allow resolution of bacterial are: *rpo*B (33), *gyr*A (5), *gyr*B (17), *tm*RDB (61), *rec*A (28), *gap*A, *gro*EL, *omp*A (52), and *hsp*60 (59). All of these genes have advantages

and disadvantages when compared with 16S, with many having a higher resolution than 16S with separation to below the species level. This resolution however is limited to very small subsections of the bacterial populations. The other major limitation for all of these alternative genes is the quantity of known sequences for these genes. The gene, *gyr*B, which encodes DNA topoisomerase II has the highest number of known sequences with 2200. This, however, is very small in comparison to the 16S rRNA gene database, which to date has in excess of 100,000 full-length sequences (http://RDP.cme.msu.edu/html/). In conclusion the 16S rRNA gene is most suited for studying total bacterial communities as it is conserved between all bacteria, with good separation of sequences across all bacterial species. The other genes are more suited to specific questions in individual bacterial species. This technique is also being used on the 18S rRNA gene for the identification of fungal isolates, a development from the traditional technique of morphological identification. However, due to the level of fungal genetic variance and the currently limited database, this technique does not allow the same levels of resolution as in bacteria.

2.1.4.2. Fatty acid methyl ester analysis.

Gas liquid chromatography of bacterial cellular fatty acid methyl esters (FAME), has routinely been used for bacterial identification in clinical microbiology (46). Fatty acids are present in all organisms, and form the main composition of cell membranes of micro-organisms. The composition of total microbial fatty acids has been demonstrated to be an important taxonomic character which, when measured quantitatively, can separate organisms at the species and even sub species level (36). Bacteria, grown under identical conditions, produce their unique spectra of FAMEs, profiled using gas chromatography. Comparisons of these to known bacterial profiles allow the rapid and highly reproducible/accurate identification of bacteria to the species level (46). This then allows the development of specific libraries to which data is constantly being added as more isolates are analysed. Detailed studies into spatial and temporal shifts in community composition have been undertaken using FAME (11, 40, 46, 48). The disadvantage of FAME is that it requires the culturing of the micro-organisms on TSBA media to allow identification.

2.1.4.3. Carbon source utilisation (BIOLOG™ plates).

BIOLOG™ (Oxoid, USA), a technique used in the identification of bacteria, consists of a 96 well microplate, with each well containing nutrients, a carbon energy source, and tetrazolium violet. Each well is identical except for the carbon energy source that it contains, with one control containing no carbon source (manufacturers manual (31)). There are numerous types of carbon sources, including: polymers, carbohydrates, esters, carboxylic acids, brominated chemicals, amides, amino acids, aromatic chemicals, amines, alcohols, and phosphorylated chemicals. Tetrazolium violet is a dye that, on oxidation by bacterial respiration, causes a build up of formazan grains in the bacteria. This results in the production of a purple pigmentation in active bacteria, indicative of the level of bacterial activity. This colour change can be logged by eye or by optical density, measured at 600 nm on a plate reader. Pure cultures of bacteria, grown on BIOLOG™ plates, produce distinct carbon utilisation profiles. By comparison to the BIOLOG™ database the identity of the bacteria can be determined. There are BIOLOG™ plate formats available for the identification of Gram-negative and Gram-positive bacteria. In addition, BIOLOG™ plates have been produced without the tetrazolium dye for the identification of fungi.

2.1.5. Community Level Physiological Profiling (CLPP).

The indigenous microflora of a particular environment are composed of a diverse range of bacteria and fungi, amongst other organisms. Research is being undertaken to identify the roles of individual organisms, and to identify organisms with particular roles. The use of BIOLOG™ plates with environmental samples for CLPP was identified as an indicator of ecosystem function, metabolic potential and functional diversity (18). BIOLOG™ plates, used with mixed environmental communities, could measure the range and scope of carbon sources a particular environmental community could utilise. Monitoring these would potentially allow the identification of functional shifts of carbon utilisation increases and decreases in metabolic activity and, hence, "metabolic" community diversity between environments. This technique was

described as "a breakthrough in unlocking the secrets of community activity and diversity in the complex soil environment" (20).

As with any technique the limitations were quickly identified. The use of environmental samples with unknown bacterial concentrations was deemed to have an important impact on colour development. The rate of colour development is dependent on inoculum density (18), but this effect is an artefact of the lag phase of growth before colour development (22). Providing samples are incubated for sufficient time there is no impact on the final colour or number of wells with colour development. This allows total community carbon utilisation profiling. This technique is limited for studies of the rate of colour development for communities if the inoculum density cannot be calibrated between samples. The sensitivity required to detect change is an important factor when used in studies designed to detect perturbations in communities. The identification of differences between soil types (60), plant types (10, 13) and even between sections of individual soil cores has been successfully shown with the use of BIOLOG™ plates (57). The use of BIOLOG™ to assess fungal communities is a new approach described in ecological context by Hobbie *et al.* (23), due to the lack of a dye for colour development specific to fungi, the growth of bacterial communities in samples need to be suppressed by the addition of antibiotics so that growth by the fungi can be measured by absorbance (OD_{600}).

2.1.6. Molecular based community analysis.

The use of molecular approaches to understand community structure has allowed the study of uncultured micro-organisms. This, therefore may provide a less biased approach to community profiling. However as with all techniques, each physical, chemical and biological step used in the analysis of an environment introduces bias (58), therefore consistent and reproducible methods are essential. The ability to extract total nucleic acid from bacteria is differential between species. Thus extraction from a mixed community, such as soil, with the potential of insufficient or preferential disruption of cells, will cause bias. Biotic and abiotic factors will also affect the ability to extract DNA. The most common techniques for disruption of soil to release

bacterial cells are: Waring blending, shaking, sonication, and chemical treatments. Each technique produces different yields. The more vigorous the technique, the higher the yield, but the more fragmented the DNA, thus a balance must be achieved (58). Providing, however, all samples are consistently processed in the same way they will have equal standing, despite these factors that can affect DNA extraction. These same techniques can be applied to fungal DNA extraction. Recent developments in DNA extractions have identified a method that successfully extract total nucleic acid (DNA and RNA) of all microbes present in soil samples (21).

Many methods used for the comparison of total environmental communities require a PCR amplification step. This can also present problems; the more a sample is processed, the more likelihood there is that influences will occur and problems will be encountered. Several problems can often be encountered with the amplification of purified environmental DNA: i) inhibition of PCR by co-extracted contaminants, ii) differential amplification of samples, iii) formation of PCR artefactual PCR products, iv) Contamination by foreign DNA, and v) 16S rRNA gene heterogeneity, thus results in unavoidable bias in community profiles (58). The inhibition of PCR amplification by co-extracted contaminates is commonly a result of $FeCl_3$, humic acids, salts and other organic compounds. The removal of these compounds can involve long, complicated and expensive purification procedures. These problems can often be overcome by the use of Bovine Serum Albumin (BSA) or T4 gene 32 protein (gp32). These proteins can reduce the inhibitory effect of contaminants between 10-1000-fold in PCR reactions (58). It is, therefore, important to optimise PCR on samples before undertaking large numbers of samples and experimentation.

2.1.6.1. Denaturing Gradient Gel Electrophoresis (DGGE).

The variation in sequence of the SSU of rRNA genes (16S or 18S) between species has resulted in the development of the technique DGGE. Muyzer *et al* (35) used this technique to detect single base mutations in DNA fragments, and discussed its adaptation for use in community profiling. PCR primers are designed to amplify over the V3 hyper-variable sequence region in the 16S rRNA gene resulting in products of

approximately 200 bp in size (35, 55). One of the primers is modified with a 5′ GC clamp. A GC clamp is a string of G and C bases 40 bp long. This clamp is important for successful functioning of DGGE. Amplified fragments are run down a polyacrylamide gel with a linear denaturing chemical gradient parallel to the electrophoresis current. Fragments migrate through the gel until they reach the point in the chemical gradient that causes their denaturation (denaturation point). On fragment denaturation, the GC clamps high melting temperature prevents complete denaturation, and holds the two strands together. This effectively doubles the size of fragments running through the gel, and thereby slows the progression of travel through the gel. This results in a distinct band produced by each amplified product.

The banding patterns produced by DGGE give a representation of the dominant PCR amplifiable organisms in the community. For the comparison between communities from different environmental conditions, samples are run in adjacent lanes on the same gel. This eliminates any between-gel variations that may occur. The comparison of community profiles can often permit a distinction by-eye of large differences in community profiles. Statistical techniques have been developed to give indications of how similar or dissimilar community profiles are.

It is important to be aware of the limitations of DGGE. Differences in 16S rRNA gene sequence do not necessarily result in changes of the denaturing point of amplified fragments. Isolates, if closely related, may not differ sufficiently to allow the separation of fragments by DGGE. However unrelated species with very different nucleotide sequences may have the same denaturing point for the amplified fragments, resulting in co-migration by DGGE. Bacteria can possess more than one copy of the rRNA operon in their genome; pseudomonads can contain up to eight, and *Clostridium paradoxum* contains the highest with fifteen (49). This has the potential of producing a different problem in community analysis by DGGE. One bacterial species may produce several bands by DGGE due to independent 16S rRNA gene evolution. The identification of species contained in bands on DGGE can be achieved by gel extraction of DNA fragments and sequencing. The data gathered is useful, but the information is limited to phylogenetic inference due to the size of fragments.

Despite this, DGGE has developed as a very powerful molecular technique for the study of population dynamics and diversity in microbial communities. In essence, DGGE allows the simultaneous analysis of a large number of samples.

2.1.6.2. Length Heterogeneity PCR (LH-PCR).

Length Heterogeneity PCR (LH-PCR) is based on the variation of the 16S rRNA gene. Apart from sequence variation, the 16S rRNA gene is variable in length due to insertions and deletions. The variance that occurs can be up to 200 bp across different species. There are no significant effects resulting from 16S rRNA gene length variations as it is an untranslated region. LH-PCR relies on this variation of length. PCR primers are designed to amplify 16S rRNA gene over hyper-variable regions, often using the same primers as in DGGE. In this technique, one primer has a fluorescent label rather than a GC clamp (32). Community samples are run through a capillary sequencer with a size standard to produce an electronic profile of fragment lengths in the given community. This gives a profile indicative of the bacterial communities present. The use of the RDP (http://RDP.cme.msu.edu/html/) allows the identification of bacterial species that produce fragments corresponding to the size of interest. This technique allows quicker and simpler detection of major shifts in community structure, but does not allow the identification of individual species directly. Database searches do, however, allow the identification of fragments to the genera / class level.

2.1.6.3. Terminal-Restriction Fragment Length Polymorphism (T-RFLP).

A third technique based on 16S rRNA gene is Terminal-Restriction Fragment Length Polymorphism (T-RFLP). This utilises the variable location of restriction endonuclease cleavage sites within the genes. A profile of different fragments lengths can be observed resulting from restriction digestion and heterogeneity of gene length. This has similarities to genomic ribotyping, but is based on the variance in restriction sites and length within an amplified region rather than the entire sequence. The full-length 16S rRNA gene is amplified by PCR for T-RFLP before the restriction digest

is undertaken. One primer has a fluorescent label for the accurate identification of the terminal fragment size using a capillary sequencer, as in LH-PCR. Different sub-populations of bacteria demonstrate higher variability with certain restriction fragments. This allows the selection of restriction digests to distinguish population changes in bacterial sub-populations. This however is limited by what is known about community members already, without the option for the identification of new community members by sequencing, if required.

2.1.6.4. Clone libraries.

The amplification, cloning and sequencing of DNA from environmental samples is a commonly used practice for the identification of genes present in the environment. The amplification of a conserved gene is typically used for the identification of organisms present. Normally the 16S ribosomal RNA gene for bacteria is used. The gene of interest is amplified from environmental DNA, and the mixed products are ligated into a suitable cloning vector. The vector is transformed into a recipient bacterial host, creating a library of clones. Clones are selected, and plasmids sequenced, for the identification of the inserted genes. Thousands of clones are required to get a true representation of the organisms present in the environment of study if rDNA is used. There are the same limitations as with DGGE analysis and PCR based techniques regarding biases. Although the sequencing of clones is now automated, with high throughput in many cases, it is still a costly and time consuming process.

2.1.6.5. rRNA gene versus rRNA for community analysis.

It was thought that the use of RNA for molecular analysis rather than DNA would give a better indication of the response of the indigenous communities to perturbations (20). This was anticipated as RNA abundance is known to be related to the physiological state of the micro-organism and in turn would bias the amplification for molecular community analysis towards the active fraction of the community (20). Studies undertaken in our laboratory have found however in the soil environment

there are no significant difference in community profiles amplified from DNA or from RNA (20). Because of this data, it was decided that analysis of community profiles would be entirely based on the DNA rather than RNA.

2.1.7. Methods to be used for community profiling.

Outlined above are several techniques that can be used for community structure analysis. Our studies were to form a baseline study on the community structure and function of three different crop species. Thus for this experimental approach it was decided to monitor the bacterial and fungal populations using plate-culturing techniques. Flow cytometry was not used, as this would only give indications of total bacterial and active counts, and would give no indication of effects on fungal populations. The additional advantage of plate count methods is the ability for the isolation of the pseudomonad subgroup. This enables direct monitoring of the impact of the BCAs addition and persistence.

The community profiling approach was CLPP with the use of BIOLOG™ plates. This would potentially give a good indication of any shifts in function of the indigenous communities that may occur. DGGE has been extensively used in environmental microbiology and there is a very good understanding of its limitations, and it is a very powerful technique for discerning differences. Recently, it has been shown that approximately 70 % of the bacteria in the rhizosphere, identified by DGGE, are culturable in the laboratory (Kowalchuk, G. A.; 2004 personal communication). In addition to DGGE, LH-PCR was undertaken to identify the potential of this new technique to distinguish between community profiles of different environments. T-RFLP was not undertaken as, although a potentially powerful technique, it would not allow the direct identification of microbes profiled. In addition the optimisation of the best restriction digestions would have to be established for community profiling could be undertaken.

2.2. Materials and Methods.

2.2.1. Laboratory micro-organism growth and storage.

2.2.1.1. Laboratory strains.

Numerous bacterial strains were used in this study and details of the strains and their origin are detailed in Table 2.1.

2.2.1.2. Laboratory micro-organisms growth conditions.

Escherichia coli cultures were grown on either Luria-Bertani Agar (LBA) (Difco, UK) at 37 °C or in Luria-Bertani Broth (LB) at 37 °C with rotation at 180 rpm overnight. Pseudomonad cultures were grown at 28 °C on Pseudomonad Selective Agar (PSA) (Difco, UK) supplemented with centrimide (10 μg ml^{-1}), fucidin (10 μg ml^{-1}), and cephalosporin (50 μg ml^{-1}) or in LB at 28 °C with rotation at 180 rpm. Antibiotic selection was added, where necessary, in the following concentrations: ampicillin 50 μg ml^{-1}, kanamycin 100 μg ml^{-1}, and rifampicin 100 μg ml^{-1}.

2.2.1.3. Storage of micro-organisms.

All bacterial isolates were stored in 70 % glycerol (v/v) and 0.85 % sodium chloride (w/v) at -70 °C. Isolates were revived by streaking on TSBA (Difco, UK) and incubated at their respective optimal growth conditions for 24 h or until colonies were visible to the naked eye.

2.2.2. Nucleic acid extraction techniques.

2.2.2.1. Extraction of total nucleic acid from cells / pure culture: CTAB method.

Bacterial total genomic DNA was extracted by a modification of the CTAB method (3). Cells (15 mg) were harvested from agar plates or pelleted from liquid culture (2 ml of liquid culture, grown overnight, spun at 14,000 x g for 2 min) and lysed by gentle resuspension in 500 µl of lysis buffer (20 µg ml^{-1} proteinase K in 0.5 % (w/v) Sodium Dodecyl Sulphate (SDS)) at 55 °C for 30 min. To this 100 µl of 5 M NaCl and 80 µl of CTAB (hexadecyltrimethylammonium bromide 10 % (w/v) in 0.7 M NaCl) pre-warmed (65 °C) were added and this solution was then incubated at 65 °C for 10 min. Chloroform:isoamyl alcohol (24:1) (680 µl) was added and, after hand shaking to form an emulsion, it was spun at 14,000 x g for 5 min. The aqueous phase was collected and precipitated with 0.6 volumes of isopropanol at 4 °C overnight. DNA was pelleted by centrifugation at 14,000 x g for 10 min, and washed with 70 % (v/v) ice cold ethanol before resuspending in 200 µl of TE pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0 (Ethylene Diamine Tetra Acetate)) or Molecular Grade H_2O (MG-H_2O).

2.2.2.2. Optimised extraction of total nucleic acid from soil: BBCTAB-freeze/thaw method.

Total DNA extractions were carried out on soil using a modification of the CTAB phenol lysis method with bead beating as described by Griffiths *et al* (21) incorporating an aditional freeze/thaw step. Half a gram of soil/roots were placed in a bead beating tube (Bio 101, inc multimix 2, 6560-215) with 500 µl of BBCTAB (120 mM K_2PO_4 buffer pH 8.0, 5 % CTAB) and shaken by hand to mix. Samples were then frozen at –20 °C for > 12 h before the addition of 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1). After shaking at 5,500 rpm for 30 s in FastPrep™ FP120, samples were spun at 4 °C for 5 min at 14,000 x g. The supernate was removed and washed with 0.5 ml chloroform:isoamyl alcohol (24:1). The

aqueous phase was precipitated with 2 volumes of 30 % PEG (30 % (w/v) polyethlene glycol 6000, 1.6 M NaCl) at 4 °C overnight. DNA was pelleted by centrifugation at 14,000 x g for 10 min at 4 °C and washed with 70 % (v/v) ice cold ethanol before resuspending in 100 µl of MG-H_2O.

2.2.2.4. Extraction of plasmid DNA.

Plasmid DNA was extracted from *E.coli* after growth overnight in LB. DNA was extracted from 2 ml of culture using the Sigma GenElute™ Plasmid Mini-Prep Kits (PLN350) according to manufacturers protocol.

2.2.2.5. DNA fragment purification.

DNA fragments < 10 kb were purified from 1 % agarose gels using Qiagen QIAquick Gel Extraction Kit (28704) as per manufacturers protocol.

2.2.2.6. PCR product purification.

PCR products were purified using Sigma GenElute™ PCR Clean-up Kits (NA 1020), or by gel extraction (section 2.2.2.5.).

2.2.2.7. Phenol:chloroform clean up.

Excess protein was removed from DNA samples, if required, using a phenol:chloroform:isoamyl alcohol clean up. DNA was diluted 5 times (100 µl to 500 µl) in TE pH 8.0 and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After shaking to form an emulsion, samples were spun at 14,000 x g for 5 min before the upper aqueous layer was removed and precipitated with 2.5 volumes of ice cold 95 % (v/v) ethanol at 20 °C for 1 h. DNA was pelleted by centrifugation at 4 °C for 10 min at 14,000 x g and washed with 70 % (v/v) ice cold ethanol before resuspending in TE pH 8.0 or MG-H_2O.

2.2.2.8. DNA precipitation.

DNA was precipitated by the addition of 2.5 volumes of 100 % ethanol with 0.1 volumes of 3 M NaAc. DNA was pelleted by centrifugation at 4 °C for 10 min at 14,000 x g, and then washed with 70 % (v/v) ice cold ethanol before resuspending in TE pH 8.0 or MG-H_2O.

2.2.3. Nucleic acid modification techniques.

2.2.3.1. PCR fragment cloning.

PCR fragments were ligated into TOPO® vector with the Invitrogen TOPO TA Cloning® Kit for sequencing (25-0276). A slight modification was made to the protocol with the ligation mix composing of: 4.5 µl of PCR product, 1 µl of salt solution, and 0.5 µl of TOPO® vector.

Plasmid transformation.

Plasmid DNA was transformed into bacteria using the Invitrogen TOPO TA Cloning® Kit for sequencing (25-0276) with a modification to the manufacturers protocol. This, in brief, was as follows: 2 µl of ligation, added to 25 µl of chemically competent cells, defrosted on ice and stabilized for 10 min. After heat shocking for 30 s at 42 °C, 250 µl of SOC media (2 % (w/v) bacto tryptone, 0.5 % (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added. This was then incubated at a temperature of 37 °C for 1 h, before 100 µl of this transformation solution was plated out on LBA selective plates, and incubated at 37 °C overnight.

2.2.3.2. Competent cells for transformation by Abdels Method (29).

Half litre cultures of *E.coli* Xl1-blue or *E.coli* DH5α, were grown up to OD_{600} of 0.4 - 0.6 then placed on ice for 30 min. Cultures were pelleted by centrifugation at 6,000 x g for 10 min at 4°C before resuspension in 150 ml of pre-chilled 100 mM $CaCl_2$. After incubation on ice for 30 min, bacteria were pelleted at 6,000 x g for 10 min at 4°C before being gently resuspended on ice in 50 ml of 50 mM $CaCl_2$ with 20 % glycerol. The bacteria were incubated on ice for 30 min, aliquoted in 500 µl volumes snap frozen and stored at –70 °C for up to 1 year.

2.2.4. DNA visualisation in agarose.

DNA was separated by agarose gel electrophoresis (10 V cm^{-1}) in 1 % (w/v) agarose, containing 0.1 µg ml^{-1} of ethidium bromide, made with 1x TBE (0.089 M Tris, 0.089 M boric acid, 0.002 EDTA, pH 8.0). Gels were visualised on a UV-transilluminator and photographed.

2.2.5. DNA amplification by PCR.

For PCR amplification of chromosomal DNA, reactions were carried out using the following protocol. Total reaction volumes were 50 µl.

The PCR Mix	
Target DNA	1-100 ng
Primers	1 p mole
dNTP's	0.2 µM
Buffer	1x (supplied with Taq)
Sigma Taq	2.5 u

Reaction cycle		
95 °C	2 min	
95 °C	1 min denaturing	x 30
X °C	1 min annealing	
72 °C	1.5 min extension	
72 °C	30 min	
10 °C	forever	

Using an MJ research, Peltier thermal cycler.

The reaction cycle was modified, as required, for specific applications with the annealing temperature (X) specified in section 2.2.5.2.

2.2.5.1. Primer design.

Primers were designed using the web based design programs, Primer Finder v 0.07 (http://eatworms.swmed.edu/~tim/primerfinder/) and Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

2.2.5.2. Primers pairs used and annealing temperatures.

Primer	Sequence	Reference
BF1	5'-tcaga(a/t)(c/t)gaacgctggcg	(55)
GC338F	5'-cgcccgccgcgccccgccccggcccgccgcccccgcccactcctacgggaggcagc	(55)
530R	5'-gtattaccgcggctgctg	(55)
1543R	5'-aaggaggtgatccagcc	(55)
EF3	5'-tcctctaaatgaccaagtttg	(41)
EF4	5'-ggaagggrtgtatttattag	(41)
Fung5	5'-gtaaaagtcctggttcccc	(41)
NS1	5'-ccagtagtcatatgcttgtc	(54)
NS2-10-GC	5'-cgcccgccgcgcgcccccgccccggcccgccgcccccgcccgaattaccgcggctggtggc	(24)
NS3-GC	5'-cgcccgccgcgcccccgccccggcccgccgcccccgcccggctgctggcaccagacttgc	(54)
NS8	5'-tccgcaggttcacctacgga	(54)

A table of the general primers used during for community amplification of rRNA genes. See figure 2.1. for location of fungal primers.

Specificity	Application	Primer pair	Annealing temperature	Product Size (approximately)	Reference
Bacterial 16S rRNA gene	DGGE	GC338F & 530R	60	200 bp	(55)
Bacterial 16S rRNA gene	General amplification	BF1 & 1543R	60	1,500 bp	(55)
Bacteria 16S rRNA gene	LH-PCR	BF1 & 530	60	500 bp	(56)
Fungi 18S rRNA gene	DGGE	EF4 & EF3	48	1400	(41)
Fungi 18S rRNA gene	DGGE	EF4 & Fung5	48	550	(41)
Fungi 18S rRNA gene	DGGE	EF4 & NS3-GC	48	450	(41)
Fungi 18S rRNA gene	DGGE	NS1 & NS8	50	1,700 bp	(24)
Fungi 18S rRNA gene	DGGE	NS1 & NS2-10-GC	55	550 bp	(24)

A table of the combination of primer pairs used for community analysis in this study.

2.2.6. DNA sequencing and analysis.

2.2.6.1. Sequencing.

Plasmids with DNA fragments cloned into them or PCR products were sequenced using the Beckman Coulter CEQ2000XL and the CEQ™ DTCS quick start kit (608120), as per manufacturers protocol. Alternatively the DNA was sequenced using the DNA sequencing facility at the Department of Biochemistry, University of Oxford.

2.2.6.2. Sequence analysis.

Sequence data was analysed using Sequencer V4.0.5 (Gene Codes, UK).

2.2.7. Soil environment and sampling conditions.

The experiments were undertaken at the University of Oxford field station, Wytham, or in laboratory based mesocosms using un-sterilised soil isolated from this location. The field soil at Wytham is classified as heavy clay, with 24.88 % sand, 21.60 % silt, and 53.52 % clay. The soil pH is 7.7 with an organic matter content of 8.57 % (47). The seeds used were the commercially available varieties; *Pisum satvium* var. *quincy* (pea), *Triticum aestivum* var. *pena wawa* (wheat), and *Beta vulgaris* var. *amythyst* (sugar beet). Sugar beet is typically produced with anti-fungal coatings. Since these experiments are aimed at natural communities, perturbations from chemicals would be undesirable, therefore sugar beet seeds were obtained from the suppliers in an uncoated form (Germain (UK) Ltd, Kings Lynn) so all seeds used were untreated.

The process of sampling is fundamentally important in any study of environmental communities. Samples have to be taken and processed in an identical manner, as a failure to adhere to this creates variance within sample time points and between sample time points. The environment under study here is the plant rhizosphere, when sampling this environment, individual plants were removed with surrounding bulk soil (non-rhizosphere soil). Non rhizosphere soil was removed before samples were

used. Three individual plants were sampled for each plant type and these plants were taken from random locations in the field experiment plot. The GM mesocosm experiments were undertaken with individual pots for each treatment and plant type. The experiment was undertaken with triplicate pots and when sampling, plants were taken from each pot.

This rhizosphere material was processed in a way that allowed all subsequent analysis to be undertaken with the same sample, to allow direct comparisons to be made. The techniques being undertaken required soil material for viable count enumeration of population densities from agar plates, bacterial suspensions with no soil for CLPP analysis, and soil suitable for DNA extraction to allow molecular based community analysis. The following method was decided upon; 1 g of material was taken (rhizosphere / bulk soil) and put in a 50 ml centrifuge tube. Ten 6 mm diameter glass beads were added together with 5 ml of 1 x phosphate buffer saline. Tubes were vortexed for 2 min to suspend the soil and bacteria from the rhizosphere in solution. From this soil suspension, samples were taken for microbe enumeration and for pelleting for DNA extraction. This soil suspension was then centrifuged at 3000 x g for 5 min, leaving bacteria still in suspension. This supernatant could then be sampled and used for CLPP analysis.

2.2.8. Culturing techniques for environmental samples.

The enumeration of bacteria and fungi in the rhizosphere was undertaken from the soil suspension created in section 2.2.7. Total heterotrophic populations were estimated on Tryptone Soya Broth Agar (TSBA) (Difco, UK), with the addition of cyclohexamide (0.1 mg ml^{-1}) for the suppression of fungal and yeast growth. This medium is rich in nutrients and allows numerous bacterial species to grow. It was therefore chosen to monitor total aerobic heterotoph counts to give a good indication of resource availability in the rhizosphere. Pseudomonad population densities were estimated on Pseudomonad Selective Agar (PSA) (Difco, UK) supplemented with cyclohexamide (0.1 mg ml^{-1}), centrimide (10 μg ml^{-1}), fucidin (10 μg ml^{-1}), and cephalosporin (50 μg ml^{-1}) giving the best possible selection for these populations. The monitoring

pseudomonad populations was undertaken to detect if there was change in population densities or displacement as a result of the inoculation of a pseudomonad biocontrol agent. Fungal population densities were estimated on Potato Dextrose Agar (PDA) (Difoco-oxoid, UK) with the addition of aueromycin™ (Cyanamid, UK) (320 $\mu g\ ml^{-1}$) for suppression of bacterial growth. A number of general fungal isolation media were equally suitable. PDA was chosen for fungal population monitoring to identify indigenous fluctuations and responses to the addition of BCAs with anti-fungal activity. Plates of all media types were incubated at 28 °C for 48 h, or until colonies were visible by eye as traditionally carried out in the laboratory.

2.2.9. Community level physiological profiling.

Analysis of community carbon utilisation profiles were undertaken using BIOLOG™ GN2 plates (Oxoid, USA). One ml of the resulting supernatant from centrifugation of the soil suspension described in section 2.2.7. was diluted into 20 ml of 1x PBS. This diluted solution was aliquoted in 100 µl per well of the BIOLOG™ plate (approximately 6.00 $\log_{10}$ bacteria) and subsequently incubated at 15 °C for 7 days. Optical densities of each well were measured at wavelength 600 nm using Rosys Anthos Lucy I plate reader (Switzerland). Previous research identified that inoculum density in BIOLOG™ affects the rate of colour development (18), but as this was identified to result from the lag phase of bacterial growth before colour development (22). Provided the inoculum densities are approximately the same, and left to incubate for sufficient time there is no resulting population density effect on final colour development.

2.2.10. Molecular community profiling techniques.

2.2.10.1. Denaturing Gradient Gel Electrophoresis (DGGE).

Once the successful extraction of environmental DNA was achieved, DNA was amplified by PCR using GC clamped primers, as describe in section 2.2.5.2. PCR products were separated by their DNA denaturation point on a polyacrylamide gel with a linear chemical denaturing gradient. Gels were poured using the Ingeny (Holland) gradient pouring system, using 27.5 ml of each denaturation solution per gel. Gels were run using the Ingeny Phor-U gel apparatus (Ingeny, Holland), with a run time of 16 h at 100 v, in 0.5 x Tris-Acetate-EDTA (TAE) buffer, heated to 60 °C.

Bacterial 16S DGGE gel properties.

Amplification of the 16S rRNA gene was undertaken from environmental DNA using the primers GC338F and 530R. These primers have been identified to bind to a very high proportion of the eubacterial community, with GC338F identified as specific to most members of the bacterial domain (55). They were therefore used to give the best representation of bacteria in the community and have previously demonstrated high levels of success in amplification and resolution of DGGE profiles (35). PCR conditions were as follows: 2 min at 95 °C for one cycle, 60 s at 95 °C, 60 s at 60 °C, and 90 s at 72 °C for 35 cycles, and 30 min at 72 °C. The final concentrations of the reaction mix a 50 µl volume were 1 pM of each primer, 0.2 µM dNTPs, 1µl Sigma taq and buffer at 1 x concentration. The reaction mix was run on a Peltier thermal cycler (MJ research, USA). PCR products were run down a 10 % acrylamide gel (10 % acrylamide/bis, 1 % TAE) with a gradient of 30 % denaturant (12 % formamide, 2.1 M urea) to 60 % denaturant (24 % formamide, 4.2 M urea) with 0.09 % fresh ammonium persulphate and 0.009 % N,N,N',N'-Tetramethylethylenediamine (TEMED) added prior to pouring to polymerise the gel.

Fungal 18S DGGE Gel properties.

Amplification of the 18S rDNA was undertaken from environmental DNA using either the primers NS1 and NS2-10GC (24), or the primers EF4, EF3, fung5, and NS3-GC (41). These two primer sets are widely described, and identification of the most suitable primer set for these environmental samples was undertaken. PCR products were run down a 6 % acrylamide gel (6 % acrylamide/bis, 1 % TAE) with a gradient optimised for the best resolution of 25 % (10 % formamide, 1.75 M urea) denaturant to 40 % (16.8 % formamide, 2.8 M urea) denaturant, with 0.09 % fresh ammonium persulphate and 0.009 % TEMED added prior to pouring to polymerise the gel.

DGGE gel visualisation and analysis

The gels were stained using 20 ml of 0.01 % SYBR® gold (Molecular Probes) for 20 min. Photographs were taken using a Bio-Rad Versa Doc system, with typical exposure times of 30-60 s, and saved as *.TIF files. For the analysis of community profiles and the determination of similarities and differences, two software packages were used: Phoretixs™ 1D Advanced gel analysis software (Ver. 5.0, Nonlinear Dynamics Ltd.) and the Multi Variable Statistic Package (MVSP) (Ver. 3.12d, Kovach Computing Services). Analysis of the diversity and temporal or spatial effects was undertaken from high quality photographs of the DGGEs. The identification of bands between lanes that correspond to the same migration properties are entered into the software. The presence and absence of bands is then identified and entered into matrix format to MVSP. This package identifies the principle components responsible for variance and plots them on a graph.

2.2.10.2. DGGE band amplification for sequencing.

In DGGE analysis, to identify operation taxonomic units from within community profiles, individual DNA bands were extracted for sequencing. Bands were cut from the DGGE gel using a BioRad spot cutter (BioRad, UK) (used for protein gels) with the utilisation of a UV-hood for visualisation of DNA bands. Extracted bands were resuspended in 10 µl molecular grade water and incubated at 4 °C overnight. Band

DNA was amplified by PCR using 5 µl of water as a template in 50 µl PCR reaction, and ligated into pCR4-TOPO before transformation into a recipient *E.coli* host (see section 2.2.3.1.). Transformed clones were then isolated on selective media. In the amplification of DNA from DGGE bands, a background of other bands are revealed as a result of the DNA contaminated nature of DGGE lanes. The identification, from this background, of the clones containing the correct band insert from this background was confirmed by running clonal colony PCR with DGGE primers. Colony PCR involved the resuspension of single colonies into 10 µl MG-H_2O. Of this bacteria water mix 5 µl was then used as template in normal 50 µl PCR reaction. Product from individual clones were then run on DGGE, against the original community profiles, for identification of the correct clones for sequencing.

2.2.10.3. Length Heterogeneity PCR.

LH-PCR was undertaken using the primers BF1 and 530RD4 (Primer 530R with 5' dyeD4 attached (Proligo, USA). The primer BF1 (35) was used, instead of 338F, to increase the size of products for analysis and to incorporate a larger region of genomic DNA for increased length fluctuations. The PCR conditions for LH-PCR, using BF1 and 530RD4, were as follows: 2 min at 95 °C for one cycle, 60 s at 95 °C, 45 s at 60 °C, and 90 s at 72 °C for 35 cycles, and 30 min at 72 °C. The reaction mix final concentrations in a 50 µl volume were 0.2 pM of each primer, 0.2 µM dNTPs, 1 unit Sigma taq and buffer at 1 x concentration. The reaction mix was run on a Peltier thermal cycler (MJ research, USA). 1 µl of product was added to 35 µl deionised-formamide with 0.5 µl of CEQ DNA size standard 600 (Beckman-Coulter, 608095), and analysed using the Beckman-Coulter CEQ2000 fragment analysis software.

2.2.10.4. Clone libraries.

Amplified PCR products were separated by agarose gel electrophoresis (10 V cm^{-1}) in 1.0 % (w/v) agarose in 1x TBE stained with ethidium bromide (0.2 μg ml^{-1} of gel), and visualised using a dark reader (GRI, UK). Bands were extracted and purified using gel extraction kit (Qiagen), before using the Invitrogen TOPO TA Cloning® Kit for sequencing (25-0276), as per manufactures protocol.

2.2.11. Statistical packages.

For statistical analysis the packages used were Microsoft Excel, and the Multi Variable Statistic Package (MVSP) (Version. 3.12d, Kovach Computing Services).

2.3. Results.

2.3.1. DNA extraction from Wytham soil.

Experiments were based at the University of Oxford field station, Wytham, or in laboratory based mesocosm experiments utilising soil from the field site. Previous work has been undertaken at this field site to investigate the bacterial and fungal population dynamics (11, 27, 47, 48). However the unculturable populations in the soil, with the isolation of environmental DNA had not been addressed. The optimisation of DNA extraction was required for successful amplification of environmental DNA. A comparison of a number of techniques was undertaken with modifications to improve DNA yield, stability during extraction, and purity for PCR amplification for DGGE analysis. This optimisation was also carried out on soil from the Sourhope research station (Scottish borders) in which the BBCTAB protocol was developed (21). In this optimisation there was a high range of DNA quality and quantity depending on technique used, see Figure 2.2. In an ideal world, the identification of protocol for both soil types would be achieved for a more universal DNA extraction technique. However this is not the case and DNA extraction has to be optimised for each soil used. The technique identified as most suited to the Wytham soil was BBCTAB (21) with an additional freeze thaw lysis step.

2.3.2. PCR amplification of environmental DNA for DGGE analysis.

2.3.2.1. PCR amplification of 16S rRNA gene.

Primers have already been designed and well-characterised for amplification of the 16S rRNA gene for the prokaryotic kingdom, the most universally known are those by Muyzer *et al.* (35). Whiteley *et al.* (55) have undertaken the modification of one of these primers, with a few base pairs shift in the primer positions on the 16S rRNA gene. This modified primer (338F) binds to a higher proportion of the prokaryotic kingdom, as tested against the RDP. The primers 338F and 530R were used for the amplification of the 16S rRNA gene for DGGE analysis. They were used in conjunction with BF1 and 1543R (35) where a nested approach is unavoidable. The bias of PCR primers was a concern when identifying the composition of uncultured environmental communities. Although primers are designed to minimise bias, it

cannot be ruled out. The use of nested primers for subsequent rounds of PCR amplification for weak environmental samples could amplify any bias that may occur. The level of bias this might cause was addressed.

The bias effect of nested PCR on DGGE profiles.

Despite the optimisation of DNA extraction, some DNA samples could not be amplified unless a nested approach was undertaken due to low yield or contaminations. Therefore, clarification of the effect of nested PCR on the community profile was required in order to determine if profiles were comparable with nested and single round amplification. Sugar beet rhizosphere samples were taken and amplified using one round of PCR with GC338F and 530R. A two round PCR was undertaken with primers BF1 and 1541R, followed by amplification using GC338F and 530R. Samples were run down a 16S DGGE gel for comparison. The community profiles produced were very similar, with no discernable differences by visual or gel analysis using Phoretixs™ 1D Advanced gel analysis software (Ver. 5.0, Nonlinear Dynamics Ltd.), See Figure 2.3. Therefore in this soil environment there appeared to be no bias affect by nested 16S rRNA gene amplification.

2.3.2.2. PCR amplification of fungal 18S rRNA gene.

Primers to amplify the specific SSU of fungi, with successful exclusion of other organisms from a mixed community sample, were more difficult to design since the 18S rRNA gene is present throughout the eukaryotic kingdom. Fungal community profiles by DGGE have been used less extensively than the equivalent in prokaryotic studies. Various primer sets have been designed, and those by Smit *et al.* (41), looked most promising from published analysis of them. Two pairs of primers were designed, with biased amplification of the fungal populations. Set one, EF4 and EF3, would amplify 78% of known fungi with one mismatch, with 95-100% coverage of Basidiomycetes, 75% of Zygomycetes, and poor coverage of Ascomycetes. Set two, EF4 and fung5, would amplify 71% of known fungi, with high coverage of Ascomycetes, and poor coverage of both Basidiomycetes and Zygomycota. The

combination of these two primer sets would allow good coverage of all the fungal taxa, except for the Chytridiomycota which are regarded as having little known importance in soil ecosystems (41). These primers have also been shown to exclude the Oomycetes (41). A second round amplification was undertaken on these products to produce samples that were suitable for DGGE analysis using, the primers EF4 and NS3 (See Figure 2.1. for locations of primers).

PCR amplification of environmental samples, using primer pairs EF4 and EF3, and EF4 and fung5 were undertaken. Nested PCR was carried out on these products using the primers EF4 and NS3, for analysis by DGGE. PCR amplification showed non-specific amplification when visualised by agarose gel electrophoresis. This was not due to PCR primers being carried over from one reaction to the next as purification of the PCR product from the first round was applied. The non-specific amplification observed would give rise to false bands on DGGE by fragments of different lengths having a GC clamp and thus mobility, with potential to provide an over estimation of diversity in the DGGE analysis. This therefore identified that these primers in the Wytham soil environment are unsuitable for fungal community analysis.

An alternative set of primers, were described by White *et al.* (54), which amplified the entire 18S rRNA gene of fungi with the primers NS1 and NS8. The primers have specificity to the fungal kingdom, with good exclusion of other eukaryotes. In tests fungal DNA could be detected in up to a ratio of 1:1000 of plant DNA (24). Using a nested approach, with the primers NS1 and NS2-10GC (24), there was an improvement in the DGGE profiles of the fungal communities.

DGGE analysis of 18S rRNA gene

Analysis of fungal samples was optimised for use with DGGE. Fungal 18S PCR products were of approximately 450 bp, compared to approximately 200 bp for 16S

analysis. Fragments were run on a 6 % polyacrylamide gel with a denaturing gradient of 25-45 %, which was optimised for separation, and clarity of bands. The profile produced spans the middle proportion of the gel (30-40 % denaturant), as found by Kowalchuk *et al.* (24) and shown in Figure 2.4. The use of a smaller gradient had no benefit in the separation of bands on DGGE, as there was decreased clarity of products.

2.3.3.1. Checking suitability of fungal primers.

The fungal primers used were specific to the fungal kingdom, which are also referred to as the "true" fungi (34). The primers did not, however, allow the amplification of the oomycetes (24). There have been debates regarding the nature of oomycetes, and whether they should be classed as fungi, as they are phylogenitically different to the other fungi (34). Oomycetes, in particular *Pythium ultimum* is the model pathogen against which the BCAs used in this study show antagonistic activity. The Oomycetes are excluded by the specificity of the NS1-NS8 primer set. This causes potential problems for analysing the impact of inoculated BCAs, as it is not possible to detect the presence of one of the target pathogens. The primer set NS1 – NS2-10 does, however, allow amplification of *Pythium* spp.

To identify the potential of using NS1 – NS2-10 directly for DGGE an investigation was undertaken on some field environmental samples (Figure 2.4.). The profile showed distinct community bands, with a common dominant band across the lower section of the gel. Six bands were cut from the DGGE for sequencing, and identification of fungi was undertaken to determine the specificity of the primers. The sequences were identified by comparison to the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and were as follows. 1) Ascomycota; pezizomycotina; sordoriomycetes; hypocreomycetidae; hypocreales; *Verticillium* spp., 2) Eukaryota; stramenopites; oomycetes pythiales; pithiaceae, *Pythium* spp., 3) Ascomycota; pezizomycotina; sordoriomycetes; hypocreomycetidae; hypocreales; *no further resolution,* 4) *Pisum savitum* (pea – rhizosphere from which samples were taken)., 5) *Mucuna* spp. (pea family) 6) *Annophilia* spp. (marram grass). This data

identifies the co-amplification of plant and fungal material from the rhizosphere of the plant host. Amplified DNA includes fragments from the Oomycetes, the fungal species of interest. The contaminating plant material, when analysed by DGGE, produced a dominant distinct band in the lower region of the DGGE profile. This indicates a different GC content (higher for the plant species) allowing the separation of plant and fungal bands. This technique was used to assess the fungal communities; bands representing plant material were excluded from analysis, to avoid bias between host plant types.

2.4. Conclusions.

Several methods for measuring indigenous rhizosphere community structure and metabolic activity have been investigated for their suitability for use with the Wytham

field soil, with reference to both culturable and molecular techniques. Considering the highly complex, active/dynamic community under investigation a selection of complementary techniques were decided upon. The techniques used were: Culturing to allow enumeration of culturable heterotrophs, pseudomonads, and fungi. Community level physiological profiling. rRNA gene analysis of 16S and 18S by DGGE. Length heterogeneity PCR analysis of 16S rRNA gene. In an ideal world, all methods known would used to achieve universal coverage of techniques. This, however, would not be practically beneficial as a point of data saturation would be reached with increased repetition potentially causing confusion and conflicting data. Methods were chosen that would allow a combined and simple protocol for sample processing for all samples. These methods have been successfully optimised for use with the soil obtained from the Wytham field site. This allows a straightforward protocol to be established for the isolation of microbes from the same soil sample for community structure and function analysis. This permits the direct comparisons between different community analysis techniques. The successful modification of the BBCTAB protocol, by the addition of a freezing lysis step, resulted in the consistent and reliable extraction and amplification of DNA. This has resulted in the successful optimisation of DGGE analysis of 16S and 18S community profiles to facilitate reproducible analyses of communities. Therefore studies into baseline community structure of the three crop species: pea, wheat, and sugar beet, were undertaken in simulated agricultural conditions at the University of Oxford field site, Wytham. In addition, Laboratory based field microcosms were undertaken to compare the impact that the inoculation of BCAs may have on these same crop species.

2.5. Figures.

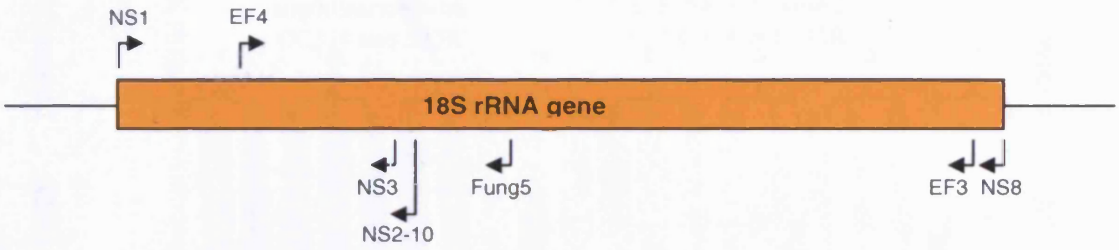
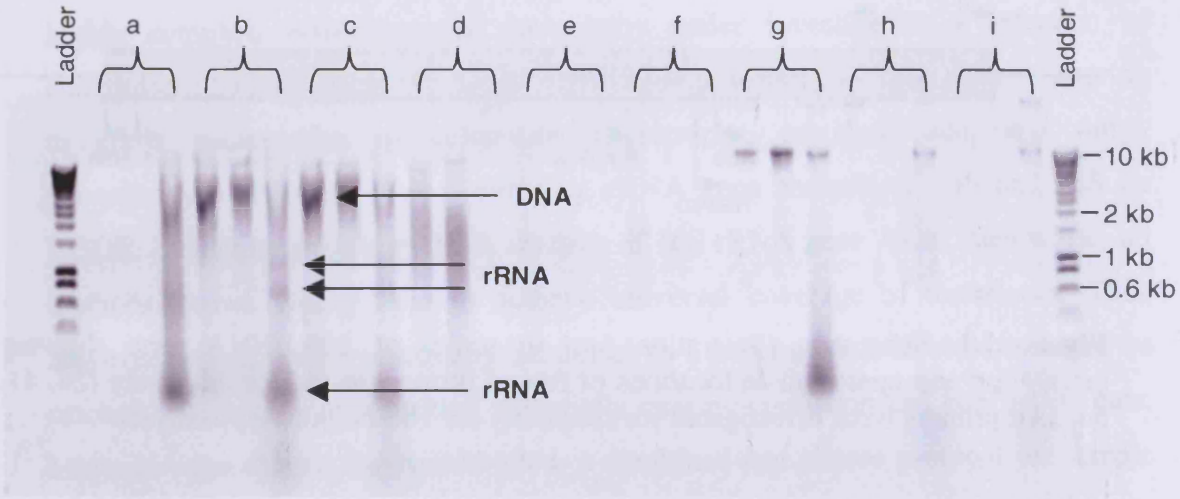


Figure 2.1. Schematic illustration (not to scale) of 18S rRNA gene showing orientation and approximate locations of fungal primers tested for this study (24, 41, 54). All primers were investigated for suitability for 18S community analysis.



Lane in Gel	DNA extraction method	Modification	Purpose of modification	PCR amplifiable	DGGE profile	Reference
a	BBCTAB	-		Unreliable	Unreliable	(21)
b	BBCTAB	Removal of CTAB from extraction buffer	Reduce precipitation of protein and humics which may co-precipitate DNA	Yes	ok	
c	BBCTAB	Addition of Freezing lysis step (see section 2.2.2.2.)	Additional lysis step	Yes	good	
d	CTAB	Sample bead beaten in Bio 101 tube (as BBCTAB protocol) prior to CTAB extraction in CTAB buffer	Additional lysis step	No	-	(3)
e	CTAB	-	-	No	-	
f	CTAB	Addition TE in lysis buffer	DNA buffering	Yes	ok	
g	CTAB	Addition of Phosphate buffer in lysis buffer	DNA buffering	Yes	ok	
h	Buckley	-	--	No	-	(6)
i	Buckley	Addition of Phe:Chl:IAA clean-up step.	DNA purification	No	-	(6)

Figure 2.2. Image of an agarose gel (1 % (w/v)) showing the comparison of total nucleic acid extraction protocols from 0.5 g of soil from three soil types. Total nucleic acid was finally resuspended in 100 µl ddH_2O of which 5 µl was loaded onto the gel shown. The protocols used are described in the table, their success in PCR and DGGE for community profiles. For each extraction protocol (a-i) samples were extracted in order: Wytham soil (high clay soil), Wytham soil spiked with Pseudomonads (7.00 $\log_{10}$ cfu g^{-1} soil), and Sourhope soil (organic rich, brown forest soil).

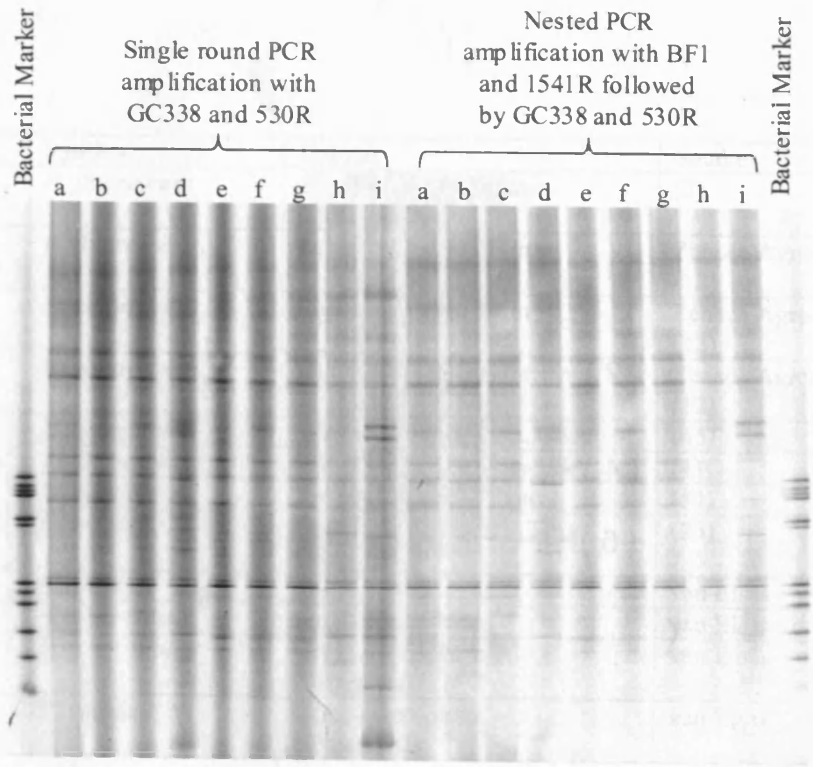


Figure 2.3. Image of a denaturing gradient gel electrophoresis gel comparing the effect of nested and single round PCR on community profiles. The gel was a 10 % Acrylamide gel with denaturing gradient 30 % to 60 %. The gel was run at 100 v for 16 h in 0.5 x TBE at 60 °C. PCR reactions were for single round, GC338F and 530R (21), for nested BF1 and 1541R, 0.5 µl of PCR product was used as template for second round PCR reaction of GC338F and 530R. DNA samples a to i were independent samples from the sugar beet rhizosphere 35 days after planting.

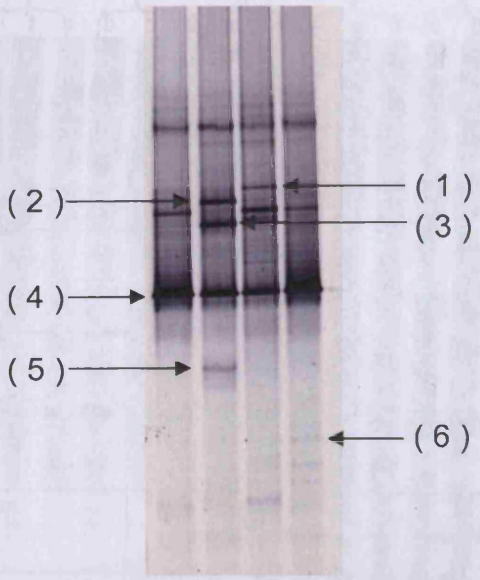


Figure 2.4. Example of fungal 18S denaturing gradient gel electrophoresis gel using primers NS1 and NS2-10GC (12). DNA Samples were from the pea rhizosphere and PCR products were run down a 6 % acylamide gels with 25-40 % denaturing gradient. Gels were run using the Ingeny Phor-U gel apparatus (Ingeny, Holland), with a run time of 16 h at 100 v, in 0.5 x TAE buffer heated to 60 °C. Numbered bands represent bands cut out and sequenced as previously described. 1) *Verticillium Spp.* 2) *Pythium Spp.* 3) *Hypocreales Spp.* 4) *Pisum savitum* (pea – rhizosphere from which samples were taken), 5) *Mucuna* (pea family), 6) *Annophilia Spp.* (marram grass).

2.6. Tables.

Isolate	Species	Habitat	Source
SBW25	*P. fluorescens*	Sugar beet phylloplane - Oxford	(3)
54/96	*P. fluorescens*	Sugar beet – Belgium	Zeneca Agrochemicals, (12)
1335	*P. chloroaphis*	Sugar beet – Belgium	Zeneca Agrochemicals, (12)
76/10	*P. chloroaphis*	Sugar beet – Belgium	Zeneca Agrochemicals, (12)
2-79	*P. marginalis*	Wheat – Washington, USA	(45)
Q2-87	*P. fluorescens*	Wheat – Washington, USA	(4)
CHA0	*P. putida*	Tobacco – Switzerland	(51)
Pf-5	*P. putida*	Cotton rhizosphere – Texas, USA	(25)
2Ps4	*P. cichorii*	Soil – Netherlands	van Elsas
GE1	*P. fluorescens*	Soil – Netherlands	van Elsas
P1	*P. chloroaphis*	Grass rhizosphere – Netherlands	van Elsas
R2F	*P. putida*	Grass rhizosphere – Netherlands	van Elsas
R12T	*P. fluorescens*	Grass rhizosphere – Netherlands	van Elsas
PH6	*P. putida*	Soyabean rhizosphere – NC, USA	(15)
M114	*P. fluorescens*	Soil – Ireland	(14)
F113	*P. savastanoi*	Soil – Ireland	(14)
A1	*P. putida*	Potato periderm – CA, USA	(16)
CR30	*P. marginalis*	Field soil – CA, USA	(16)
ML5	*P. chloroaphis*	Beet Spermosphere – CA, USA	(37)
PGS12	*P. chloroaphis*	Unknown	(19)
R20	*P. savastanoi*	Bean rhizosphere- CA, USA	(37)
UWC1	*P. putida*	Unknown	Cardiff collection
PGSB1500	*P. fluorescens*	Sugar beet rhizosphere – Belgium	Plant genetic systems
PGSB5589	*P. savastanoi*	Sugar beet rhizosphere – Belgium	Plant genetic systems
PGSB8456	-	Sugar beet rhizosphere – Belgium	Plant genetic systems
7SR1	*P. aeruginosa*	Unknown	(7)
A214	*P. viridflava*	Unknown	(7)
B10	*P. cichorii*	Unknown	(7)
Pf0-1	*P. fluorescens*	Soil	(2)
KT2442	*P. putida*	Soil	(50)
NCIMB 11764	*P. fluorescens*	Unknown	CEH- collection
PAO1	*P. aeruginosa*	Patient wound infection	(43)
-	*B. pumilis*	Unknown	CEH- collection
-	*C. violaceum*	Unknown	CEH- collection
23:10	*P.f luorescens* SBW25::Ptac-*Phz*ABCDEFG-Km	GM of SBW25 for use in biocontrol	(3)

Table 2.1. Bacterial strains used in this study.

2.7. References.

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Chapter Three: Baseline study of microbial community structure and function in three crop species under field conditions.

3.1. Introduction.

An investigation into the function and structure of the indigenous bacterial and fungal communities was undertaken. The effects of growth stage and plant type were studied for the three United Kingdom grown crops, pea (*Pisium satvium* var. *quincy*), wheat (*Triticum aestivum* var. *pena wawa*), and sugar beet (*Beta vulgaris* var. *amythyst*) under simulated agricultural conditions. This was undertaken to provide base line data to evaluate the sensitivity of community profiling techniques and to determine whether natural perturbations in indigenous soil communities could be detected. These data are vital to the overall objective of evaluating whether the use of biologically active inocula for fungal disease control, genetically modified biological control agents (GM-BCAs), have a discernable impact on rhizosphere microbiology.

Many environments have been studied in respect to their indigenous bacterial populations. The monitoring of fluctuations that occur in these populations has been researched in an attempt to gain a greater understanding of the microbiology. Microbial diversity and ecosystem function studies are being undertaken for fungal populations but to date this research has not been as comprehensive (2, 15, 37, 41). Research into the soil environment has attempted to identify the effect of soil type, the influence of roots, plant species and seasonal effects that may cause an impact on the indigenous bacterial communities (17, 27 , 35 , 38). Originally the majority of these studies were based on culture dependant techniques monitoring bacterial population dynamics and composition by plating, community level physiological profiling, FAME analysis of community isolates, and related bacterial identification techniques for isolates (9, 10, 22, 23, 39). Culturable populations account for 1-5 % of the indigenous communities in bulk soil so studies on functional diversity, active and inactive populations and community structures limited to this subsection of the population would give potentially distorted results; techniques which can potentially address the remaining 95-99 % as well would potentially give a better representation of community structure and function.

The advent of molecular based analysis has allowed investigations into the total communities to provide a greater understanding of microbial diversity and population

dynamics. These molecular microbial methods have allowed the study of the high diversity environments at increasingly smaller scales (1). The principle technique that improved our understanding of microbial diversity was the development of denaturing gradient gel electrophoresis (DGGE) of the conserved small subunit ribosomal RNA gene of individual organisms including bacteria and fungi (31) and potentially higher eukaryotes. Various techniques now utilise the heterogeneity of the ribosomal RNA gene sequence for community profiling and micro-organism identification, these include LH-PCR and T-RFLP (as previously described), amplified ribosomal DNA restriction analysis (ARDRA), and the many variations of these basic PCR based approaches.

The introduction of plants to the soil affects the soil environment and its microbiology. The majority of chemical compounds present in plants are also known to be present in the rhizosphere. This rhizodeposition includes amino acids, hormones (auxins), organic acids, sugars and vitamins, the secretion of polysaccharides and enzymes etc. In addition it also includes plant cell lysates, which comprise of whole roots, sloughed off cells, cell walls, and volatile compounds (4) as well as decomposing plant material. This source of nutrients supports increased microbial growth in comparison to that of the bulk soil communities. These increased population densities result from the enrichment of micro-organisms from the bulk soil (6, 36), this phenomenon is often referred to as the "rhizosphere effect".

It has been demonstrated that different soil types support highly diverse microbial communities and functions (13, 46). The introduction of plants into this environment will further affect the indigenous communities. This may be a result of the plant supplying a source of inocula or by encouraging or suppressing the growth of indigenous bacteria and fungi by their presence. The size of this perturbation is currently under debate. Both soil type and the presence of plants significantly affect the functional diversity of soil and plant associated microbial communities. Some studies have concluded that soil type has the greatest effect on community structure (3, 13) whereas others have shown that plants have the greatest effect (17). Marschner *et al.*, (28) suggests that the complex interactions between plant species and soil types might not be easily identified by the study of soil and plants as independent variables. This may be the cause of the conflicting reports on the significance of these

parameters on community structure. In addition the sampling procedures adopted by researchers and definitions of the soil/rhizosphere environment can cause differences. Regardless of the dominating effect on community structure, plants clearly have a significant effect (6, 28, 36, 43). Recent data confirm the logical suggestions that the observed seasonal variations in the activity and relative abundance of rhizosphere microbial populations are plant dependent (6, 21, 36). The composition of the microbial community present in the rhizosphere also changes over time in response to the changing root exudation patterns that vary over the life cycle and seasonal response of plants (6). Enrichments of bacterial populations have been identified when the plants begin physiological changes as a result of nutrient redirection in their growth or maturation to harvest (seed set) as a part of their natural life cycle (6, 36). Comparisons between plant species have demonstrated that there are distinct differences between plant species when comparisons of community structure and function are undertaken with the highest variability occurring in young plants (16, 36).

All of these factors need to be considered when undertaking a good study of plant microbiology to define the microbial ecology of this highly specialised and relatively ubiquitous habitat. In this study we have decided to use rhizopshere microbial dynamics to provide reproducible measures of ecosystem responses to the use of GM bacteria for the control of plant fungal diseases. These data are critical for improved BCA effect; but also in evaluating approaches to assess the impact (real or potential) of the use of GM organisms in agriculture or horticulture.

3.1.1. Aims.

The aim of this research is to monitor changes in the population dynamics and measure any effects on the community function of the rhizosphere microbial communities in a growing season for three crop plants grown under field conditions. Populations will be monitored using a number of complimentary techniques and applied simultaneously to gain a more robust understanding of the population dynamics of the indigenous communities. This research simultaneously monitored bacterial and fungal communities by culturable techniques, and culture independent methods. The hypothesis being tested were:

1) Different plant species enrich different bacterial and fungal species from the indigenous soil microbial communities.

2) The selection effect of plant species on rhizosphere microbiology is dynamic and changes over the life cycle of a particular plant variety/type.

3) Individual plant species enrichment differences between growth stages will have a greater effect on the diversity and function of indigenous communities over seasonal effects in bulk soil.

3.2 Materials and Methods.

3.2.1 Field design.

Field experiments were undertaken at the University of Oxford field station, Wytham. The field soil is classified as heavy clay with 24.88 % sand, 21.60 % silt, and 53.52 % clay. The soil pH is 7.7 with an organic matter content of 8.57 %. The seeds used were the commercially available varieties; *Pisium satvium* var. *quincy* (pea), *Triticum aestivum* var. *pena wawa* (wheat), *Beta vulgaris* var. *amythyst* (sugar beet). Sugar beet typically is produced in pelleted form with anti-fungal coating. This experiment was aimed at the baseline natural communities without perturbations from chemicals. Sugar beet seed was obtained from the suppliers in an uncoated form (Germain (UK) Ltd, Kings Lynn.).

Three 2 m by 2 m plots were created for the planting of crops. Pea and wheat were sown in rows 15 cm apart with approximately 3 cm between seeds at a depth of 3 cm. Sugar beet seeds were sown 15 cm apart at a depth of 3 cm. Plots were weeded by hand with irrigation as required. Seeds were sown in June 2002.

3.2.2. Sampling

For each plant species, three plants were sampled as independent replicates. Rhizosphere samples were collected at five time points, to represent the different growth stages of the plant. Growth Stage one (GS1) represented young seedling; GS2 represented the growing plant, approximate midpoint of rapid growth; GS3 represented flowering/mature plant; GS4 represented fruiting plant (seed set) at point of harvest; and GS5 represented post-harvest plant rhizosphere. This scheme was only for the pea and wheat plants as sugar beet does not flower until the following year (it is illegal to allow sugar beet to flower and set seed). Therefore GS3 for sugar beet represents the large plant before it begins to lay down its root reserves (tuber expansion). GS4 represents tuber expansion; and GS5 represents the plant as its growth slows and enters senescence for over wintering. For each plant species, the days after sowing that correspond to GS1-GS5 samples are as follows: Pea, 14, 30,

57, 70, and 85 days; Wheat, 12, 48, 76, 103 and 132 days; and Sugar beet, 16, 44, 78, 99, and 120 days.

Samples were taken at 9 am on each sampling day to limit diurnal effects. For each species being sampled three plants were taken at random from the plot with adhering soil. Samples were individually bagged and returned to the laboratory for processing (<1 hr). Loosely adhering soil was removed and 1 g of root with rhizosphere material was collected and put into sterile 50 ml centrifuge tubes. As sugar beet tubers increased in size (>4 mm diameter), tuber peelings (approximately 2 mm thick) were taken as material from the rhizosphere. To each tube 5 ml of phosphate buffer saline was added with ten 6 mm glass beads and vortexed for 2 min. From this soil mixture 1 ml was put into a 2 ml centrifuge tube spun down at 16000 x g for 2 min for DNA extraction. Two hundred microlitres were removed for serial 1:10 dilutions for enumeration of culturable population numbers. The 50 ml tubes were then spun at 3000 x g for 5 min and 1 ml was removed for CLLP analysis.

3.2.3. Culturable population counts.

Populations were estimated using 1:10 serial dilutions of the soil/PBS mixture from the root rhizosphere (section 3.2.2.). One hundred microlitre of dilutions were plated out onto three media types. Tryptone Soya Broth Agar (TSBA) (Difco-Oxoid, UK), with the addition of cyclohexamide (0.1 mg ml^{-1}) for the suppression of eukaryote growth. Pseudomonad population densities were estimated on Pseudomonad Selective Agar (PSA) (Difco-Oxoid, UK) supplemented with cyclohexamide (0.1 mg ml^{-1}), centrimide (10 μg ml^{-1}), fucidin (10 μg ml^{-1}), and cephalosporin (50 μg ml^{-1}). Fungal population densities were estimated on Potato Dextrose Agar (PDA) (Difco-Oxoid, UK) with the addition of aueromycin™ (Cyanamid, UK) (320 μg ml^{-1}) for suppression of bacterial growth. Plates were incubated at 28 °C for 2 days.

3.2.4. Extraction of total nucleic acid from environmental samples.

DNA extractions were carried out from soil pellets, from 1 ml soil/PBS mixture, harvested by centrifugation (section 3.2.2.). The protocol used was BBCTAB (19) with the modification of an additional freeze/thaw lysis step section 2.2.2.2. DNA was resuspended in 100 µl ddH_2O (Yield ~150 ng μl^{-1}) and stored at – 20 °C for PCR amplification.

3.2.5. Community level physiological profiling.

The 50 ml centrifuge tubes containing soil/PBS mixture (section 3.2.2.) were centrifuged at 3000 x g for 5 min before 1 ml of supernatant was diluted into 20 ml 1x PBS. One hundred mictolitres of this dilution were added per well of a BIOLOG™ GN2 plate and were incubated at 15°C for 7 days. This translates as approximately 6.00 $\log_{10}$ bacteria inoculated per well. These densities are confirmed in total count data. Optical densities of each well were measured on a Rosys Anthos Lucy I plate reader (Switzerland) at a wavelength of 600 nm.

3.2.6. Denaturing gradient gel electrophoresis (DGGE).

Community analysis was evaluated using 16S and 18S rRNA genes as optimised in section 2.2.10.1.

3.2.7. Sequencing of 16S and 18S DNA fragments.

Bands were extracted from DGGE gels, purified, amplified by PCR and inserted into cloning vectors for sequencing, as detailed in section 2.2.10.2. Vector inserts were sequenced using the Beckman Coulter CEQ2000XL and the CEQ Dye terminator cycle sequencing kit as per manufacturers protocol, or by the DNA sequencing facility, Dept. of Biochemistry, University of Oxford.

3.2.8. LH-PCR.

LH-PCR was undertaken as described in section 2.2.10.3.

3.3. Results.

Plants grown at the field site had a general healthy appearance with no obvious signs of disease. Plants were sampled at different time points to correspond to equivalent stages in their growth. These comparative stages were chosen as indicators of significant points in the plants life cycle. These stages were as follows:

Growth Stage	Description	Pea plant age (days)	Wheat plant age (days)	Sugar beet plant age (days)
GS1	Young seedling	14	12	16
GS2	Growing plant, midpoint of rapid growth	30	48	44
GS3	Flowering/mature plant	57	76	78
GS4	Fruiting plant, point of harvest (sugar beet tuber expansion)	70	103	99
GS5	Post-harvest plant rhizosphere (sugar beet senescence / point of harvest)	85	132	120

3.3.1. Population dynamics in the plant rhizosphere of bacteria and fungi.

The enumeration of culturable bacteria and fungi was undertaken between plant growth stages. This assessed whether there were significant growth stage dependent population fluctuations, and if significant differences in the rhizosphere carrying capacity of micro-organisms occurred between different plant species. This was compared to population densities enumerated in bulk soil at four time points throughout the plant growing season, day 12, 76, 111, and 153 after sowing of the plants. The population densities in the bulk soil were highly stable with no significant seasonal variation. Therefore seasonal average population densities were calculated to allow direct comparison to population densities of the bacteria and fungi in the plant rhizosphere.

3.3.1.1. Pea rhizosphere population dynamics.

The enumeration of indigenous communities was undertaken for the pea rhizosphere at five growth stages: GS1 = 14 days, GS2 = 30 days, GS3 = 57 days, GS4 = 70 days, and GS5 = 85 days (Figure 3.1.). Average population densities in rhizosphere for total culturable heterotrophs varied between 9.01 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS5) and 10.14 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4). This was significantly higher than identified in bulk soil, 7.46 $\log_{10}$ cfu g^{-1} bulk soil. Growth stage comparisons identified no significant difference in total culturable heterotroph populations (Table 3.1). Pseudomonad population densities in the pea rhizosphere varied between 7.84 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS5) and 9.59 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3), see Figure 3.2. This was significantly higher than those identified in bulk soil, 5.66 $\log_{10}$ cfu g^{-1} bulk soil. GS3 population densities were significantly higher than GS1 (8.21 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS5 (Table 3.1). GS4 (9.14 $\log_{10}$ cfu g^{-1}wet weight rhizosphere) was significantly higher than GS5 but not to any other growth stage. The enumeration of fungal populations was undertaken with potato dextrose agar (PDA) with densities varying between 5.15 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3) and 5.90 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4) see Figure 3.3. This was similar to that identified in bulk soil, 5.13 $\log_{10}$ cfu g^{-1} bulk soil. The fungal population densities at GS3 were significantly lower than that at GS1 (5.80 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) and GS4 (Table 3.1). The population density at GS4 was also significantly higher than at GS5 (5.36 $\log_{10}$ pfu g^{-1} wet weight rhizosphere).

3.3.1.2. Wheat rhizosphere population dynamics.

The enumeration of culturable populations were undertaken in the wheat rhizosphere at five growth stages: GS1 = 12 days, GS2 = 48 days, GS3 = 76 days, GS4 = 103 days, and GS5 = 132 days (Figure 3.1.). Total culturable heterotroph densities varied between 8.64 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) and 9.18 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS2). This was significantly higher than those identified in bulk soil, 7.46 $\log_{10}$ cfu g^{-1} bulk soil. The difference between GS2 and GS3 was the only significant difference between all growth stages (Table 3.2). Pseudomonad population

densities varied between 7.05 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) and 8.59 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1), see Figur.e 3.2. This was significantly higher than those identified in bulk soil, 5.66 $\log_{10}$ cfu g^{-1} bulk soil. There was no significant difference identified between growth stages. Estimates of fungal population densities lie between 5.15 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3) and 5.91 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4) (Figure 3.3.) This was similar to that identified in bulk soil, 5.13 $\log_{10}$ cfu g^{-1} bulk soil. No significant differences between growth stages were identified. This data is summarised in Table 3.2.

3.3.1.3. Sugar beet rhizosphere population dynamics.

Culturable populations in the sugar beet rhizosphere were enumerated at five growth stages. They correspond to the days after sowing as follows: GS1 = 16 days, GS2 = 44 days, GS3 = 78 days, GS4 = 99 days, and GS5 = 120 days (Figure 3.3). Total heterotroph population densities vary between 10.37 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4) and 7.81 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) (Figure 3.1.). This was higher than those identified in bulk soil, 7.46 $\log_{10}$ cfu g^{-1} bulk soil. GS1 (8.12 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) was significantly lower than GS2 (9.63 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS4. GS4 and GS2 had population densities significantly higher than GS3 and GS5 (8.06 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) (Table 3.3). Pseudomonad population densities in the sugar beet rhizosphere varied between 6.60 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 7.32 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS5), see Figure 3.2. This was higher than that identified in bulk soil, 5.66 $\log_{10}$ cfu g^{-1} bulk soil. GS1 and GS4 (6.43 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) had significantly lower population densities than GS3 (6.98 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS5 (Table 3.3). Total Fungal population densities for sugar beet varied between 4.17 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4) and 5.74 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) (Figure 3.3.). This was similar to that identified in bulk soil, 5.13 $\log_{10}$ cfu g^{-1} bulk soil. Population densities at GS1 were significantly higher than all other growth stages with GS4 significantly lower than GS2 and GS5 (4.78 and 5.00 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively) (Table 3.3).

3.3.2. Plant species effects on the culturable population dynamics.

A comparison was undertaken to identify plant specific effects on the populations of bacteria and fungi in the three plants under study, pea, wheat and sugar beet. This comparative analysis was undertaken for individual growth stages.

3.3.2.1. Total culturable heterotroph dynamics between plant rhizospheres (TSBA).

At GS1 there were no significant differences between pea and wheat population densities (9.50 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 9.10 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively). Both had significantly higher population densities than the sugar beet populations (8.12 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) (Figure 3.1. and Table 3.4). At GS2 there were no significant differences between plant species on population counts. At GS3 a similar effect to that at GS1 was identified. Pea and wheat population densities were not significantly different to each other (9.63 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 8.64 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively), but both were significantly larger than the sugar beet (7.86 $\log_{10}$ cfu g^{-1} wet weight rhizosphere). At GS4 the population densities of wheat (8.76 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) were significantly lower than that of pea and sugar beet (10.14 and 10.37 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively). At GS5 there were no significant differences between plant types.

3.3.2.2. Total pseudomonad population dynamics between plant rhizospheres (PSA).

At GS1 sugar beet population densities (6.60 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) were significantly lower than that for pea (8.21 $\log_{10}$ cfu g^{-1} wet weight rhizosphere). There were no significant differences between wheat population densities (8.57 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) compared to pea and sugar beet (Figure 3.2. and Table 3.4). At GS2 there were no significant differences between plant types. At GS3, pea total pseudomonad population densities (9.60 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) were significantly higher than wheat and sugar beet (7.05 and 6.98 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively). At GS4, pea, wheat and sugar beet population densities (9.14 $\log_{10}$ cfu g^{-1} wet weight rhizosphere, 7.08 $\log_{10}$ cfu g^{-1} wet weight

rhizosphere, and 6.43 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively) were all significantly different to each other. At GS5 there were no significant differences between plant types.

3.3.2.3. Total fungal population dynamics between plant rhizospheres (PDA).

Total fungal population densities in the wheat rhizosphere (5.26 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) were significantly lower than those shown in pea and sugar beet (5.80 and 3.74 $\log_{10}$ pfu g^{-1} wet weight rhizosphere respectively) at GS1 (Table 3.4. and Figure 3.3). At GS2 sugar beet fungal population densities were significantly lower than that in wheat (4.78 and 5.59 $\log_{10}$ pfu g^{-1} wet weight rhizosphere respectively). There were no identifiable significant differences at GS3. At GS4 there was significant difference in fungal populations for pea, wheat and sugar beet (5.90, 5.34, and 4.17 $\log_{10}$ pfu g^{-1} wet weight rhizosphere respectively). There were no significant differences between plant types at GS5.

3.3.3. Community level physiological profiling.

Community level physiological profiling (CLPP) was undertaken on rhizosphere material from the three plant types, pea, wheat and sugar beet and bulk soil to evaluate variability in functional diversity of substrate utilisation. Plant rhizosphere samples were taken at five growth stages and bulk soil at four time points. BIOLOG™ GN2 plates were used on these samples with substrate utilisation measured at 600 nm. Analysis of the results was undertaken using the statistical package MVSP, and data (n = 3) presented by principle component analysis, see Figure 3.4. Statistical tests were undertaken using paired two-tailed *t*-test on the average well colour of substrates between treatments (Figure 3.5.). It is important to remember that this statistical test measures the difference between all substrates, giving an overall indication of difference. Principle component analysis identifies the principle components that account for the greatest variation between data samples, not the complete data set of all substrates.

3.3.3.1. CLLP between plants and bulk soil.

The data from all growth stages of plant in comparison to the bulk soil demonstrated that there is marked seasonal variance between growth stages of plant species and that there is some variation in the bulk soil carbon utilisation profile over time. (Figure 3.4a). These data are spread along axis 1 (70 % of the variance) with distinct clusters represented by early and late growth stages of plants, the soil samples spread across the entire axis with partial separation along axis 2 from the plant profiles. Comparisons at individual time point highlight that although there is separation of soil CLPP between time points, the effect of plants cause distinct separation of plant species CLPP from bulk soil. As a result of this plant species data will be compared excluding the bulk soil data.

3.3.3.2. CLPP between plants for all growth stages.

The data from all growth stages and plants was analysed to identify if there were plant species dependent effects. There was a distinct separation of growth stages independent of plant type. GS1 and GS2 for pea and sugar beet, and GS2 for wheat had strong separation from the remaining growth stages along axis 1 (74.3 % of the variance). GS1 for wheat had distinct separation from the other growth stages on axis 2 (6.3 % of the variance; Figure 3.4b). CLPP analysis was undertaken for plant species to identify growth stage effect for individual plant types.

3.3.3.3. CLPP analysis of the individual plant rhizospheres.

CLPP of the pea rhizosphere.

The community profiles for the pea rhizosphere demonstrated very distinct and significant separation of GS1 and GS2 from GS3, GS4 and GS5 along axis 1 (84.7 % of the variance; Table 3.5). The CLPP for GS1 has tight internal clustering adjacent to GS2. GS1 and GS2 are significantly different to each other. GS3, GS4 and GS5 data points cluster tightly to themselves with the slight separation of each growth stage.

GS4 and GS5 were not significantly different to each other, whereas all other growth stages were (Table 3.5).

CLPP of the wheat rhizosphere.

The comparison between growth stages for the wheat rhizosphere CLPP demonstrated similar separation to that identified in CLPP comparison between all plant types. GS2 separates to one extreme of axis 1 from the other growth stages that are clustered near the origin (70.1 % of the variance). GS1 separates from GS3, GS4 and GS5 on axis 2 (13.7 % of the variance) with some contribution of axis 1. GS3, GS4 and GS5 cluster closely together but remain significantly different. All growth stages were significantly different from each other (Table 3.5).

CLPP of the sugar beet rhizosphere.

Significant patterns were demonstrated in sugar beet CLPP comparisons between growth stages. These patterns were similar to those found when comparing the CLPP of the three plant species between growth stages. GS1 and GS2 separated to one end of axis 1 (77.2 % of the variance) with GS3, GS4 and GS5 having significant separation to the other end (Table 3.5). GS1 and GS2 both demonstrated tight internal clustering with a small, but significant, degree of separation to each other. GS3 and GS5 cluster to the same location with no significant difference.

3.3.3.4. Plant species effect on CLPP at individual growth stages.

The analysis of CLPP at individual growth stages was undertaken to identify the significance of plant species in carbon utilisation profiles. The wheat plants CLPP profile at GS1 separated from the pea and sugar beet CLPP along axis 1 (94.7 % of the variance), with all demonstrating tight internal clustering (Table 3.6). At GS2 and GS3, all plant types separated equally around the origin to a significant level with even distribution on axis 1 and 2 (35.7 and 17.2 % of the variance respectively for GS2 and 38.7 % and 26.1 % of the variance respectively for GS3). At GS4 the CLPP of each plant type separated along axis 1 (47.5 % of the variance) with wheat centring on the origin. There is partial overlap of data between wheat and sugar beet but all

were significantly different by *t*-test. At GS5 the distribution of plant species are demonstrated around the origin. The internal clustering of data is not as tight as previously demonstrated, with data points for the pea and sugar beet being closer to each other than to the wheat. *T*-test analysis identified that pea and sugar beet were not significantly different to one another despite distinct clustering (Table 3.6).

3.3.4. Average well colour (AWC) for community level physiological profiling.

Data collected from BIOLOG™ plates were used to calculate an average well colour for each plate. This was used to make comparisons between the five growth stages and plant species (Figure 3.6).

3.3.4.1. AWC for individual plants.

AWC for the pea rhizosphere.

The AWC for the pea rhizosphere for GS1 and GS2 (OD_{600} = 0.448 and 0.602 respectively) were not significantly different, however they were significantly different to GS3, GS4 and GS5 (OD_{600} = 1.183, 1.054, and 1.116 respectively; Table 3.7). The AWC for GS3, GS4 and GS5 were not significantly different to each other.

AWC for the wheat rhizosphere.

Results indicate the AWC for GS1 (OD_{600} = 1.246) was significantly higher than that of all other growth stages (Table 3.7). GS2 (OD_{600} = 0.440) had a significantly lower AWC in comparison to all other growth stages. GS3, GS4 and GS5 were not significantly different to one another (OD_{600} = 0.892, 0.994, and 0.813 respectively).

AWC for the sugar beet rhizosphere.

In the sugar beet rhizosphere the AWC for GS1 (OD_{600} = 0.429) was significantly lower in comparison to all other growth stages (Figure 3.7). GS2 (OD_{600} = 0.552) had a slightly higher AWC than GS1, however it was still significantly lower than the

remaining growth stages. GS3 is not significantly different to GS4 and GS5, but GS4 and GS5 are significantly different to each other.

3.3.4.2. Growth stage comparison of AWC between plant species.

At GS1 there was no significant difference in the values of AWC between pea (OD_{600} = 0.489) and sugar beet (OD_{600} = 0.429). Both plants were significantly lower in their AWC than wheat (OD_{600} = 1.246; Table 3.8). At GS2 the pea AWC (OD_{600} = 0.602) was significantly higher than in wheat (OD_{600} = 0.440), but there were no significant differences from either to sugar beet (OD_{600} = 0.552). At GS3 pea (OD_{600} = 1.177) had a significantly higher AWC than wheat, but not sugar beet (OD_{600} = 0.892 and 1.099 respectively). There was a significant difference between wheat and sugar beet. At GS4 the wheat AWC (OD_{600} = 0.994) was not significantly different to either pea or sugar beet (OD_{600} = 1.123 and 0.903 respectively). Despite this, the AWC for pea and sugar beet were significantly different to each other. At GS5 pea and sugar beet AWC (OD_{600} = 1.116 and 1.093) were significantly different to each other. Wheat (OD_{600} = 0.813) had a significantly lower AWC than both the pea and sugar beet, with probabilities 0.02 and <0.01.

3.3.5. Community analysis of the rhizosphere of three crop plants by DGGE.

3.3.5.1. 16S community analysis of the bulk soil.

PCR amplification of DNA was carried out using the universal eubacterial primers where possible (31, 44). A single round approach was employed however, when there was weak amplification a nested approach was used with the primers BF1 and 1543R (31) this did not introduce unacceptable bias, see chapter two. PCR products were run down standard bacterial DGGE gel as described in section 2.2.4.2. The comparisons between time points in bulk soil identified no seasonal changes in community profile with highly reproducible profiles, see Figure 3.6.

3.3.5.2. 16S community analysis of the rhizosphere of three crop plants by DGGE.

PCR amplification of plant rhizosphere DNA was carried out as described above (Figure 3.7.). Comparisons were made between the DGGE community profiles of the three plant species for all time points. Principle component analysis resulted in a large spread of data points (Figure 3.8.). With axis 1 and 2 representing 27.0 % and 17.2 % of the variance respectively. There was a slight shift in the wheat data, with tighter clustering in comparison to the other two plants. This shift was contributed equally by axis 1 and 2. Too many variables were being simultaneously compared to identify any trends, therefore principle component analysis was undertaken on subsections of the data set.

16S community analysis of the pea rhizosphere by DGGE.

Growth stage comparisons were undertaken for the pea rhizosphere. Individual growth stages clustered independently to one another with the principle separation along axis 1 (27.3 % of the variance). There is an oscillation along axis 2 and back with sequential growth stages (16.6 % of the variance). Each progressive growth stage time point clusters get closer to the next time point in spatial separation, with GS4 and GS5 clustering to the same point. Visual inspection of DGGE profiles did not identify any seasonal changes in the number of dominant bands.

16S community analysis of the wheat rhizosphere by DGGE.

The wheat rhizosphere communities demonstrated high internal growth stage clustering, with the major separation of growth stages on axis 1 (18.1 % of the variance). A small level of separation was represented on axis 2 (16.0 % of the variance). Replicate one of data at GS2 was separated from the other replicates along this axis. The data for each growth stage shifted sequentially along axis 1, with GS5 returning to a location situated between growth stages two and three. The number of dominant bands on DGGE appeared to decrease with subsequent growth stages.

16S community analysis of the sugar beet rhizosphere by DGGE.

In the sugar beet rhizosphere there was high internal clustering of growth stage data with the distinct separation of growth stages. This separation was principally along axis 1 (31.1 % of the variance). The data separation on axis 2 (19.8 % of the variance) was represented by an oscillation of growth stages along this axis. This shift in growth stages was sequential from GS2-GS5 with GS1 located next to GS4. Visual inspection of DGGE profiles did not identify any seasonal changes in the number of dominant bands.

16S community analysis of individual growth stages by DGGE.

For the identification of plant species effects on the DGGE community profiles the comparison of individual growth stages was undertaken. At GS1 the distinct separation of the three plant species was visible with the separation of the sugar beet profile from pea and wheat along axis 1 (32.2 % of the variance). The pea and wheat community profiles were more closely positioned to each other by principle component analysis with separation dominated by the influence of axis 2 (22.9 % of the variance). At GS2 a higher degree of variation within growth stages was identifiable with the overlap between pea and wheat data points and separation from sugar beet on axis 1 (33.3 % of the variance). At GS3 the community profile of pea demonstrated higher internal variance in comparison to the wheat and sugar beet profiles. Plant species were separated with the contribution of axes one and two (32.4 % and 26.6 % of the variance respectively). The separation at GS4 demonstrated a similar effect with the separation of pea from wheat and sugar beet on axis 1 (33.8 % of the variance) and the separation of wheat and sugar beet on axis 2 (17.0 % of the variance). At GS5 the profiles for each plant type were distinct and different with the separation contributed equally by axis 1 and axis 2 (33.1 % and 27.7 % of the variance respectively).

3.3.5.3. 18S community analysis of bulk soil.

18S DGGE community analysis was undertaken using the primers described by Kowalchuk *et al.* (25). DNA was preferentially amplified with a single PCR reaction

using the primers NS1 and NS2-10GC. In the event of weak DNA amplification, a nested approach was used. PCR amplified DNA was run down a fungal DGGE as optimised in section 2.3.2.2. Community analysis of the bulk soil environment demonstrated the same as with bacterial communities. There were no differences between community profiles within or between time points sampled (data not shown).

3.3.5.4. 18S community analysis of the rhizosphere of three crop plants by DGGE.

PCR amplification of plant rhizosphere DNA was carried out as described above (Figure 3.9.). The clarity of 18S DGGE gels in comparison to 16S can be attributed to the lower diversity of target fungal communities/populations present in the environmental samples. Initial analysis of 18S community structure was undertaken with alignment of bands present in the community profile included for all three plant types. This resulted in the principle component analysis plot in Figure 3.10a. There was distinct separation of the three plant types with the axes representing 18.7 % and 13.5 % of the variance. It has been previously identified that the host plant 18S rRNA gene can be amplified and will be represented as bands on DGGE profiles. The resulting effect would have high levels of plant bias in the interpretation of the presence or absence of bands. It has also been recognised that nematode and beetle DNA can be successfully amplified in such samples. With the identification of these contaminates and their zone of migration on DGGE profiles it is possible to successfully exclude them from analysis. The removal of these bands allows only true plant driven effects on fungal rhizosphere communities to be identified.

The DGGE profiles were re-analysed with the removal of the known bands corresponding to non-fungal DNA. This resulted in the principle component analysis in Figure 3.10b. These data demonstrate that the significant plant effects on the fungal communities with the differential clustering of plant types. The pea and sugar beet profiles separate to either end of axis 1 (16.2 % of the variance), while the wheat plant profiles are distributed around the origin, overlapping both other plant types.

At this scale, the resolution of principle component analysis was insufficient to identify if there was any significant separation of fungal community profiles between plant growth stages. Data was therefore analysed for plants individually.

18S Community analysis of the pea rhizosphere by DGGE.

The pea rhizosphere demonstrated a gradual sequential shift of DGGE profiles between growth stages along axis 1 (23.0 % of the variance).

18S Community analysis of the wheat rhizosphere by DGGE.

No clear separation of growth stages was identified in the wheat rhizosphere, with data clustering around the origin. GS1 and GS5 cluster together, and it appears that there is a possible gradual oscillation of intermediate growth stages along axis 1. The axes one and two represented 25.7 % and 16.3 % of the variance respectively.

18S community analysis of the sugar beet rhizosphere by DGGE.

The community profiles of sugar beet demonstrated similar separation as identified in the wheat rhizosphere. GS1 and GS5 clustered together with the shift of growth stages away from GS1 to GS3 located the furthest away, before returning to the location of GS5. Axes one and two represented 23.8 % and 17.5 % of the variance respectively.

18S community analysis of individual growth stages by DGGE.

At GS1 the community profile of the pea rhizosphere separated away from the wheat and sugar beet profiles along axis 1 (29.4 % of the variance), with a high level of internal variance demonstrated by axis 2 (20.4 %). At GS2 there is high internal variance of the wheat and pea data in comparison to the sugar beet, which is tightly clustered and separated from the other two plant species. This separation is contributed equally by both axis 1 and 2 (25.3 and 18.9 % of the variance). At GS3, GS4 and GS5 there is the distinct separation of all growth stages contributed equally by both axes.

3.3.6. Community analysis of the rhizosphere of three crop species using length heterogeneity PCR.

Bacterial rhizosphere community analysis was undertaken using length heterogeneity PCR of 16S rRNA gene. Rhizosphere DNA from the three crop species, pea, wheat and sugar beet was used. Samples were taken at the same five growth stages sampled for plate counts, CLPP, and DGGE. Samples were analysed over the 16S hyper-variable region as describe in section 2.2.4.3., using primers described by Whiteley and Bailey (44). The 16S rRNA gene varies in length as well as GC content, and LH-PCR utilises the variability in length to produce community profiles. One of the primers has a fluorescent tag from which, when samples were run through a capillary sequencer with size standard, a community profile of fragment lengths was produced (Figure 3.11.). This profile was analysed using the software program CEQ8000 (Beckman-Coulter, USA) from the DNA capillary sequencer used. The data was exported in binary format representing the presence/absence of fragments. Analysis of profile similarities was undertaken using MSVP (Ver. 3.12d, Kovach Computing Services) (Figure 3.12).

3.3.6.1. 16S community profile comparison of three plant species at all growth stages.

The comparison of LH-PCR community profiles between all plant types and growth stages was undertaken. Analysis demonstrated a high level of variation within each plant species with no distinct separation of plant types (Figure 3.12.). Axis 1 and 2 represented 10.9 % and 10.5 % of the variance respectively. For the identification of potential growth stage dependent effects on community profiles, individual plant types were analysed separately. There was incomplete amplification of certain replicates in some growth stages.

Community analysis of the pea rhizosphere by LH-PCR.

The comparison of community profiles between growth stages was undertaken for the pea rhizosphere. It was identified that GS4 separated from the other growth stages along axis 1 (18.2 % of the variance). The remaining growth stages clustered around the origin with no distinct separation.

Community analysis of the wheat rhizosphere by LH-PCR.

The comparison of community profiles between growth stages for the wheat rhizosphere identified GS4 to separate from the other growth stages along axis 1 (20.7 % of the variance). GS4 had tight clustering in comparison to the other growth stages. The remaining growth stages clustered around the origin with no distinct separation.

Community analysis of the sugar beet rhizosphere by LH-PCR.

The comparison of community profiles in the sugar beet rhizosphere by LH-PCR identified greater separation of the growth stages than shown in the other plants. GS1 clustered away from the other growth stages on axis 1 (21.4 % of the variance). In addition GS4 had partial separation, lying between the main group and GS1 along axis 1. GS2, GS3 and GS5 clustered together with no distinct separation.

Comparison of LH-PCR community profiles at individual growth stages.

The comparison of LH-PCR profiles between plant species at individual growth stages was undertaken. At GS1 there was high variability within the pea and wheat rhizosphere profiles, with a large degree of spread along axis 1 (28.7 % of the variance). The sugar beet community profiles clustered tightly, separating from the other plant types on axis 2 (20.7 % of the variance). At GS2 the pea community profile was distinct from the other plant types, with partial separation of the wheat and sugar beet profiles. Axis 1 and 2 represented 23.9 % and 18.1 % of the variance respectively. At GS3 there was a high level of internal variance for each plant type with no distinct separation of data sets. At GS4 each plant type had distinct separation with tight internal clustering. The data sets separated equally by a combination of both axes. Axis 1 represented 32.0 % of the variance and axis 2 21.6 % of the variance. Plant species at GS5 showed no discernable separation.

3.4. Discussion.

This research was undertaken to monitor the population dynamics and measure the effect on community structure and function in the rhizosphere of three crop species over a growing season in field conditions with comparison to bulk soil. It was found that bulk soil was highly stable and little variation between time points was identified. This has been previously shown in the study by Griffiths (18). The plant rhizosphere has significant effects on community structure and function this is known as the "rhizosphere effect" (6, 36). I found that culturable populations of micro-organisms had high stability in the plant rhizosphere except in the sugar beet rhizosphere whose populations demonstrated more seasonal variation. Different plant species had different carrying capacities of indigenous micro-organisms. The comparison of CLPP demonstrated distinct separation of profiles between young plant rhizospheres and those of older plants, each plant species having its own unique profile. This effect was also identified in the AWC calculations as indications of bacterial activity but was not as a response of changes in bacterial population densities in the rhizosphere. Molecular community analysis by DGGE by the amplification of the 16S rRNA gene demonstrated strong growth stage dependant shifts in bacterial community profiles with the separation of plant species. Fungal (18S rRNA gene) community analysis demonstrated gradual seasonal shifts of growth stages with very strong distinct separation of plant types. The community profiles of bacterial populations by LH-PCR did not demonstrate enough resolution in this environment to determine any plant species or growth stage effects.

The three plants species were sampled at five growth stages throughout their life cycle. GS1 represented the small seedling stage; this was approximately when the root rhizosphere was approximately 1 g or more in total to allow enough for sampling. GS2 represented growing plant, approximately midpoint of its most rapid growth. GS3 was at the point where plants had matured/at the point of flowering. For pea and wheat, GS4 represented plants that had fruited and were at the point of harvest and GS5 post harvest plant rhizosphere. The sugar beet's growth takes considerably longer to mature as it over winters before flowering, however it is cropped within the first year. It was therefore decided that GS4 would represent the sugar beet as it

begins to lay down its sugar reserves, its point of maturation, and GS5 where the plant enters senescence for over wintering (the point where the plant is commercially harvested).

Studies of the culturable populations were undertaken as it is considered a valuable tool for evaluating community diversity and structure in presence of plants. Recently research has predominantly focused on molecular techniques that allows data from the unculturable fraction as well as culturable to be analysed. It is important to have base line culturable population data for comparisons to data generated by other techniques, particularly if the release of BCAs is to be undertaken, so that effects of population dynamics can be factored into any changes identified. Recent research has highlighted the relevance of this by showing that up to 70 % of rhizosphere communities identified by molecular techniques can actually be isolated on laboratory media (Kowalchuk, personal communication).

Total heterotroph population densities (per unit gram of plant material) in the pea rhizosphere did not significantly differ between growth stages indicating a stable carrying capacity of the pea rhizosphere of the indigenous bacteria. Pseudomonad populations gradually increased in density from early seedling to flowering stage, before returning to the same population densities as in early seedlings after harvest. This increase in population density was a result of population structure changes as total heterotroph populations were stable. Fungal population densities reduced as the pseudomonads increased which may have been a result of the suppressive effect the pseudomonads may have on the indigenous fungi populations (11, 24, 26, 29, 33, 34, 42).

Population densities in the wheat rhizosphere of the culturable fraction also demonstrated high stability. Total heterotroph densities were stable throughout the entire growing season, with a similar effect in the pseudomonad population densities, except for a transient reduction in the population at flowering (GS3). The fungal populations were stable with no significant difference over the growing season. Densities were lower, however, for heterotrophs and pseudomonads in comparison to the pea rhizosphere.

The sugar beet rhizosphere was not a stable environment in comparison to the pea and wheat rhizospheres with significant fluctuations in population densities. Total heterotroph densities fluctuated between growth stages with peaks at GS2 and GS4. This was also reflected in the pseudomonad densities but not to the same extent. This indicates there were factors at GS2, point of rapid growth, and GS4, point crop maturation, that caused an increase in the bacterial carrying capacity of the sugar beet rhizosphere. This increase in bacterial carrying capacity may have been as a result of either the moisture content in the rhizosphere of plant (would expect to see effect in other plants if that was the case) or the sugar beets growth stage. At GS2 the plant would be at mid point of rapid growth, where there is the rapid increase in growth rate of the roots and penetration of the long taproot down into the soil strata (7). This may increase the availability of nutrients, increased cell sloughing and exudation at the root tip (4), and provide a better water supply to the plant, resulting in an increase in exudates available for growth in the rhizosphere. At GS4, where the sugar beet is laying down carbohydrate reserves with the rapid expansion of root mass (7), there is the same increase in population densities. This may be a response to the sugar beets storage of carbohydrate reserves in the taproot. This would naturally result in an increase in root exudation causing a concentration gradient across the root cell membranes (7). The fungal populations appear to have a very gradual decline in density to GS4, before a slight increase. However there does not appear to be any direct link to the population fluctuations as seen in the bacterial populations. The densities identified in the sugar beet were significantly lower than those identified in pea for all culturables, and for wheat densities of the heterotrophs and fungal communities.

Population density data indicates that the pea rhizosphere consistently supports larger populations of bacteria and fungi throughout the growing season in comparison to the other plant species. Sugar beet, on the other hand, consistently supports fewer micro-organisms than the other plant species, with the wheat plants fluctuating between the high and low micro-organism population densities depending on the organisms being measured. In comparison to bulk soil, all plant species enrich the populations of culturable bacteria. This "rhizosphere effect" has been attributed to the high level of nutrients available to the micro-organisms inhabiting this environment. From this data it can be inferred that pea plants have the highest level of available niches for

rhizosphere colonisation of the three plant species followed by wheat and then sugar beet. This is reflected in the populations of micro-organisms that they can support. This data when compared to other studies in our lab (40) where in laboratory microcosms found that the pea rhizosphere supported higher populations than wheat. This data, however, indicated that sugar beet had the highest population densities in its rhizosphere not the lowest as our data suggested. This conflicting data may be a result of the soil environment for plant growth, my experiments were undertaken in natural field conditions where as the others were undertaken using commercial topsoil in growth chambers (40). The field conditions may result in less favourable conditions for bacterial colonisation or be a result of the high variability identified in the sugar beet rhizosphere. This data is important to correlate with data from CLPP and AWC to prove the effects identified were not as a result of changes in populations, and to understand the communities so that the impact of release BCAs can be identified.

The comparison between growth stages and plant type identified a strong and significant plant growth stage effect in the CLPP. These effects were greater than the background variance identified in bulk soil. Young plant and early growth (GS1 and GS2) for all plant species were distinct from the final growth stages (GS3-GS5). As seasonal shift in CLPP has been previously demonstrated with the change in profiles in response to sampling date (32, 38), however this variance is not always the case (30, 5). In young seedlings (GS1) there was a difference between the wheat CLPP and that of the other plants. This may have been a result of root type in the early growth stages. Wheat plants have fibrous roots compared to the taproots of pea and sugar beet, this could potentially result in the difference for early growth and rhizosphere colonisation. For the remaining growth stages the same shifts in carbon utilisation profiles were demonstrated for all plant types. In young seedling all plant species were significantly different to each other in their CLPP with the greatest separation being between the wheat and the other two plants. All subsequent growth stages demonstrated equal separation between plant species. This would demonstrate that the plant species has a significant effect on the selection of specific communities, which is reflected in the carbon utilisation profile of the microbial communities. This impact of plant species has been demonstrated before (8, 10, 30), my data demonstrated that the greatest effect on the rhizosphere CLPP is growth stage.

The measuring of the rate of colour development in BIOLOG™ plates for CLPP as an indication of relative activity is wrought with problems (12, 20). However the use of AWC at the end of the incubation (7d) as a crude but simple indication of metabolic potential for growth on BIOLOG™ substrates was considered. These data proved to be very interesting showing that for the pea and sugar beet rhizosphere AWC was significantly lower in early growth (GS1 and GS2) in comparison to later growth stages (GS3-GS5). This effect has similar separation as identified with CLPP for the influence of growth stages. This would indicate lower relative bacterial activity in the early growth stages of the two plants. However this effect was not a result of population densities in the rhizosphere. The wheat rhizosphere showed comparable effects to those identified in CLPP. GS1 was found to have a high AWC. A rapid drop to a value similar to that of the pea plants was seen at GS2 with a subsequent increase.

There appeared to be a general trend in the changes in bacterial population activities, with only one exception. Plants (pea and sugar beet) at the beginning of their life (GS1 and GS2) had low rhizosphere activity on the BIOLOG™ substrates. A sharp increase in activity was then seen up to when the plant matures (GS3) where bacterial activity plateaus and stabilises. The exception was in the wheat rhizosphere where the highest level of bacterial activity was found at GS1. Although there were significant differences at individual growth stages between plant species, similar bacterial activity was seen on pea and sugar beet plants. In the pea rhizosphere four out of five growth stages were significantly higher than the AWC of wheat. Comparison of this data to population densities identified that the bacterial activity in the rhizosphere was independent of the population densities. No correlations between population activity and total bacterial, pseudomonad, or fungal population densities were identified. This would suggest that they are responses to the level of microbial activity in the rhizosphere which may be a result of plant exudation stimulating bacteria.

Analysis of the 16S rRNA gene DGGE analysis identified plant specific effects when the three plant species were compared. The comparison between growth stages for individual plant species identified that there were strong growth stage effects on the bacterial community structure. The comparison of each plants species community profiles at individual growth stages confirmed the plant effects on the community structure. The separation of plant types by principle component analysis was distinct

at each growth stage, demonstrating a higher degree of separation than was indicated by looking at overall communities. The analysis of community structure by 16S rRNA gene DGGE analysis has been wildly undertaken in which there have been many conclusions with support or contradict this data. Some researchers have found little effects of plant on community structure (5), whereas others have found significant shifts (16, 28, 36, 38). These conflicting data may be a result of the highly complex and diverse nature of the soil environment. Different soils may have higher resilience to perturbations, which include planting, in comparison to other due to their composition, Soil type has been shown to be influential in the effects of rhizosphere colonisation (27). It was therefore vital to understand the level of significance that the plant species have in the soil environment understudy to allow further research into the potential impact of BCAs and the effect that they might have.

The 18S rRNA gene DGGE community analysis comparisons between the three plant species identified significant plant effects on the fungal community structure. All known non fungal bands were not included in analysis, therefore this effect was not a result of 18S rRNA genes amplified from the host plant or other amplified non-fungal 18S rRNA gene sequences. This proves that the effects seen on the community structure are a direct result of plant interactions as no variation was identified in bulk soil. The comparison of growth stages for individual plant species identified gradual shifts between growth stages of the fungal community structure. This would suggest a gradual succession of fungal colonisers of the rhizosphere in response to the plant. The study of fungal populations by DGGE in rhizosphere environment is relatively new. Plant enrichment and seasonal shifts have recently been reported in the maize rhizosphere with limited differences in bulk soil (15). Molecular analysis of fungal communities has be undertaken by other techniques (14, 37) but no measures of seasonal variation were undertaken. Comparison of each plant species community profile at individual growth stages confirmed the plant effects on the community structure. The separation of plant species by principle component analysis increased from young seedlings with each subsequent growth stage. This indicates a changing but increasingly plant species-specific effect on the fungal community.

The 16S rRNA gene community analysis of the rhizosphere of three crop plants by LH-PCR identified no discernable plant specific effects with the comparison of all

growth stages. The comparison between growth stages for individual plants identified only minor shifts in community profiles with growth stage. These shifts comprised of the separation of GS4 away from the other growth stages although confidence in this shift is low due to the highly variable nature of the results. In the sugar beet profile there was the additional separation of the young seedling (GS1). The comparison of plant community profiles at individual growth stages by LH-PCR increased the level of separation identifiable between plant species but there was no the confidence or clarity that could be achieved by DGGE analysis. This indicates that the technique of LH-PCR, which has been successful in other environmental situations (45), does not have the resolution to separate complex bacterial community profiles for growth stage or plant effects in this soil environment. and only partially for plant effects.

In conclusion, I found:

Different plant species had different carrying capacities on indigenous micro-organisms.

Each plant species has its own unique profile when analysed by CLPP.

Bacterial activity shifts in CLPP were not a response of changes in bacterial population densities in the rhizosphere.

Strong growth stage dependant shifts with the separation of plant species by DGGE.

With the confirmation of the following hypothesis:

1) Different plant species enrich different bacterial and fungal species from the indigenous soil microbial communities.

2) The selection effect of plant species on rhizosphere microbiology is dynamic and changes over the life cycle of a particular plant variety/type.

3) Individual plant species enrichment differences between growth stages will have a greater effect on the diversity and function of indigenous communities over seasonal effects in bulk soil.

With reference to hypothesis 3, the bulk soil environment had no seasonal effects when communities were analysed by bacterial or fungal DGGE or by other culturable techniques.

The techniques chosen for this study compliment each other and are highly suitable for analysis of rhizosphere communities. These methods demonstrate the significance of growth stage and plant species in rhizosphere function and diversity. These methods were: culturable population density enumeration, CLPP, AWC, and 16S and 18S DGGE. LH-PCR did not add any significant data to compliment the other techniques, so would not be used further in community analysis. These methods provided a good overall understanding of the microbial communities seen in the rhizosphere of pea wheat and sugar beet. We now have a baseline understanding of the diversity and changes seen throughout the growing season of these crop species. This will help in the evaluation of the impact of BCA inocula for the next chapter.

3.5 Figures.

Microbial population dynamics in the rhizosphere of three field crop plants.

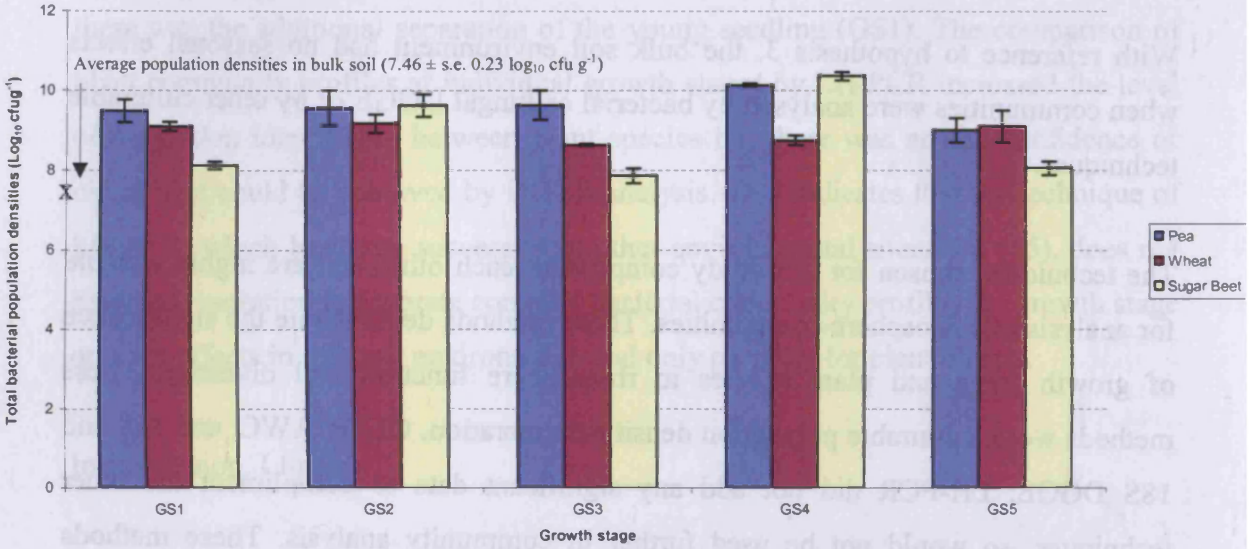


Figure 3.1 Total bacterial population counts in the rhizosphere of the three crop types. Populations were measured at five growth stages and were estimated on tryptone soya broth agar. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n =3) I = standard error of samples.

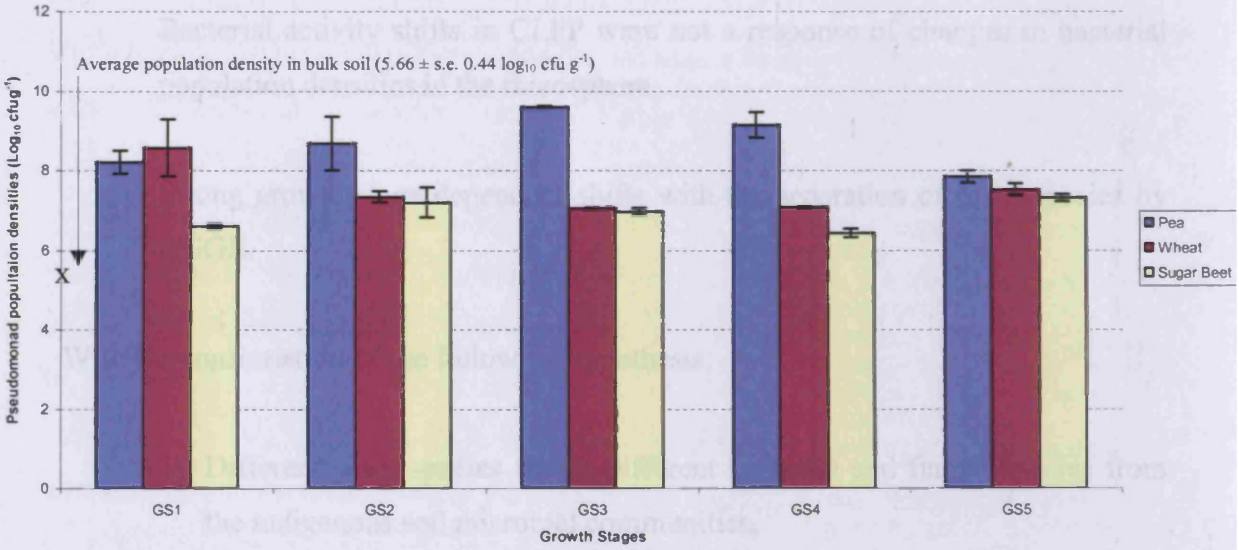


Figure 3.2 Pseudomonad population counts in the rhizosphere of the three crop types. Populations were measured at five growth stages and were estimated on pseudomonad selective agar. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n =3) I = standard error of samples.

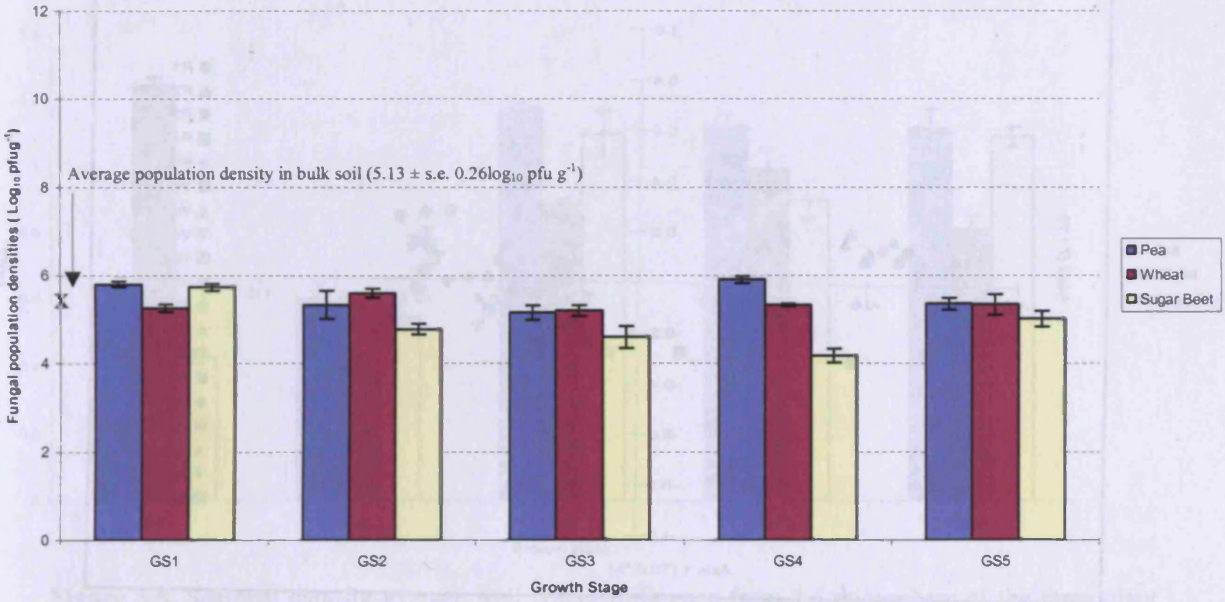
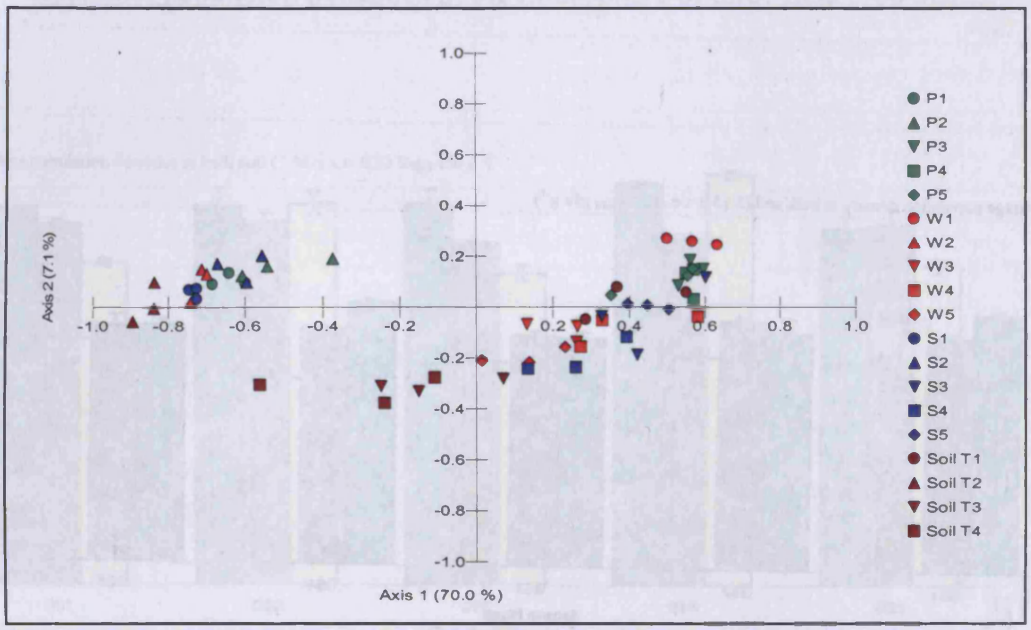


Figure 3.3 Fungal population counts in the rhizosphere of the three crop types. Populations were measured at five growth stages and were estimated on potato dextrose agar. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n =3) I = standard error of samples.

CLPP and calculation of AWC in the rhizosphere of three field crop plants.

(a)



(b)

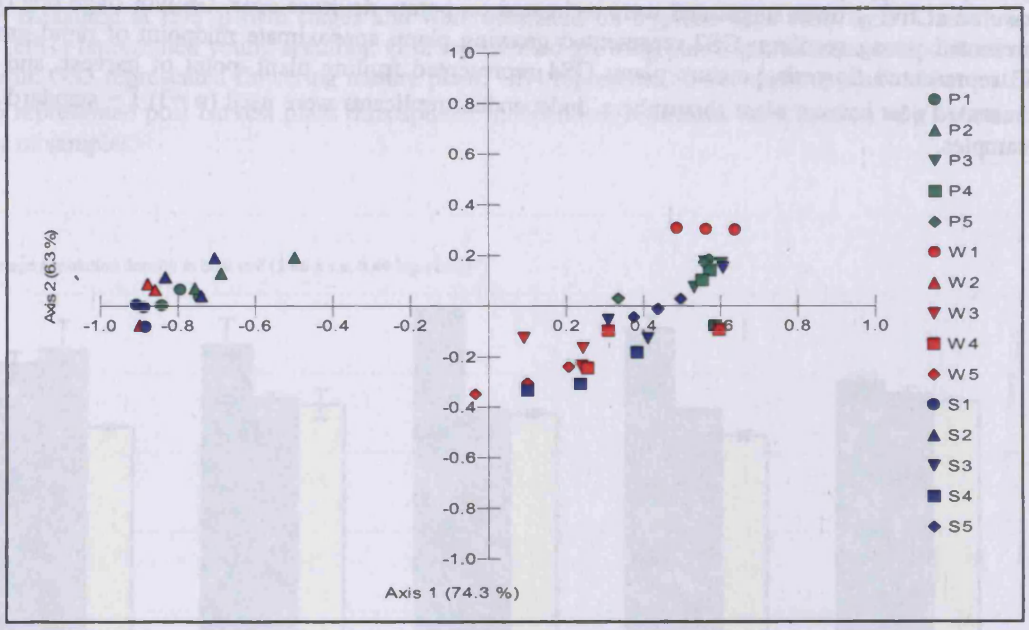


Figure 3.4. (a) Seasonal data for the community level physiological profiling of the rhizosphere of the three plant types using Biolog™ GN2 plates in comparison to bulk soil. **3.4 (b)** Seasonal data for the community level physiological profiling of the rhizosphere of the three plant types using BIOLOG™ GN2 plates Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for plant types are represented in the legend by P = pea, W = wheat, S = sugar beet (23:10). Growth stages are indicated by the numbers 1, 2, 3, 4, and 5 suffix to treatment in legend. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Soil was sampled at four time points T1-T4. Independent replicates were used (n = 3).

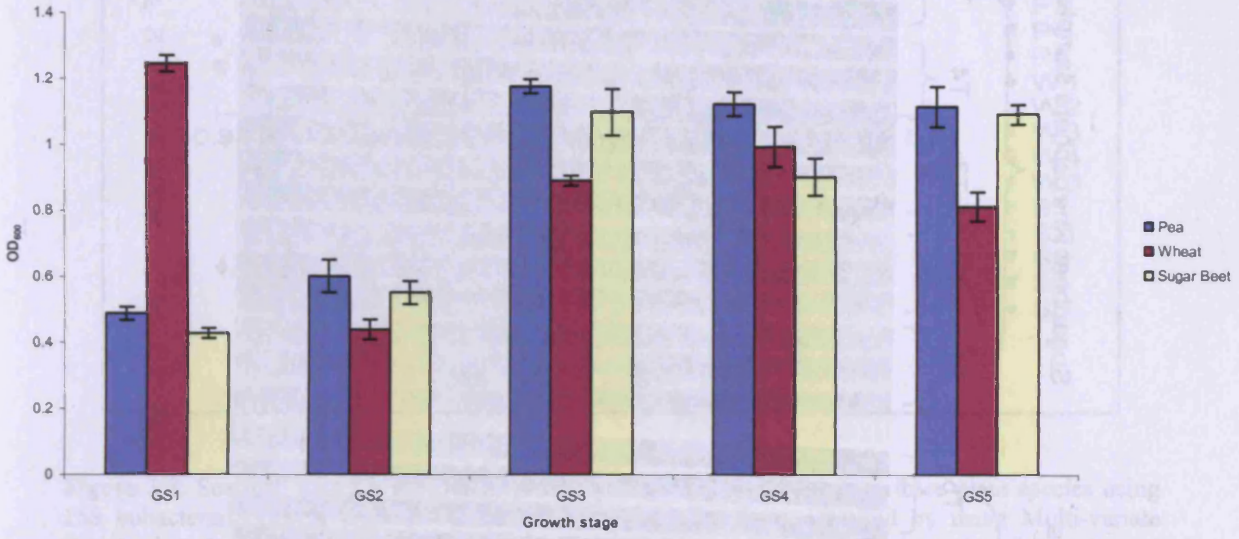


Figure 3.5. Seasonal data for average well colour difference from the rhizosphere of the three plant types using BIOLOG™ GN2 plates. Plate's optical densities measured for each well and an average value was calculated for each plate. This value was used for average well colour difference comparisons. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3).

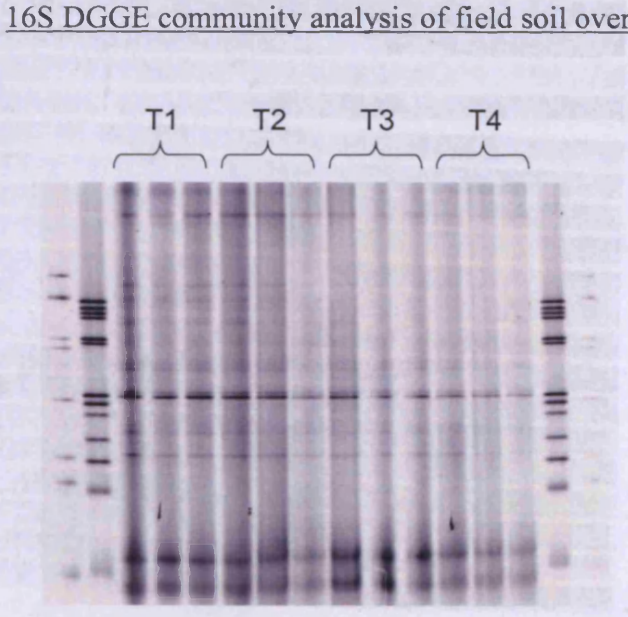


Figure 3.6. DGGE profiling of bulk soil using 16S eubacterial primers GC338F and 530R (44). Time points are indicated by the numbers T1, T2, T3, T4, and T5. Independent replicates were used (n = 3).

16S DGGE community analysis in the rhizosphere of three field crop plants.

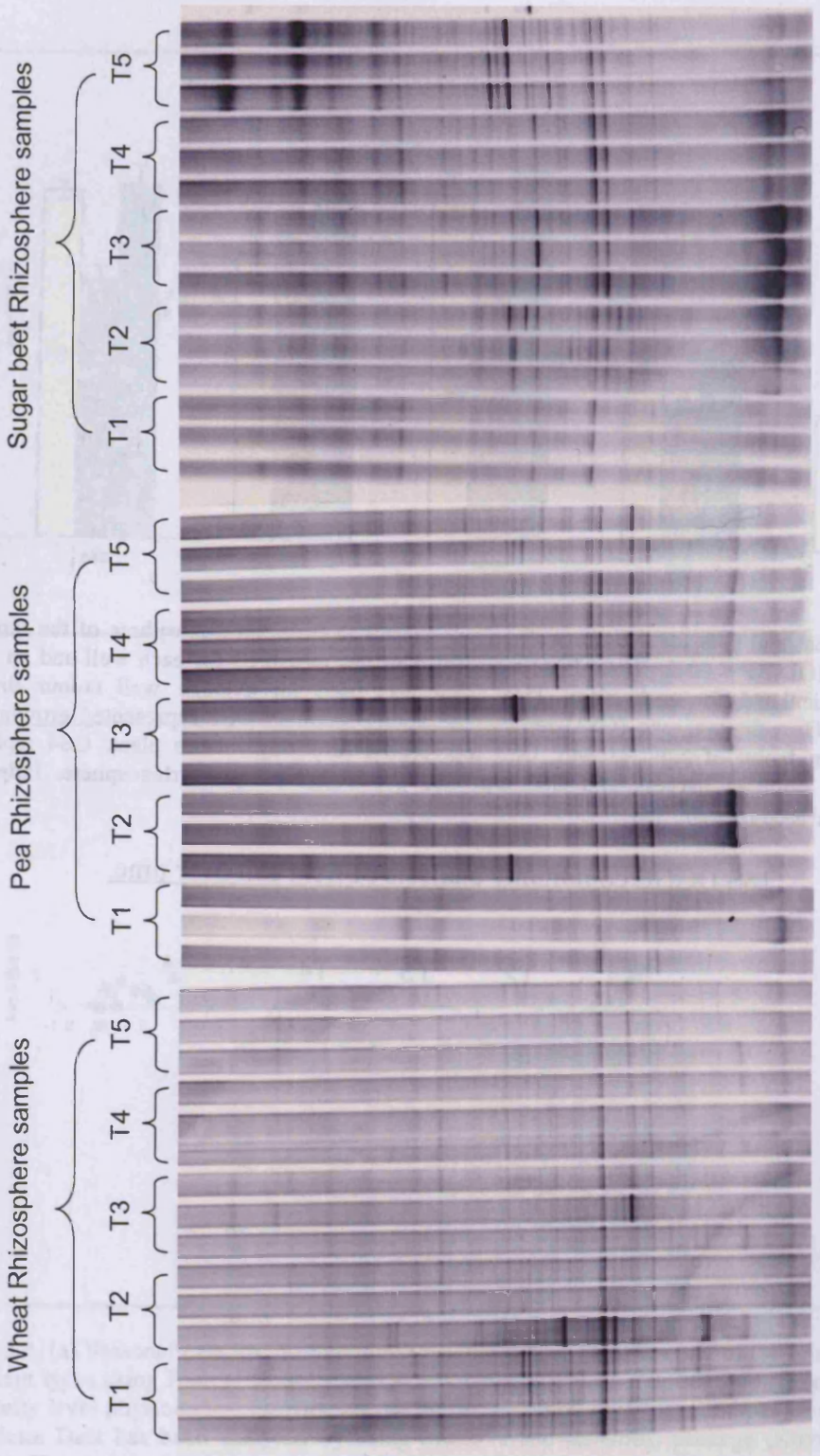


Figure 3.7. DGGE profiling of the rhizosphere of the three plant species using 16S eubacterial primers GC338F and 530R (44). Growth stages are indicated by the numbers 1, 2, 3, 4, and 5 suffix to treatment in legend. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3).

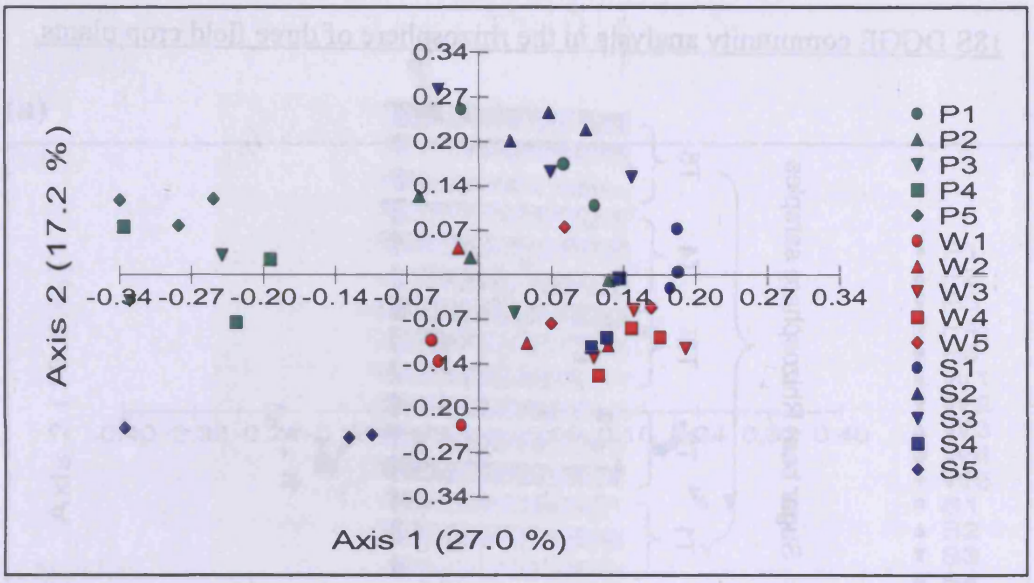


Figure 3.8. Seasonal data for the DGGE profiling of the rhizosphere of the three plant species using 16S eubacterial primers GC338F and 530R (44). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for plants species are represented in the legend by P = pea, W = wheat and S = sugar beet. Growth stages are indicated by the numbers 1, 2, 3, 4, and 5 suffix to treatment in legend. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3).

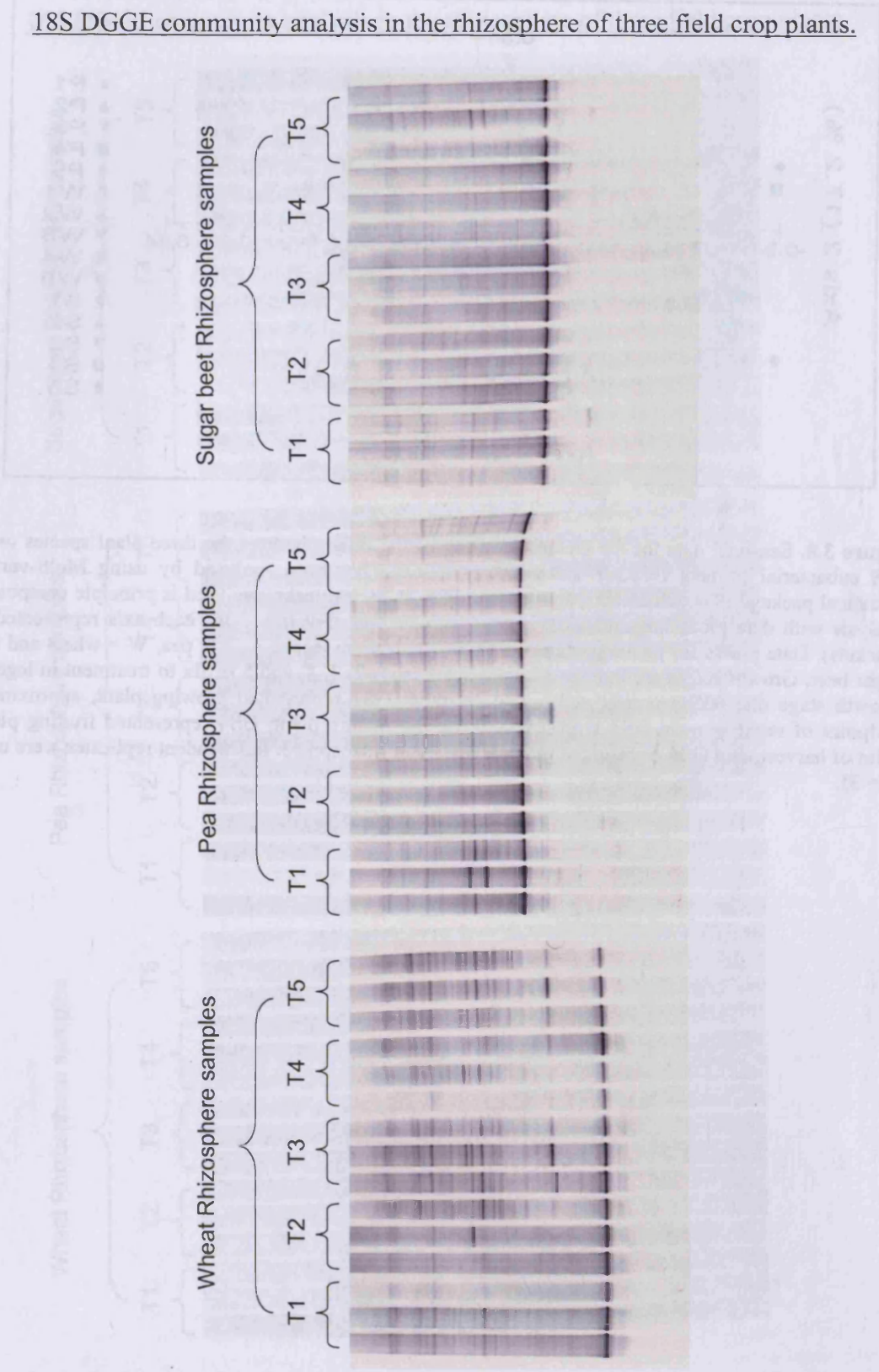
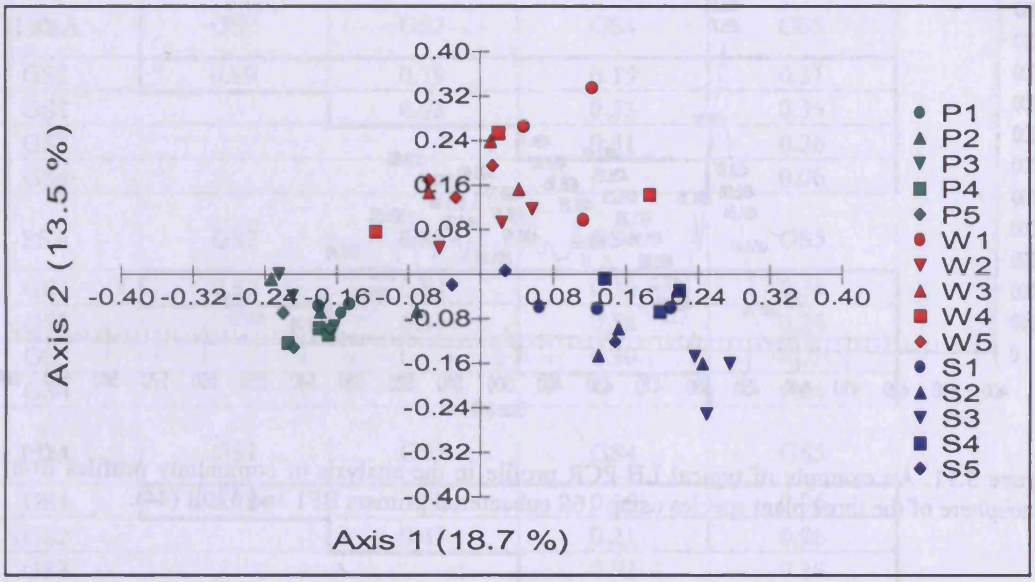


Figure 3.9. DGGE profiling of the rhizosphere of the three plant species using 18S primers NS1 and NS2-10GC (25). Dominant band in each plant species represents plant 18S. Growth stages are indicated by the numbers 1, 2, 3, 4, and 5. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3).

(a)



(b)

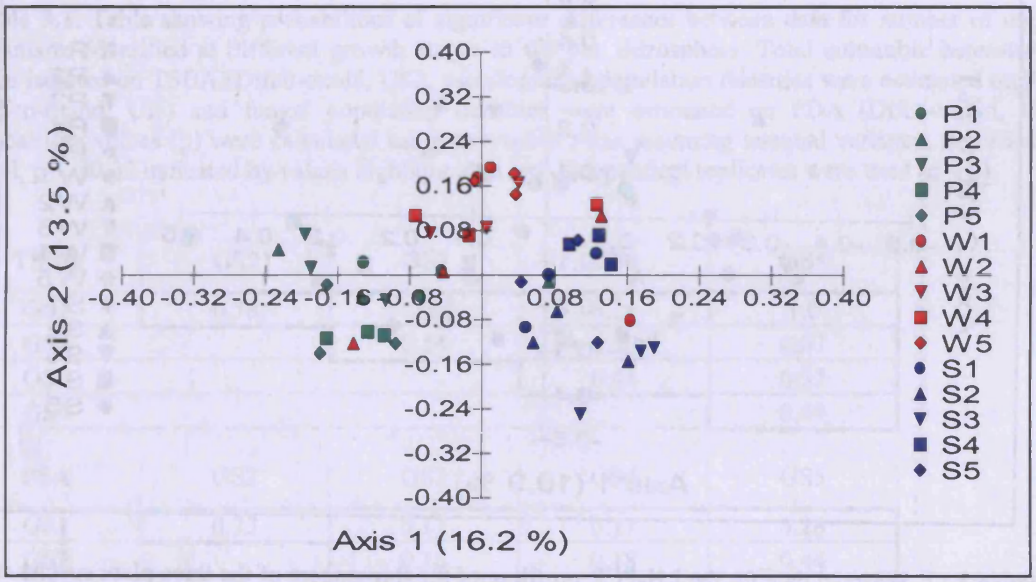


Figure 3.10. Seasonal data for the DGGE profiling of the rhizosphere of the three plant species using 18S primers NS1 and NS2-10GC (25). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for plants species are represented in the legend by P = pea, W = wheat and S = sugar beet. Growth stages are indicated by the numbers 1, 2, 3, 4, and 5 suffix to treatment in legend. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3). **Figure 3.9. (a)** Data analysed including all 18S bands on DGGE profiles, including host plant. **Figure 3.9. (b)** Data analysed excluding all known non-fungal 18S bands on DGGE profiles.

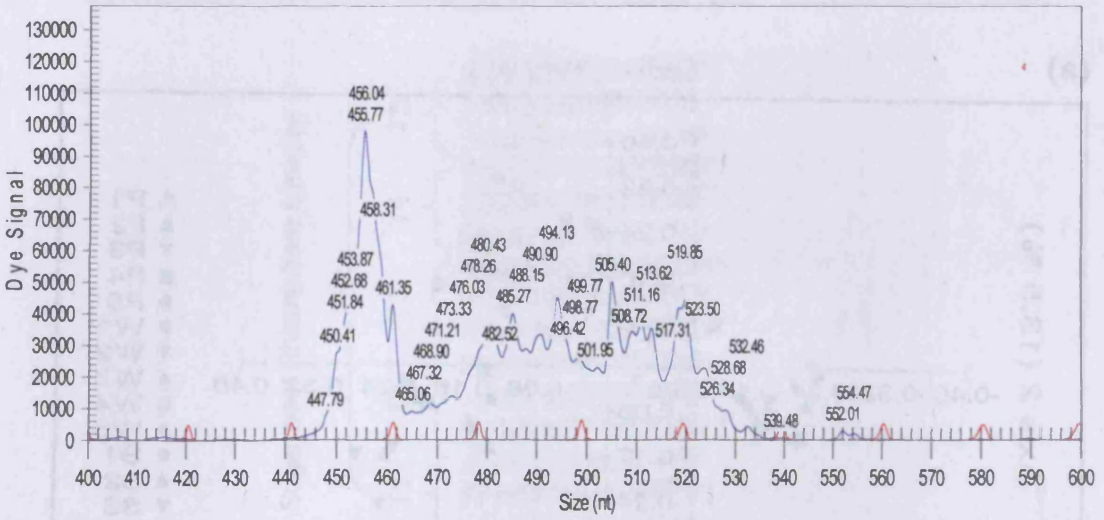


Figure 3.11. An example of typical LH-PCR profile in the analysis of community profiles from the rhizosphere of the three plant species using 16S eubacterial primers BF1 and 530R (44).

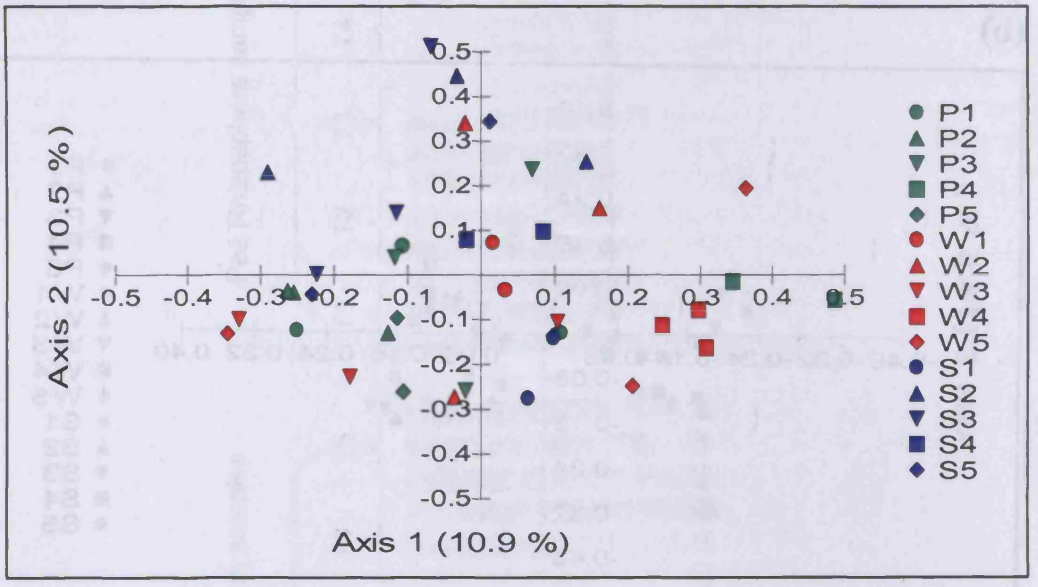


Figure 3.12. Seasonal data for the LH-PCR profiling of the rhizosphere of the three plant species using 16S eubacterial primers BF1 and 530R (44). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for plants species are represented in the legend by P = pea, W = wheat and S = sugar beet. Growth stages are indicated by the numbers 1, 2, 3, 4, and 5 suffix to treatment in legend. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; GS4 represented fruiting plant, point of harvest; and GS5 represented post harvest plant rhizosphere. Independent replicates were used (n = 3).

3.6 Tables

TSBA	GS2	GS3	GS4	GS5
GS1	0.89	0.79	0.15	0.31
GS2		0.93	0.33	0.35
GS3			0.31	0.26
GS4				0.06

PSA	GS2	GS3	GS4	GS5
GS1	0.57	0.04	0.10	0.34
GS2		0.31	0.58	0.34
GS3			0.30	<0.01
GS4				0.04

PDA	GS2	GS3	GS4	GS5
GS1	0.28	0.04	0.35	0.06
GS2		0.65	0.21	0.96
GS3			0.03	0.38
GS4				0.04

Table 3.1. Table showing probabilities of significant differences between data for number of micro-organisms identified at different growth stages in the pea rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) and fungal population densities were estimated on PDA (Difco-oxoid, UK). Probability values (p) were calculated using two-tailed *t*-test assuming unequal variance. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

TSBA	GS2	GS3	GS4	GS5
GS1	0.78	<0.05	0.11	1.0
GS2		0.14	0.20	0.87
GS3			0.44	0.37
GS4				0.49

PSA	GS2	GS3	GS4	GS5
GS1	0.23	0.17	0.17	0.28
GS2		0.16	0.18	0.44
GS3			0.43	0.09
GS4				0.10

PDA	GS2	GS3	GS4	GS5
GS1	0.07	0.71	0.52	0.77
GS2		0.07	0.11	0.40
GS3			0.40	0.63
GS4				0.96

Table 3.2. Table showing probabilities of significant differences between data for number of micro-organisms identified at different growth stages in the wheat rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) and fungal population densities were estimated on PDA (Difco-oxoid, UK). Probability values (p) were calculated using two-tailed *t*-test assuming unequal variance. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

TSBA	GS2	GS3	GS4	GS5
GS1	0.03	0.31	<0.01	0.76
GS2		<0.01	0.10	0.01
GS3			<0.01	0.48
GS4				<0.01

PSA	GS2	GS3	GS4	GS5
GS1	0.25	0.04	0.28	<0.01
GS2		0.62	0.17	0.79
GS3			0.02	0.06
GS4				<0.01

PDA	GS2	GS3	GS4	GS5
GS1	<0.01	0.04	<0.01	0.04
GS2		0.56	0.04	0.36
GS3			0.24	0.26
GS4				0.03

Table 3.3. Table showing probabilities of significant differences between data for number of micro-organisms identified at different growth stages in the sugar beet rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) and fungal population densities were estimated on PDA (Difco-oxoid, UK). Probability values (p) were calculated using two-tailed *t*-test assuming unequal variance. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Growth Stage	Media	Pea versus wheat	Pea versus sugar beet	Wheat versus sugar beet
GS1	TSBA	0.31	0.03	<0.01
GS2	TSBA	0.49	0.92	0.29
GS3	TSBA	0.12	0.02	<0.05
GS4	TSBA	<0.01	0.15	<0.01
GS5	TSBA	0.86	0.07	0.11
GS1	PSA	0.68	0.03	0.11
GS2	PSA	0.19	0.15	0.75
GS3	PSA	<0.01	<0.01	0.55
GS4	PSA	0.02	<0.01	0.02
GS5	PSA	0.22	0.05	0.33
GS1	PDA	<0.01	0.56	0.02
GS2	PDA	0.51	0.22	<0.01
GS3	PDA	0.84	0.15	0.12
GS4	PDA	<0.01	<0.01	0.01
GS5	PDA	0.95	0.21	0.34

Table 3.4. Table showing probabilities of significant differences between data for number of micro-organisms identified in comparison between the pea, wheat and sugar beet rhizosphere at individual growth stages. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) and fungal population densities were estimated on PDA (Difco-oxoid, UK). Probability values (p) were calculated using two-tailed *t*-test assuming unequal variance. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Pea	GS2	GS3	GS4	GS5
GS1	<0.01	<0.01	<0.01	<0.01
GS2		<0.01	<0.01	<0.01
GS3			0.01	0.01
GS4				0.77

Wheat	GS2	GS3	GS4	GS5
GS1	<0.01	<0.01	<0.01	<0.01
GS2		<0.01	<0.01	<0.01
GS3			<0.01	<0.01
GS4				<0.01

Sugar beet	GS2	GS3	GS4	GS5
GS1	<0.01	<0.01	<0.01	<0.01
GS2		<0.01	<0.01	<0.01
GS3			<0.01	0.76
GS4				<0.01

Table 3.5. Table showing probabilities of significant differences between BIOLOG™ data identified at different growth stages in the pea wheat and sugar beet rhizosphere. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Growth Stage	Pea versus wheat	Pea versus sugar beet	Wheat versus sugar beet
GS1	<0.01	<0.01	<0.01
GS2	<0.01	<0.01	<0.01
GS3	<0.01	<0.01	<0.01
GS4	<0.01	<0.01	<0.01
GS5	<0.01	0.36	<0.01

Table 3.6. Table showing probabilities of significant differences between BIOLOG™ data identified between the different plant species, pea wheat and sugar beet rhizosphere at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Pea	GS2	GS3	GS4	GS5
GS1	0.10	<0.01	<0.01	<0.01
GS2		<0.01	<0.01	<0.01
GS3			0.27	0.40
GS4				0.92

Wheat	GS2	GS3	GS4	GS5
GS1	<0.01	<0.01	0.02	<0.01
GS2		<0.01	<0.01	0.02
GS3			0.18	0.16
GS4				0.07

Sugar beet	GS2	GS3	GS4	GS5
GS1	0.03	<0.01	<0.01	<0.01
GS2		<0.01	<0.01	<0.01
GS3			0.10	0.93
GS4				0.04

Table 3.7. Table showing probabilities of significant differences between average well colour for each BIOLOG™ plate used for comparison between identified at different growth stages in the pea wheat and sugar beet rhizosphere. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Growth Stage	Pea versus wheat	Pea versus sugar beet	Wheat versus sugar beet
GS1	<0.01	0.07	<0.01
GS2	<0.05	0.45	0.08
GS3	<0.01	0.35	0.04
GS4	0.15	0.03	0.34
GS5	0.02	0.75	<0.01

Table 3.8. Table showing probabilities of significant differences between average well colour for each BIOLOG™ plate used for comparison between the different plant species, pea wheat and sugar beet rhizosphere at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

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Chapter Four: Strategies for impact assessment, evaluating the environmental impact of fungal disease suppressing GM bacteria on non-target species.

4.1. Introduction.

The potential of bacterial biological control agents for effective disease suppression has been widely discussed (50) and although some specific antagonistic isolates have been identified because of specialised secondary metabolite biosynthesis (15, 21, 23, 27, 32, 33, 49), for the majority of BCAs the mode of action is multifactorial in the disease control of crop plants. It has been widely accepted that for specific environments, the isolation of an indigenous bacterium with biological control potential would be competitively advantageous (1); in combination with the choice of the correct isolate for genetic modification our understanding would also be significantly enhanced by a more complete study of the population dynamics of the potential target, e.g. the plant rhizosphere.

4.1.1. Isolation of a biological control agent for environmental monitoring.

In 1990 experiments were begun to study the phenotypic and genetic diversity of fluorescent pseudomonads isolated form the phyllosphere of sugar beet (36, 42, 43). The purpose of this study was to improve the understanding of the structure and diversity of these bacterial communities over a complete growing season. In addition the identification of the variability of population densities was assessed. The potential isolation and selection of a suitable candidate for genetic modification and subsequent field release was also undertaken. This research concluded that much greater information was required on pseudomonad population structure in the natural environment.

Consequently as a result of this research a bacterium was identified in a collection of 502 isolates from the phytosphere of sugar beet. These were common, and some were identified in both the soil environment and detected in leaf samples throughout the study period (43). Of a number investigated in more detail, one Pseuodomonad demonstrated good phyllosphere and rhizosphere colonisation abilities in a number of plants and had the potential to be a suitable biological control agent. The bacterium identified was *Pseudomonas fluoresecens* SBW25 (originally classified as *P. aureofaciens*) (1, 35).

The knowledge of how a chosen inoculant strain survives, persists and colonises an environmental habitat is an essential requirement when considering a candidate as a biological control agent. This background information is important for future impact assessments, particularly if you are developing a genetically modified biological control agent (GM-BCA).

An assessment was undertaken by Bailey *et al.* (1) to insert marker genes in to the chromosome of *P. fluorescens* SBW25. This was undertaken to generate a bacterium suitable for environmental release to answer the following questions:

1) Assess the ability of the GMM when introduced to survive and disseminate in the environment.

2) Monitor gene transfer of novel genetic elements to related indigenous populations.

3) Determine the impact of the deliberate released recombinant microbial inoculum on the indigenous populations.

The genes used to mark the bacterium were decided upon by choosing phenotypes that were common from the prokaryotic kingdom but absent from this bacterium. Kanamycin resistance (Kan^r), β-galactosidase activity (*lac*ZY), and catechol cleavage (*xyl*E) were the three genes used. Two gene cassettes were created, one containing *lac*ZY and the other with the Kan^r and *xyl*E. They were inserted into chromosomal fragments of SBW25 contained in a mobilisation suicide integration vector and transferred in to SBW25. These fragments were then incorporated into the chromosomal DNA by homologous recombination. The two cassettes were inserted independently in locations approximately 1 M bp apart (15 % of genome). These locations were chosen as neutral areas of the chromosome and for the ease of identification of transfer or loss of one or other of the cassettes.

In vitro competition experiments identified that there were no significant differences in fitness of the WT and GM inoculum and under continual sub culture and there was no detectable loss or transfer of genes from one bacterium to another. When experiments were undertaken in the plant rhizosphere, persistence of the GM was identified. The WT and GM bacteria were also shown to competitively exclude the enterobacteriaceae. The WT bacteria was shown to have a greater impact on the community diversity than the GM indicating that the modification had made the bacteria less competitive than the WT. This impact has been demonstrated but the effect is transient and with no long term affects (44).

P. fluorescens SBW25 has been shown to survive well in the rhizosphere and is an effective biocontrol agent against a number of plant pathogens including *Pythium spp.* (14). Analysis of SBW25 by transposon mutagenesis identified several loci involved with its biocontrol ability, however no single common locus was identified (12). SBW25 does not produce any of the typical identified antifungal metabolites including phenazine, pyoluteorin, hydrogen cyanide, or 2,4-diacetylphloroglucinol (14). SBW25 was chosen as a suitable bacterium for genetic modification because of the absence of any known secondary metabolites with anti fungal properties and because of its good plant colonisation abilities (45). The genetic modification undertaken was the introduction of a phenazine biosynthesis locus; an important antifungal gene cluster often found in pseudomonads (14, 26, 45).

4.1.2. Phenazine biosynthesis.

The phenazine biosynthesis pathway has been well characterised, expression of the pathway results in the production of the secondary metabolite PCA whose with antibiotic characteristics are effective against a number of plant pathogens. Phenazines are low molecular weight heterocyclic nitrogen containing pigments (27). They were first identified as crystalline precipitates in media of cultured pseudomonas and since their identification over fifty naturally occurring phenazines are now known (47). Phenazines are exclusively produced by a limited number of bacteria, which apart from pseudomonas include *Burkholderia, Brevibacterium,* and *Streptomyces* (27).

The majority of known phenazines demonstrate broad spectrum antagonistic activity against various species of bacteria, fungi, some higher plants and animals (47). This is a result of the transformation of phenazine compounds by oxidation-reduction reactions, to toxic superoxide radicals in the target cells harmful to the organism (27). The adverse affect on plant metabolism that some phenazines cause has been hypothesised to induce the plants own defence mechanism resulting in an increase in the host's resistance to disease (34).

The importance of phenazine-1-carboxylic acid (PCA) production in the control of take-all disease of wheat caused by *Gaemannomyces graminis* var. *tritici* has been demonstrated. The spontaneous accumulation of microflora antagonistic to take-all can occur after several years of crop monoculture, a phenomena known as take-all decline (TAD). Bacteria with the ability to produce phenazine-1-carboxylic acid and control take-all disease of wheat have been identified, they include *Pseudomonas fluorescens* 2-79 (28) and *Pseudomonas aureofaciens* 30-84 (34). *P. fluorescens* 2-79 suppresses *G. graminis* and other fungal phytopathogens *in vitro* with concentrations of phenazine-1-carboxylic acid at less than 1 $\mu g\ ml^{-1}$ (28). The detection of phenazine-1-carboxylic acid by HPLC has been identified in the rhizosphere of crop plants with success (5). Estimates have been made that 55-80 mg of root associated PCA would significantly suppress disease in seedling for a hectare of land (28).

Investigations with *Pseudomonad fluorescens* 2-79, a well characterised PCA producing strain showed a strong correlation between the population size of 2-79 in the rhizosphere and suppression of root lesions caused by take-all in early pathogenesis (4). By the use of Phz^+ and Phz^- mutants in biocontrol assays, phenazine biosynthesis was confirmed to be the trait responsible for this control (27). The Phz^+ mutant persisted in the rhizosphere environment in significantly higher levels over 5 consecutive harvest cycles compared to the Phz^- mutants, this effect is of little importance during rapid growth of the Phz^+ and Phz^- mutants. Differences in the composition and substrate availability may result in the increased survival of the Phz^- mutant in the presence of *G. graminis* infections (27).

4.1.2.1. Enzymatic pathway for phenazine biosynthesis.

Phenazine biosynthesis has been linked to the shikimic acid synthesis pathway, responsible for the biosynthesis of aromatic amino acids. The structure of shikimic acid is a suitable precursor to phenazine biosynthesis. This was investigated using ^{14}C-shikimic acid added to *P. aureofaciens*, of which 16 % was recovered in the form of phenazine (47). Phenazine 1,6-dicarboxylic acid is thought to be the first phenazine produced from which the rest are derived however the precursors required for this and the mode of synthesis has not yet been confirmed.

A greater understanding of phenazine biosynthesis came with the identification of the *phz*ABCDEFG operon allowing further studies of the biosynthetic pathway. The gene cluster responsible for phenazine biosynthesis was first described by Pierson *et al.* (34) in *P. aureofaciens* 30-84 and by Mavrodi *et al.* (26) in *P. fluorescens* 2-79. Gene expression studies were undertaken using cosmid libraries, mutation analysis and promoterless gene fusion studies (26, 34). It was determined that the phenazine biosynthesis was the result of the seven gene locus *phz*ABCDEFG under the control of *phz*I and *phz*R in *P. fluorescens* 2-79 (26). The identification of the potential gene function was carried out using the blast database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The genes *phz*A and *phz*B have no homologs except to *phz*X and *phz*Y in *P. auerofaciens* 30-84. The gene *phz*C is homologous to *agr*G 3-deocy-d-arabino-heptulosonate-7-phosphate (DAHP) synthase the primary enzyme in the shikimic acid pathway. The *E.coli* gene *ent*B involved in iron chelating and transport shares homologies to *phz*D. The gene *phz*E has similarities to a large group of enzymes including the anthranilate synthase. The gene *phz*F has no known functional homologs, just to hypothetical proteins and *phz*G is homologous to *pdx*H involved in vitamin B_6 production. Mutation studies on the importance of these genes could not identify any mutants in *phz* A, B, or F. Mutations in any of the other genes resulted in termination of phenazine synthesis except in *phz*G where expression was reduced to 1.3% of the expected level. From this information a potential biosynthesis pathway was generated, see Figure 4.1.

The identification of the gene involved in phenazine production and properties of biocontrol allowed its identification as a potential operon to use in the improvement of BCAs.

Phenazine-1-carboxylic acid biosynthesis pathway was the chosen as a suitable antifungal biosynthesis operon for insertion into the SBW25 genome. The genetic modification of this bacterium to contain the *phz*ABCDEFG operon was undertaken with expression of the gene under the control of the constitutive P*tac* promoter. This biosynthesis pathway was inserted using a disarmed mini-Tn*5* vector, containing a kanamycin resistance gene, to introduce a single copy in the bacterial chromosome. The resulting variant *P. fluorescens* 23.10 was chosen after careful fitness and BCA ability checks and was shown to have improved biological control ability when compared to the wild type SBW25 (45). The inoculum persists well in the phytosphere of several crop plants and can suppress *Pythium* infestation up to 100 times normal levels (46).

4.1.3. Aims.

The aim of this research is to monitor the population dynamics and community function of the rhizosphere of three crop species over a growing season in mesocosm experiments using normal (non-sterile) field soil. These populations were monitored for their responses to inoculation of the wild type SBW25, and the GM-BCA 23.10 (Phz+). Complimentary techniques were undertaken tried to provide a robust evaluation of impact on the indigenous communities. This research monitored bacterial and fungal population responses by culturable techniques, and culture independent molecular based approaches. The techniques used are described in detail in chapter two and applied in the same way described in Chapter 3, with the omission of LH-PCR. The hypothesis to be tested are:

1) The impact on indigenous bacterial and fungal communities after the inoculation of the WT and GM-BCA bacteria will be small.

2) The impact on the indigenous bacterial and fungal communities as a result of the inoculation of the WT and GM-BCA will be transient.

3) Any impact observed on the indigenous fungal communities will be greater after inoculation of the microbial GM-BCA than that of the WT bacterium.

4) The impact on subsections of the indigenous bacterial communities as a result of the inocula will be most identifiable in the pseudomonad populations.

5) Plant species and growth stage effects will dominate any impact that the inocula have.

4.2. Materials and Methods.

4.2.1. Experimental mesocosm design.

Mesocosm experiments were undertaken using field soil from the University of Oxford field station, Wytham. The seeds used were untreated and commercially available; *Psium satvium* var. *quincy* (pea), *Triticum aestivum* var. *pena wawa* (wheat), *Beta vulgaris* var. *amythyst* (sugar beet). Pots of 200 mm diameter were used containing 2 kg of soil. Each was planted with approximately 50 seeds per pot equally spaced. After planting, 100 g of sterile general purpose compost was spread over the top of each pot to aid moisture retention during germination. Pots were inoculated by a soil drench with one of the each of the following treatments; water control, WT bacterium SBW25, and GM bacterium 23.10. This was carried out for each of the three plant species with each treatment in triplicate. Bacteria were inoculated to achieve a final concentration of 7.00 $\log_{10}$ cfu g^{-1} soil. Mesocosms experiments were maintained at a constant temperature in a growth room at 22 °C with a light/dark cycle of 16 h / 8 h respectively.

4.2.2. Sampling.

For each plant species, three plants were sampled from each of the three pots as independent replicates for rhizosphere material. Rhizosphere samples were taken for each plant species at three or four time points, depending on plant type, to represent the different growth stages of the plant. Growth Stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering/maturing plant; GS4 represented mature plant. For each plant species, the days after sowing that correspond to GS1-GS4 samples are as follows: Pea, days 10, 41, and 110 (only GS1-GS3 taken); wheat, days 5, 48, 100 and 117; and sugar beet, days 35, 96, 133, and 167.

For each plant sampled, the roots were removed from the pot intact with adhering soil. Loosely adhering soil was removed and the root with rhizosphere material was

collected and put into sterile 50 ml centrifuge tubes weighed and processed as described in section 2.2.7.

4.2.3. Culturable population counts.

Populations were estimated using 1:10 serial dilutions of the soil/PBS mixture from the root rhizosphere (section 4.2.2.). One hundred microlitres of dilutions were plated out onto four media types. Tryptone Soya Broth Agar (TSBA) (Difco, UK) to enumerate total heterotrophs, with the addition of cyclohexamide (0.1 mg ml^{-1}) for the suppression of eukaryote growth. Pseudomonad population densities were estimated on Pseudomonad Selective Agar (PSA) (Difco, UK) supplemented with cyclohexamide (0.1 mg ml^{-1}), centrimide (10 μg ml^{-1}), fucidin (10 μg ml^{-1}), and cephalosporin (50 μg ml^{-1}). Fungal population densities were estimated on Potato Dextrose Agar (PDA) (Difco, UK) with the addition of aeuromycin™ (Cyanamid, UK) (320 μg ml^{-1}) for suppression of bacterial growth. For identification of the inoculum population densities, PSA was used as described above, supplemented with rifampicin (100 μg ml^{-1}) and if selecting for 23.10, rifampicin (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) was added . Plates were incubated at 28 °C for 2 days.

4.2.4. Extraction of total nucleic acid from environmental samples.

DNA extractions were carried out from soil pellets (1 ml soil/PBS mixture spun down when sampling (section 4.2.2.)). The protocol used was BBCTAB (19) with the modification of an additional freeze/thaw lysis step after resuspension in the BBCTAB buffer. Extracted DNA samples were resuspended in 100 μl MG-H_2O and stored at -20 °C for PCR amplification.

4.2.5. Community level physiological profiling.

The 50 ml centrifuge tube containing soil/PBS mixture (section 4.2.2.) were centrifuged at 3000 x g for 5 min before 1 ml of supernatant was diluted into 20 ml 1x PBS. One hundred μl of this bacterial suspension was added to each well of a BIOLOG™ GN2 plate and was incubated at 15°C for 7 days. This translates as an

inoculation rate of approximately 6.00 log_{10} bacteria per well. Optical densities of each well were measured on a Rosys Anthos Lucy I plate reader (Switzerland) at a wavelength of 600 nm at the 7 day point.

4.2.6. Denaturing gradient gel electrophoresis (DGGE).

4.2.6.1.General primers.

16S and 18S community analysis was undertaken as optimised in Chapter 2.

4.2.6.2. Group specific primers.

PCR amplification of the α-proteobacteria was undertaken using the primers F203α and R1494, see table below (18). PCR conditions were as follows: 5 min at 95°C for one cycle, 60 s at 95 °C, 60 s at 56 °C, and 60 s at 72 °C for 30 cycles, and 30 min at 72 °C. The reaction mix final concentrations in a 50 µl volume were 1 pM of each primer, 0.2 µM dNTPs, 1U Sigma taq and buffer at 1 x concentration. The reaction mix was run on a MJ research, Peltier thermal cycler. PCR amplification of the Pseudomonad proteobacteria was undertaken using the primers Ps-For and Ps-Rev, see table below (53). The same reaction conditions were used apart from an annealing temperature of 65 °C. A second round PCR reaction was carried out to make amplified products suitable for comparative DGGE analysis. 1µl of previous PCR reaction was used as template for the second round 16S DGGE using primers as described in section 2.2.4.2.

Group	Primers	Sequence	Annealing temperature	Reference
General 16S / Second round of group specific	GC338F	5'-cgcccgccgcgcccccgcccccggcccgccgcccccgc ccactcctacgggaggcagc	60 °C	(52)
	530R	5'-gtattaccgcggctgctg		(29)
16S α-proteobacteria	F203α	5'-ccgcatacgccctacgggggaaagatttat	56 °C	(18)
	R1494	5'-ctacgg(c/t)taccttgttacgac		(18)
16S Pseudomonad	Ps-For	5'-ggtctgagaggatgatcagt	65 °C	(53).
	Ps-Rev	5'-ttagctccacctcgcggc		(53).
18S Fungal	NS1	5'-ccagtagtcatatgcttgtc	55 °C	(22)
	NS2-10GC	5'-cgcccgccgcgcgcccccgccccggcccgccgcccc cgcccgaattaccgcggctggtggc		(22)

4.2.7. Clone libraries.

After producing DGGE profiles with group specific primers, their specificity was confirmed by the generation of small cloned libraries from community amplified PCR products. PCR products were purified by gel extraction using the QIAquick Gel extraction kit (28704). These were ligated into pCR4-TOPO and transformed into recipient *E.coli* hosts using the Invitrogen TOPO TA Cloning® Kit for sequencing (25-0276) as per manufacturer's protocol.

4.2.8. Sequencing.

Plasmids with chromosomal DNA fragments cloned into them, or PCR products were sequenced using the Beckman-Coulter CEQ2000XL and the CEQ™ DTCS quick start kit (608120) as per manufactures protocol, or by the DNA sequencing facility, Dept of Biochemistry, University of Oxford.

4.2.9. Statistical analysis.

Statistical analysis was undertaken as in section 2.2.11.

4.3. Results.

4.3.1. Enumeration of populations in the plant rhizosphere.

4.3.1.1 Pea rhizosphere culturable population densities.

Fate and persistence of inocula on pea seedlings.

The survival of the inocula is represented in Figure 4.2 and at first glance the data indicates that the inocula population densities decline with subsequent growth stages in the rhizosphere of peas growing in mesocosm containing field soil. Initially the bacteria SBW25 and 23.10 were individually added to mesocosms to achieve an inoculation level of 10^7 cfu g^{-1} soil. At the young seedling stage (GS1) of pea (10 days after planting) the inoculum density in the pea rhizosphere was 9.03 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 9.22 $\log_{10}$ cfu g^{-1} wet weight rhizosphere for the WT and GM bacteria respectively. This shows the initial colonisation of these inocula is very similar. At each successive growth stage there was a progressive reduction in numbers of inocula isolated. At GS2 (41 days) population densities were 6.05 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 6.73 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (WT and GM inoculations respectively) which were significantly lower than at GS1, see Table 4.1. At GS3 (110 days) population densities had reduced to 4.33 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 4.87 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (WT and GM inoculations respectively). This was significantly lower than GS1 and GS2 for the GM treatments. Whereas no significant difference between GS2 and GS3 for WT treated populations were identified. When comparing bacterial inoculum survival at each growth stage there was no significant difference in the population densities of the WT inoculant strain levels compared to the GM inoculant strain levels at each growth stage. No inocula were detected in the water treatments as expected.

Total culturable heterotroph population densities on pea seedlings.

Total culturable heterotrophs were enumerated in the three treatments described and compared through the three growth stages of the pea as indicated, represented by

Figure 4.3. As expected, the addition of inocula, and with plant growth, higher total population densities in the WT and GM treated mesocosms were seen than the water control at the later growth stages. In the control pots, which were treated with water alone, the average population densities of total culturable heterotrophs were 6.59 x 10^8 cfu g^{-1} wet weight rhizosphere. Although these densities were significantly different at GS3 when compared to GS1 and GS2, see Table 4.1. Mesocosms inoculated with WT bacteria demonstrated population densities between 9.72 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.82 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). GS1 heterotroph population densities were significantly higher than GS2 (8.79 $\log_{10}$ cfu g^{-1} wet weight rhizosphere). There were no significant differences between GS3 and GS1 or GS2. The GM bacterial treatment heterotroph population densities varied between 9.46 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.87 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). There were no significant differences seen between populations at all growth stages. When comparing treatments within growth stages a significant difference was only observed at GS2. The WT and GM treatment had significantly higher population densities of heterotrophs in comparison to the water control, see Table 4.2.

Pseudomonad population densities in the pea rhizosphere.

Pseudomonad population densities in the pea rhizosphere are represented in Figure 4.4. The general appearance of the graph indicates a decline in population densities for all treatments with successive growth stages, slightly higher densities were observed in the inoculated mesocosms. Total pseudomonad populations for the water control pots showed a gradual decline in population density from 9.72 $\log_{10}$ cfu g^{-1} wet weight rhizosphere to 5.95 $\log_{10}$ cfu g^{-1} wet weight rhizosphere from GS1 to GS3 (over a 100 days). The decline between each growth stage was to a significant level, see Table 4.1. There was a reduction in the WT treated mesocosm pseudomonad populations from 9.53 $\log_{10}$ cfu g^{-1} wet weight rhizosphere to 7.15 $\log_{10}$ cfu g^{-1} wet weight rhizosphere. The population density in young seedlings (GS1) was significantly higher in comparison to GS2 (7.82 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS3. There were no significant differences between GS2 and GS3. The GM treatment pseudomonad population densities varied between 9.20 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 7.07 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3).

Population densities at GS1 were significantly higher than in GS2 (7.74 $\log_{10}$ cfu g^{-1} wet weight rhizosphere). There were no significant differences between GS2 and GS3 (Table 4.1.). The comparison within growth stages between treatments identified no significant differences (Table 4.2.). The proportion the inoculum represent in the total pseudomonad populations is represented by a * on Figure 4.4. This data indicates the pseudomonad populations recovered at GS1 in the treatments was almost entirely made up of the inoculum with out any significant shifts in pseudomonad population density in comparison to the water control. The proportion of the pseudomonad population represented by the inoculum reduced with each subsequent growth stage with no differences between the WT and GM.

Fungal population densities on pea seedlings.

The fungal population densities in the pea rhizosphere are shown in Figure 4.5. The data shows no real trends in population densities as a result of treatments or growth stages. Fungal population densities for the water control varied between 5.69 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3) and 6.36 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS2). There were no significant differences between the populations of fungi enumerated across the three growth stages studied, although a significant difference was identified between GS1 and GS3, see Table 4.1. For the WT bacterial treatment the population densities enumerated were between 5.57 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3) and 6.49 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS2). There was no significance between GS1 (6.36 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) and GS2, however both were significantly different to GS3. Population densities for the GM treatment varied between 5.71 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3) and 6.93 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS1). GS1 and GS2 (6.83 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) were not significantly different to each other but were significantly different to GS3. The comparison between treatments within growth stages identified a significant difference in young seedling (GS1). The water control and WT treatment were not significantly different to each other but were significantly different to the GM inoculation, see Figure 4.5. and Table 4.2.

4.3.1.2. Wheat rhizosphere culturable population densities.

Fate and persistence of inocula on wheat seedlings.

The survival of the inocula in the wheat seedlings is represented in Figure 4.6. The data shows similar survival and persistence of both the WT and GM inoculum with a gradual decline in numbers with successive growth stages. The bacterial inocula SBW25 and 23.10 were individually added to mesocosms to achieve an inoculation level of 7.00 $\log_{10}$ cfu g^{-1} soil. In young seedlings (GS1, 5 days after planting) inoculum density in the rhizosphere was 7.97 $\log_{10}$ cfu g^{-1} wet weight rhizosphere and 7.21 $\log_{10}$ cfu g^{-1} wet weight rhizosphere, for the WT and GM bacteria respectively. With each successive growth stage there was a reduction in inocula densities. At GS2 population densities were 6.69 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (WT) and 7.09 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GM) with a significant reduction in WT population densities but not in the GM from GS1, see Table 4.3. At GS3 population densities for the WT and GM inoculum had reduced to 5.46 and 5.43 $\log_{10}$ cfu g^{-1} wet weight rhizosphere respectively. This reduction was significant in both the WT and GM treatment. Population densities at GS4 had reduced to 5.16 and 4.95 $\log_{10}$ cfu g^{-1} wet weight rhizosphere for WT and GM respectively. The GM treatment had no significant difference to the previous growth stage unlike the WT treatment that had reduced further. No inocula were detected in the water control mesocosms as expected. Within growth stages, variance between treatments was undertaken for inocula density in the wheat rhizosphere. There were no significant differences in the population densities in the WT compared to the GM inoculum at each growth stage, see Table 4.4.

Total heterotroph population densities on wheat seedlings.

Total heterotroph population densities in wheat rhizospheres are represented in Figure 4.7. The general appearance of the data gives an indication of stable population densities with no influence of growth stage or treatments. Total bacterial population densities varied between 7.53 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.69 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4) for the water control over the growing

season. Bacterial densities at GS1 and GS2 (7.91 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) were not significantly different. However, GS1 was significantly different to GS3 (8.08 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS4. GS2, GS3 and GS4 were not significantly different to one another, see Table 4.3. For the WT treated mesocosms population densities varied between 8.19 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.53 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4), there were no significant differences between all growth stages. The GM treatment demonstrated population densities that varied between 7.62 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.41 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS2). The only significant difference identified was between GS2 and GS3 (7.99 $\log_{10}$ cfu g^{-1} wet weight rhizosphere). The comparison within growth stages between treatments identified no significant differences (Table 4.4.).

Pseudomonad population densities in the wheat rhizosphere.

First impressions of pseudomonad population densities show stable communities in the two treatments and between growth stages, see Figure 4.8. Pseudomonad population densities for the water control varied between 7.08 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1 and GS4) and 7.48 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). There were no significant differences between growth stages, see Table 4.3. Population densities for the WT bacterial inoculation varied between 7.17 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS2) and 8.06 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1), no significant variation between growth stages was detected. Population densities in the GM bacterial treatment varied between 7.57 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 7.98 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4). GS4 was demonstrated to be significantly different to GS1 and GS3. The comparison within growth stages between treatments identified a significant difference at GS1 between water control and WT treatment, see Table 4.4 and Figure 4.8. The proportion of the pseudomonads populations that are represented by the inoculum is indicated by a * in Figure 4.8. This indicates the pseudomonad population at GS1 in the treatments was almost entirely replaced by the inoculum. This was shown as a significant increase in the WT population density compared to the water control. The proportion of pseudomonads represented by the inocula decreased over time.

Fungal population densities on wheat seedlings.

Fungal population densities are represented in Figure 4.9. The bar chart shows that fungal populations remain stable regardless of treatment or plant growth stage. Detailed analysis identified that fungal population densities in the water control mesocosms varied between 5.10 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS1) and 6.16 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS2). There were no significant differences between growth stages, see Table 4.3. Population densities in the WT treatment varied between 5.30 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS1) and 6.44 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4). GS1 was significantly lower than GS2 (5.96 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) and GS4. No other significant differences in population density were identified. The GM treatment population densities varied between 5.42 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS1) and 6.37 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS2). The population density at GS1 was significantly lower than at GS2 and GS4 (6.26 $\log_{10}$ pfu g^{-1} wet weight rhizosphere). The comparison between treatments within growth stages identified no significant differences, see Table 4.4.

4.3.1.3. Sugar beet rhizosphere culturable population densities.

Fate and persistence of inocula on sugar beet seedlings.

Inocula survival in the sugar beet rhizosphere is represented in Figure 4.10. Initial trends indicate a steady decline with growth stages for both WT and GM inocula, except at GS4 for WT in which there appears to be a subsequent increase. The bacterial inocula SBW25 and 23.10 were individually added to mesocosms to achieve an inoculation level of 10^7 cfu g^{-1} soil. The WT inoculum population densities varied between 2.35 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) and 6.97 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1). There was a significant decline in population numbers in successive growth stages from GS1 to GS3. GS4 was not significantly different to GS1 and GS2 although it was significantly higher than GS3 (Table 4.5.). In the GM treatment there was a decline in population density of the inoculum from 6.79 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) to 0.57 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4). Each subsequent growth stage had a significant reduction in population densities apart from GS2 and GS3, see Table 4.5. The comparison between treatments

within growth stages identified a significant treatment effect at GS4 between populations of the WT and GM inoculum, see Table 4.6. No inocula were detectable in the water control as expected.

Total heterotroph population densities on sugar beet seedlings.

Heterotroph populations densities are represented in Figure 4.11. The data indicates no distinct effects of treatments or growth stages. In detail the water control mesocosm population densities varied between 7.79 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS2) and 8.80 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). GS1 (7.80 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) was not significantly different to GS2 with both significantly different to GS3 (8.80 $\log_{10}$ cfu g^{-1} wet weight rhizosphere), see Table 4.5. There was no significance difference in GS4 to all other growth stages. Total heterotroph densities on the WT treated sugar beet varied between 7.70 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1) and 8.78 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). There were no significant variations in the total population densities between all growth stages. The GM treatment population densities varied between 7.57 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS4) and 8.83 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3). The only significant difference identified in population densities was between GS2 and GS3. The comparison between treatments within growth stages identified a significant treatment effect at GS2 between population densities in the water control and the GM inoculum treatment (Table 4.6.).

Pseudomonad population densities in the sugar beet rhizosphere.

Pseudomonad population densities in the sugar beet rhizosphere are represented in Figure 4.12. At first glance the data indicates the populations are less stable than total heterotrophs but do not demonstrate any identifiable trends. The pseudomonad population densities of the water control varied between 6.33 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) and 7.16 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1). GS1 population densities were significantly higher than those at GS2 (6.36 $\log_{10}$ cfu g^{-1} wet weight rhizosphere) and GS3. There were no other detectable significant differences in population densities for all other growth stages. For the WT treatment mesocosms, population densities varied between 6.04 $\log_{10}$ cfu g^{-1} wet weight

rhizosphere (GS2) and 7.39 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1). There were no significant differences detectable in pseudomonad population densities for all growth stages. For the GM treatment, population densities varied between 5.49 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS3) and 7.44 $\log_{10}$ cfu g^{-1} wet weight rhizosphere (GS1). GS1 and GS3 were the only samples that were significantly different to each other, see Table 4.5. The comparison within growth stages between treatments identified a significant treatment effect at GS3 between populations in the water control and the GM inoculum treatment, see Table 4.6. The proportion of the pseudomonads population that are represented by the inocula is indicated by a * in Figure 4.12. This indicates the recovered pseudomonad population is almost entirely that of the inocula in GS1. In subsequent growth stages the proportion of the inocula declines except in GS4 for the WT treatment where it increases again. This did not affect total population densities.

Fungal population densities on sugar beet seedlings.

At first glance there appears to be some variation in fungal densities in the sugar beet rhizosphere. There are however no distinct trends, although at GS4 populations are slightly lower than in the other three stages sampled, see Figure 4.13. Fungal population densities for the water control vary between 5.36 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4) and 6.20 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS1). GS1, GS2 (6.05 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) and GS3 (6.05 $\log_{10}$ pfu g^{-1} wet weight rhizosphere) were not significantly different to each other in their population densities, but they have significantly larger population densities than GS4. The WT treatment population densities vary between 5.28 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4) and 6.32 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS3). There were no significant differences between growth stages. The GM treatment population densities varied between 5.10 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS4) and 6.22 $\log_{10}$ pfu g^{-1} wet weight rhizosphere (GS2). There were also no significant differences between growth stages, see Table 4.5. The comparison between treatments within growth stages identified a significant treatment effect at GS2 between populations in the water control and the WT inoculum treatment, see Table 4.6.

4.3.2. Community level physiological profiling (CLPP).

4.3.2.1. Pea rhizosphere CLPP analysis.

Comparison of treatment effect at growth stages on pea.

Data from CLPP is presented in Figure 4.14 as a principle components analysis (PCA) scatter plot. The plot shows distinct clustering and separation of data based on growth stage and not treatment indicating that bacterial communities are clearly changing in the rhizosphere through pea plant development and not with the application of inocula (GM or WT). This comparison of all growth stages with treatments shows good separation along axis 1 (59.71 % of the variance) with GS1 and GS2 at opposite ends. GS3 generally centres in the middle with partial separation on axis 2 (8.9 % of the variance) (Figure 4.14.). At this scale it is not possible to identify if there were any significant treatment effect on carbon utilisation profiles. Further, more specific, analysis was undertaken for individual growth stages to identify any treatment effect. At GS1, axes one and two represented 32.3 % and 19.3 % of the variance respectively. Separation of data was primarily by axis 1, with the GM treatment being significantly separated from the WT and water treatments (Table 4.7). The WT treatment was not significantly different to the water control. At GS2, axis 1represented 45.5 % of the variance and axis 2 represented 22.3 % of the variance. The separation of the GM treatment from the WT and water treatment was less distinct than in GS1 although still significant. One replicate of the GM had higher similarity to the WT treatment than to the GM. The water and WT treatments are significantly different to one another despite only partial separation on axis 2. At GS3 there was distinct clustering of the water treatment replicates in comparison to the WT and GM inoculation in which there was greater variance. Axis 1(32.6 % of the variance) separates the water control from the GM treatment to a significant level. A combination of axis 1 and axis 2 (23.415 % of the variance) separates water treatment with WT treatment, however this is not significant according to *t*-test. There is partial overlap between WT and GM treatment data sets, although they are significantly different.

Differences within treatments between growth stages on pea.

The comparisons of treatments over time was undertaken to identify any variance due to growth stage. For each treatment there was similarity in separation as identified in Figure 4.14. GS1 and GS2 separated to either end of axis 1, with GS3 centred on axis 1 but with separation by axis 2. Significances are summarised in Table 4.8.

4.3.2.2. Wheat rhizosphere CLPP analysis.

Comparison of treatment effect at growth stages on wheat.

Data from CLPP is presented in Figure 4.15. as a PCA scatter plot. First impressions show a distinct pattern caused by changing communities in the rhizosphere through growth stages rather than a result of treatments. This comparison of all treatments between growth stages demonstrates that treatments clustered with growth stage. The shift between growth stages was sequentially along axis 1 (36.0 % of the variance) with axis 2 (13.4 % of the variance) contributing to some of GS1 variance (Figure 4.15.). As with pea plant BIOLOG^{TM} data the resolution on this scale was not sufficient for identification of treatment effect on carbon utilisation profiles; analysis was undertaken at growth stage level. At GS1 each treatment clustered separately on the PCA scatter plot. The water control and GM treatments separated on axis 1 (33.4 % of the variance) with the WT treatment located on the origin of axis 1 with separation by axis 2 (18.2 % of the variance). This separation was significant for all treatments, see Table 4.9. At GS2 there was high internal variance for the water control on axis 1 (38.2 % of the variance). WT and GM treatments clustered together at the origin of axis 1 with partial separation on axis 2 (21.4 % of the variance). By *t*-test there were no significant differences. At GS3 the treatments cluster to themselves with significant separation along axis 1 (47.1 % of the variance) in order water, WT, and GM. Axis 2 (16.4 % of the variance) contributed little to treatment separation. This separation was significant by *t*-test analysis. At GS4 the water treatment had high internal variance distributed over axis 1 (54.2 % of the variance) with WT and GM treatment clustering at the origin of the scatter plot. The WT and GM treatments were not significantly different to each other but were to the water control.

Differences within treatments between growth stages in the wheat rhizosphere.

The comparison of the effects of inocuum treatments between growth stages was undertaken to identify the variance due to plant development. For each of the two inoculum treatments, each growth stage separated away from other growth stages. For the water control, differences were significant for all growth stages, but in the WT and GM treatment; GS1 and GS2 were not significantly different to one another, see Table 4.10.

4.3.2.3. Sugar beet rhizosphere CLPP analysis.

Comparison of treatment effect at growth stages on sugar beet.

CLPP data from the sugar beet rhizosphere is represented in Figure 4.16. as a PCA scatter plot. It shows the data has high variability with less distinct changes in communities than identified in other plant species between growth stages. The comparison between all treatments for all growth stages demonstrated there was no easily identifiable separation between them (Figure 4.16.). Analysis was undertaken for individual growth stages to identify if there were any significant treatment effects. At GS1 there was high variation within samples for each treatment; the water control separated from WT with partial overlap on axis 1 (41.8 % of the variance). The GM treatment separated, with overlap, from WT treatment on axis 2 (20.2 % of the variance). According to *t*-test, the WT and GM treatments were not significantly different to each other, but were significantly different to the water control, see Table 4.11. At GS2 there appears to be no separation between samples on axis 1 or two (45.0 % and 16.2 % of the variance respectively). *T*-test analysis identified that the water control and GM treatment were not significantly different to one another but were significantly different to the WT treatment. At GS3 there is partial separation of WT from GM on axis 1 (34.1 % of the variance), and partial separation of the WT treatment from the water control along axis 2 (17.6 % of the variance). These separations are weak due to the large variance of the data sets. *T*-test analysis identified that the water control and GM treatments are not significantly different to one another but both are different to the WT. At GS4 the three treatments overlap

with spread over both axis (axis 1, 40.1 % and axis 2, 19.3 % of the variance). Despite this, *t*-test analysis found all treatments to be significantly different.

Differences within treatments between growth stages in the sugar beet rhizosphere.

The comparison of individual treatments between growth stages was undertaken to identify variance due to growth stage. For the water control, internal variance is represented by axis 1 for all growth stages (39.2 % of the variance). There is separation on axis 2 (18.4 % of the variance) of the individual growth stages with the tight clustering of GS4. For the WT treatment there is no identifiable separation with time although some significant differences were identified by *t*-test: GS1 was not significantly different to any other growth stages, with GS2 not significantly different to GS3 and GS4. GS3 and GS4 were significantly different to one another. For the GM treatment, GS1, GS2 and GS3 cluster together separated along axis 1 (34.708 % of the variance) from GS4. All growth stages are significantly different to each other apart from GS1 and GS3.

This data clearly identifies differences between plant type specially in the sugar beet rhizosphere with communities being more distinct.

4.3.3. Results for average well colour (AWC).

Community level physiological profiling data was analysed using the average well colour for individual BIOLOG™ plates. Given that all BIOLOG™ plates were inoculated with the same levels of bacteria it was assumed that AWC could be used as an indicator of activity. This data was used to compare treatments and growth stages for each of the plant species.

4.3.3.1 Pea rhizosphere AWC analysis.

The bar chart for average well colour (Figure 4.17) shows that the activity of the communities isolated from different inoculated and control treatments at each growth stage is very similar in the pea plant. But levels of activity changed between growth stages. There is almost a 50 % reduction between GS1 and GS2 which appears to be recovered by GS3. The comparison of the water control between growth stages identified significant differences between GS1 and GS2 (OD_{600} = 1.119 and 0.620 respectively), and between GS1 and GS3 (OD_{600} = 0.847), see Figure 4.17 and Table 4.13. There was no significant difference between GS2 and GS3. The WT bacteria inoculation treatment demonstrated that GS2 was significantly different to the other growth stages. GS1 and GS3 (OD_{600} = 0.902) were not significantly different to one another Significant differences were identified in the GM treatment between all growth stages.

The comparison within growth stages between treatments demonstrated no significant difference at GS1 between water control and WT treatment (OD_{600} = 1.119 and 1.138 respectively). Although there were significant differences between water control and GM (OD_{600} = 1.271). WT and GM were not significantly different to each other. At GS2 and GS3 there were no significant differences between treatments, see Table 4.14.

4.3.3.2. Wheat rhizosphere AWC analysis.

The AWC for the bacterial communities of wheat seedlings was plotted as a bar chat (Figure 4.18). Firstly the major growth stage changes in AWC seen in the pea rhizosphere bacterial communities are not seen in the data generated for wheat rhizosphere bacterial communities. There is however greater variation in the effect of treatments. The water controls were not significantly different to each other in all growth stages (GS1-GS4, OD_{600} = 1.079, 1.237, 0.976 and 0.843 respectively) (Figure 4.18 and Table 4.15.). In the WT treatment there was no significant difference between GS1 and GS2 (OD_{600} = 1.194 and 1.192 respectively). GS1 and GS2 were identified as significantly different to GS3 and GS4 (OD_{600} = 1.043 and 0.946 respectively). There was also a significant difference identified between GS3 and GS4

($p = <0.05$). For the GM inoculated treatment there was no significant difference between GS1 and GS2 (OD_{600} = 1.263 and 1.212). GS1 was significantly different to GS3 and GS4 (OD_{600} = 1.128 and 0.964). GS2 and GS3 were not significantly different to each other, neither were GS3 and GS4 there was however, significant difference between GS2 and GS4, see Table 4.15.

The comparison of data between treatments within growth stages was also undertaken. This identified that at GS1 in the wheat rhizosphere there were significant differences between all treatments. At GS2, GS3 and GS4 there were no significant differences between the treatments, see Table 4.16.

4.3.3.3. Sugar beet rhizosphere AWC analysis.

The sugar beet bacterial communities AWC on BIOLOG™ GN2 plates were plotted on a bar chart (Figure 4.19) showing high treatment variability with no growth stage variability. For AWC in sugar beet there were no significant differences with treatments between growth stages. There were also no significant differences within growth stages between treatments, see Figure 4.19., Table 4.17. and 4.18.

4.3.4. DGGE analysis of community structure.

Total nucleic acid was extracted from the rhizosphere samples using a slight modification of the BBCTAB protocol (Griffiths *et al.*, 2000) as detailed in Chapter 4. DNA was subjected to PCR amplification for eubacteria using 16S as described by Whiteley and Bailey (52) (section 2.3.2.1) and 18S (22) as described in section 2.3.2.2 for community analysis by DGGE. Amplification of 16S α-proteobacteria was undertaken using the primers F203α and R1494 by Gomes *et al* (18) and pseudomonads using the primers Ps-for and Ps-rev by Windmer *et al.* (53). For the analysis of community profiles and determination of similarities and differences, two software packages were used. Phoretics™ 1D Advanced gel analysis software (Version 5.0, Nonlinear Dynamics Ltd.) and the Multi Variate Statistical Package (MVSP) (Version 3.12d, Kovach Computing Services).

Analysis of community structure was undertaken for comparison of treatment effects and growth stage influence within plant species. In Chapter 3 the comparisons were made between plant species and growth stage influence. It was shown that there were significant plant and growth stage effects on the on the indigenous community structure. DGGEs were not run for comparisons between plant species but a composite figure has been created (Figure 4.20.) to demonstrate the differences between plant species are still applicable in this data set. This figure clearly demonstrates the differences between the profiles. The pea and wheat community profiles show some similarity although they are still quite different in comparison to each other. In comparison to the sugar beet community profile which shows a more unique community profile in comparison to the other two plant species. This indicates how different plant species enrich different bacteria species from the indigenous soil communities for the colonisation of the rhizosphere.

4.3.4.1. DGGE analysis of the pea rhizosphere.

16S DGGE analysis of the pea rhizosphere.

The bacterial community structure of the plant rhizosphere was analysed by DGGE using three different primer sets, biased to specific community profile as described in section 4.2.6. These primers were the general eubacterial primers (52), the α-proteobacterial specific (18), and the pseudomonad specific (53). Each primer set produced different profiles in DGGE analysis distinct to the organisms amplified. Figure 4.21. shows a comparison of the three primer sets and their PCR products amplified for GS1 in the pea rhizosphere. This clearly demonstrates the successful bias of the PCR reactions.

16S eubacterial DGGE analysis of the pea rhizosphere.

Data from 16S DGGE analysis using the primers GC338F and 530R is presented in Figure 4.22. as a PCA scatter plot. At a glance the scatter plot shows a distinct pattern, where communities are clearly changing in the rhizosphere with growth stages and not with treatment. This comparison of seasonal eubacterial community DGGE data

from analysis of the pea rhizosphere demonstrated distinct separation of GS1 along axis 1 (18.0 % of the variance) from GS2 and GS3 perhaps indicating a greater perturbation in the young seedling which is less apparent as the plant develops. The data from GS2 and GS3 had no distinct separation from one another (Figure 4.22.). Analysis of data at GS1 demonstrated no distinct separation of the treatments from one another although high levels of variation over both axes were demonstrated (25.0 % and 19.0 % for axis 1 and 2 respectively). At GS2 the separation of the WT and GM treatments from the water control along axis 1 was observed (23.7 % of the variance). WT and GM treatments separated along axis 2 (18.4 % of the variance), but not so significantly. At GS3 the GM treatment was still separated from the water control along axis 1 (30.9 % of the variance), however the WT inoculation clustered with the water control. There was partial separation of the WT and water control over axis 2 (15.5 % of the variance) but the data points were partially overlapped. For the comparison of seasonal effects, individual treatments were compared over time. Each treatment demonstrated the same effect with each growth stage being distinctly separated from each other.

α-proteobacteria biased DGGE analysis of the pea rhizosphere.

DGGE data for α-proteobacteria biased PCR is presented in Figure 4.23. as a PCA scatter plot. This scatter plot shows a seasonal separation of growth stages with no distinct treatment effects. GS1 and GS2 separated from GS3, along axis 1 (16.0 % of the variance) in which there was clear separation. The data points of GS1 and GS2 overlap, but have partial separation on axis 2 (11.5 % of the variance). Data was analysed at individual growth stages to identify significant treatment effects. At GS1 replicates within treatments clustered tightly together with the distinct separation of treatments. WT and GM treatments separated to either end of axis 1 (30.7 % of the variance) with separation from the water control on axis 2 (25.8 % of the variance). At GS2 the WT and GM treatments were clustered together, away from the water control with separation as a combination of both axis 1 and 2 (25.5 % and 22.4 % of the variance respectively). The water control demonstrated higher internal variance within the treatment compared to the bacterial inoculations. At GS3 there was no distinct separation of the treatments with high variance between replicates for all. For the

comparison of seasonal effects, individual treatments were compared over time. Each treatment demonstrated the same distinct separation of growth stages.

Pseudomonad (γ-proteobacteria) biased DGGE analysis of the pea rhizosphere.

DGGE data for pseudomonad biased PCR is presented in Figure 4.24 as a PCA scatter plot. This data showed no discernable separation of growth stages or treatments for the comparison of seasonal effect for all treatments with the pseudomonad specific primers. Data was analysed at individual growth stages to identify if there were any treatment effects. At GS1 there was distinct separation of the GM treatment from WT and water control along axis 1 (27.1 % of the variance). The WT and water control showed partial separation on axis 2 (16.4 % of the variance), although there still was a slight overlap in data points. At GS2 separation of the GM treatment from WT and water control on axis 2 was observed (18.6 % of the variance). The WT and water control were not significantly different to each other. A high level of internal variance in the water control was represented by axis 1 (24.6 % of the variance). At GS3 there was still separation of the GM treatment from the WT and water control on axis 1 (26.2 % of the variance) with the overlap of the water control and WT treatment on axis 1. There was no separation on axis 2 (21.1 % of the variance). For the comparison of seasonal effect, treatments were plotted over time. Both water and WT treatments had separation of GS1 from GS2 and GS3, with GS2 and GS3 data points overlapping. The GM treatment demonstrated all growth stages separating from each other.

18S DGGE analysis of the pea rhizosphere.

Amplification of the 18S rRNA gene for DGGE analysis of the pea rhizosphere was undertaken using the primers NS1 and NS2-10GC. Figure 4.25. represents a typical 18S DGGE. This gel was analysed and the data is represented in Figure 4.26. as a PCA scatter plot. Comparison of seasonal fungal community data from analysis of the pea rhizosphere demonstrated large variance within growth stages and with partial separation of data from individual growth stages (Figure 4.26.). Axis 1 represents 15.9 % of the data with GS1 clustering to one end, with large variance over axis 2 (12.6 %

of the variance). GS2 and GS3 have no distinct clustering, with large variance over both axes. For the identification of potential treatment affects growth stages were analysed individually. There was large data variance over axis 1 (32.0 %) which did not contribute to any separation between treatments. At GS1 the separation of treatments identified was on axis 2 (16.8 % of the variance) with the WT treatment separating vertically from water and GM treatments. At GS2 there is distinct separation of the WT and GM from the water control over axis 1 (38.7 % of the variance), with the partial independent separation of WT and GM treatments from each other. The water control contributed to the main variance observed on axis 2 (16.3 % of the variance), with the WT and GM treatments clustering closely to the origin. At GS3 the separation of each treatment was by a combination of axis 1 and 2 (31.8 and 20.9 % of the variance respectively). WT and GM treatments cluster closer together away from the water control, whilst remaining distinct. For the comparison of seasonal effects individual treatments were compared over time. For the water treatment there were distinct growth stage effects with approximately equal variance represented by axis 1 and 2 (23.0 % and 21.7 % respectively). For WT treatment a similar effect was identified with separation over axis 1 and 2 (23.7 % and 22.5 % of the variance respectively). For the GM treatment, GS1 and GS2 cluster together but away from GS3 using a diagonal combination of axis 1 and 2 (30.3 % and 22.9 % of the variance respectively).

4.3.4.2. DGGE analysis of the wheat rhizosphere.

Eubacterial DGGE analysis of wheat rhizosphere.

Data produced by 16S DGGE analysis of the wheat rhizosphere, using the primers GC338F and 530R is represented in Figure 4.27. Comparison of eubacterial community data from analysis of the wheat rhizosphere community profiles showed a distinct clustering of each growth stage regardless of treatments. GS1, GS2 and GS3 were sequentially separated along the axis 2 (11.0 % of the variance) to one end of axis 1 (14.4 % of the variance). GS4 separated away from the other growth stages along axis 1 (Figure 4.27.). Analysis was undertaken for each growth stage individually for the identification of treatment effects. At GS1 internal variance of each treatment was demonstrated by axis 1 (24.0 % of the variance) with the

separation of treatment on axis 2 (17.0 % of the variance). The water control flanked the origin of axis 2 with the bacterial treatments either side. At GS2 the water control demonstrated variance along axis 2 (21.0 % of the variance) with overlap of data points with the WT treatment. The WT treatment was close to the origin of both axes with its separation from the GM treatment along axis 1 (27.3 % of the variance). There was overlap between the WT and GM treatment, but not between water control and GM treatment. At GS3 there was overlap between all treatments clustered around the origin of both axis. GS4 demonstrated a similar effect to GS3, but there was greater separation of data points with no overlap of the water control to the other data. Analysis of treatments over time demonstrated a similar effect to the first analysis (Figure 4.27) with definite growth stage dependent shifts.

α-proteobacteria biased DGGE analysis of the wheat rhizosphere.

Data produced by α-proteobacteria DGGE analysis of the wheat rhizosphere is represented as a PCA scatter plot in Figure 4.28. Strong seasonal effects were demonstrated in the comparison of all treatments between growth stages. GS1 separated from GS2, GS3 and GS4 along axis 1 (28.7 % of the variance). GS2, GS3 and GS4 had higher similarity to one other and were sequentially separated along axis 2 (10.6 % of the variance), with data for each growth stage overlapping. The comparison of treatments at individual growth stages demonstrated at GS1 the GM treatment separation from the water control was contributed by axis 1 and 2 (29.9 and 19.3 % of the variance respectively). The WT treatment centred on the origin of both axes overlapping the other two treatments. At GS2 there were no discernable differences between treatments. At GS3 the WT treatment separated away from the water control and the GM treatment. The GM treatment centred on the origin with overlap to water control along axis 1 (27.8 % of variance). The WT treatment separates away from the other treatments in a combination of both axes to the lower left quadrant. At GS4 the water control and WT treatment cluster with distinct separation from the GM treatment. All samples were highly variable.

Pseudomonad (γ-proteobacteria) biased DGGE analysis of the wheat rhizosphere.

Data for γ-proteobacteria DGGE analysis is represented in Figure 4.29. as a PCA scatter plot. No clear dominating effects could be identified from first impressions. The comparison of all treatments between growth stages demonstrated that there was high variance in the data and no clearly identifiable seasonal effect (Figure 4.29.). The comparison of all treatments at individual growth stages identified that at GS1 there was the distinct separation of treatments. The water control had high internal variance around the origin of the graph with the separation of the tightly clustered GM treatment data on axis 1 (29.3 % of variance). The WT treatment separated from the water control by a combination of axis 1 and 2 (24.1 % of variance). At GS2 there was distinct separation of the three treatments. Separation was by axis 1 (27.2 % of variance) with the GM treatment flanking the origin and the WT and water control separated in either direction along axis 1. At GS3 the water control and WT treatment clustered with distinct separation from the GM treatment. The separation of the two groups was by a combination of both axis 1 and 2 (27.7 and 24.1 % of variance respectively). At GS4 the water control and WT treatment were separated, but still remained in close proximity to one another. The GM treatment separated along axis 1 (27.4 % of the variance) to the other two treatments. In the original comparison of all data there was no distinction of any seasonal effects, so comparisons of individual treatments was undertaken. For the water control there was the separation of growth stages by a combination of axis 1 and 2 (19.3% and 15.4 % respectively) with GS1 and GS4 adjacent to each other the same with GS2 and GS3, and an overlap of data points between GS1 and GS3. The WT treatment also demonstrated the separation of individual growth stages. GS1 separated distinctly to the other growth stages along axis 1 (28.0 % of variance) with the others clustered to the same point on axis 1 with separation along axis 2 (17.5 % of variance). For GS2, GS3, and GS4 there was a sequential shift of growth stages with each having partial overlap to adjacent growth stages. The GM treatment demonstrated very distinct growth stage differences with GS3 centred on the origin and the radial separation of GS1, GS2 and GS4 around the origin. The variance represented by axis 1 and 2 was 22.4 % and 15.0 % respectively.

18S DGGE analysis of the wheat rhizosphere.

Amplification of the 18S rRNA gene for DGGE analysis of the wheat rhizosphere was undertaken using the primer NS1 and NS2-10GC, and is represented in Figure 4.30 as a PCA scatter plot. Comparison of fungal community data from analysis of the wheat rhizosphere showed a progressive shift in community structure with subsequent growth stages. Axis 1 and 2 represented 20.6 and 9.8 % of the variance respectively, for which the major growth stage separation was by axis 1 (Figure 4.30.). The data for each growth stage overlapped with the next, *i.e.* GS1 with GS2, GS2 with GS3 *e.t.c.*, but not with any other, *i.e.* GS1 with GS3 or GS4. The comparison within growth stages between treatments was undertaken for the identification of treatment effects. At GS1 there is distinct separation of the GM treatment from WT and water control with separation on axis 1 (29.1 % of the variance). The WT and water control are similar to each other with tight clustering. At GS2 the WT and water control cluster together with high internal variance. There is separation from the GM treatment along axis 1 (31.3 % of the variance). At GS3 the WT and GM clusters away from the water control along axis 1 (34.7 % of the variance) where they cluster together with overlap. At GS4 there is distinct separation of the three treatments along axis 1 representing 30.4 % of the variance.

4.3.4.3. DGGE analysis of the sugar beet rhizosphere.

Eubacterial DGGE analysis of the sugar beet rhizosphere.

Data produced by 16S DGGE community analysis of the sugar beet rhizosphere using the primers GC338F and 503R is represented in Figure 4.31. as a PCA scatter plot. The initial comparison of all treatments between growth stages demonstrated no distinct separation of growth stages or treatments (Figure 4.31.). The comparison of individual growth stages was undertaken for the identification of any treatment effects. GS1 demonstrated the separation of the three treatments principally along axis 1 (26.4 % of variance). The water controls separation from the WT treatment is also contributed by axis 2 (18.9 % of variance). At GS2 the three treatments are distinctly clustered with separation of the WT and GM treatment from the water control on axis 2 (21.0 % of the variance). The WT and GM treatment are separated along axis 1

(27.3 % of the variance). At GS3 there is no distinct separation of the treatments. This is reflected in GS4 with only the partial separation of the GM treatment from the water control with overlap from the WT for both treatments. The comparison of growth stages for individual treatments identified clear growth stages effects.

α-proteobacteria biased DGGE analysis of sugar beet rhizosphere.

Data produced by α-proteobacteria DGGE community analysis is represented in Figure 4.32 as a PCA scatter graph. First indications show very strong growth stage effects. The initial comparison of all treatments between growth stages demonstrated the sequential shift in data points from GS1 to GS4 with overlap by neighbouring growth stages. This shift incorporates both axis 1 and 2 (15.9 and 12.1 % of variance respectively) (Figure 4.32.). For the identification of any potential treatment effects, individual growth stages were analysed. At GS1 the WT and GM treatments clustered together, with separation from the water control dominated by axis 2 (21.8 % of the variance). At GS2 there was no discernable separation between treatments. GS3 demonstrated high variation within treatments with the separation of the WT and water treatments on axis 2 (17.0 % of the variance). GS4 demonstrated no distinct separation of the treatments. Individual analysis of treatments over time confirmed the sequential shift in community profiles for all treatments.

Pseudomonad (γ-proteobacteria) biased DGGE analysis of the sugar beet rhizosphere.

Data produced by pseudomonad bias PCR DGGE analysis is represented in Figure 4.33. Initial comparison shows strong growth stage effect with little treatment effect. This was represented by a sequential shift in community profiles between growth stages, predominately along axis 1 (15.2 % of variation), with approximately equal internal variance contributed by axis 1 and 2 (11.0 % of variation) (Figure 4.33.). At GS1 there are no differences between treatments. At GS2 there is the distinct separation of the three treatments with the water control and WT treatment separated by axis 1 (22.9 % of variation). The GM treatment lies on the origin of axis 1, but has a downward shift in comparison to the other two treatments by axis 2 (19.0 % of

variance). At GS3 there was no clear separation of the treatments although distribution of data hints at a possible separation of the GM and water treatments along axis 2 (18.7 % of variance). At GS4 the water and WT treatment demonstrated high variance with spread along axis 1 (23.0 % of variance), and the separation from the GM treatment on axis 2 (19.8 % of the variance). Individual analysis of treatments over time confirmed the sequential shift of data with growth stages.

18S DGGE analysis of the sugar beet rhizosphere.

Amplification of the 18S rRNA gene for DGGE analysis for the sugar beet rhizosphere was undertaken using the primers NS1 and NS2-10GC, and is represented in Figure 4.34. as a PCA scatter plot. The comparison of seasonal data for all treatments of the fungal community analysis identified that there were no dominating effects on community structure by treatments or growth stages. Analysis was undertaken at individual growth stages to identify if there were any significant treatment effects. At GS1 there was high variance in the data with the only possible separation of WT and water control by a combination of axis 1 and 2 (24.5 and 17.0 % of variance respectively), the GM treatment data over lapped both data sets. A similar effect was identified at GS2 with the GM and WT treatments separating and the water control having high variance overlapping both data sets. At GS3 there were no distinct differences between treatments. At GS4 the water control data was positioned at the lower end of axis 2 (19.2 % of variance) with the WT and GM treatments demonstrating overlapping linear perpendicular variance by a combination of axis 1 (26.7 % of the variance) and two. In the original comparison of all data there were no seasonal effects identifiable, so the comparisons of individual treatments was undertaken over growth stages. The water control demonstrated distinct separation of GS1, GS2 and GS4 from each other around the origin. GS3 had high variance with large separation of data points along axis 1 (19.1 % of variance), overlapping GS1 and GS2. For the WT treatment the separation of growth stages was a result of both axis 1 and 2 (29.2 % and 16.4 % respectively) with GS1 and GS4 clustering together with partial overlap, the same occurring with GS2 and GS3. There was distinct separation of the two clusters. The general movement of data points in the GM treatment was a sequential shift by a combination of both axes.

4.3.5. Confirmation of specificity of group specific primers.

Small clone libraries were created from PCR products amplified for DGGE analysis of the pea rhizosphere for confirmation of primer specificity. For each library 20 clones were sequenced. The α-proteobacterial primers were 100% specific to the alpha subgroup with 41.2 % of the library represented in the order rhizobiales, 29.4 % in the order caulobacterales, 23.5 % were identified as uncultured α-proteobacteria, and 5.9 % of the clones in the order rhodobacteriacae. The pseudomonad specific primers were not 100 % specific to the pseudomonadales but were 100 % specific to the γ-proteobacteria. The proportions represented were 66.7 % in the order enterobacteriales, 11.1 % in the order xanthomonadales, 11.1 % in the order pseudomonadales, and 5.6 % were uncultured γ-proteobacterium.

4.4. Discussion.

4.4.1. Colonisation abilities in the plant rhizosphere.

The bacterial inoculum was introduced to the soil at density of 7.00 $\log_{10}$ cfu g^{-1} soil. This increased to 9.03 $\log_{10}$ cfu g^{-1} for the WT and 9.22 $\log_{10}$ cfu g^{-1} for the GM in the pea rhizosphere after ten days of growth. *Pseudomonas fluorescens* SBW25 has previously been demonstrated as highly prolific with the ability to colonise the rhizosphere rapidly and aggressively (1). The data in this chapter supports the colonisation abilities of SBW25 WT and GM inoculum as described previously (45, 46). Increases in population densities from the background inoculation were also observed in the wheat rhizosphere. Population densities five days after inoculation had increased to 7.97 $\log_{10}$ cfu g^{-1} for the WT inoculum and 7.21 $\log_{10}$ cfu g^{-1} for the GM inoculum. The sugar beet population densities for the bacterial inocula were not different from the initial inoculation with populations of 6.97 $\log_{10}$ cfu g^{-1} for the WT and 6.79 $\log_{10}$ cfu g^{-1} for the GM at day thirty five. This demonstrates a differential colonisation ability of the inocula in the rhizosphere of different plants in relation to the plants growth stage. These data also supports the view that there is no detriment to colonisation ability of the GM-BCA following the insertion of the phenazine biosynthesis operon when compared to the WT (34, 45, 48). It has previously been demonstrated that different plants produce exudates with characteristic signatures to the plant (37). This and the differential community profiles produced by different plant species (Chapter 3, (38, 40) or even different cultivars of plant species (10, 39) strongly indicates that different plants select/enrich their own specific microbial populations. These data also indicate differential rhizosphere carrying capacities and survival of the introduced inocula on different plant species. This is consistent with the findings in Chapter 3 with the identification of differential carrying capacities for all micro-organisms in the three crop types when grown in the field rather than mesocosms. Pea plants had the highest population densities when compared to wheat and sugar beet, with sugar beet supporting the lowest population densities of the inoculum.

4.4.2. Fate and persistence of inocula.

In all plant species studied there was the decline in inocula population numbers from the initial inoculation. The introduced inocula could not maintain high population densities for the entire growing season although its persistence was demonstrated. This is a common feature that biological control agents encounter, and has been demonstrated in both laboratory and field experiments (16, 20, 24, 44). The only occasion there was a significant increase in the population densities of the WT inoculum was between GS3 and GS4 in sugar beet. The increase in population numbers is of interest, as it has not been observed before within a growing season. Previous studies have reported the reoccurrence of strains in consecutive seasons (13). The reason behind the observed reoccurrence may be a result of the physical and biological properties of the mesocosm grown sugar beet. Sugar beet naturally grows to a large size in the open field environment determined by the initial growth of root and lying down of cells. Once the root has developed sugar beet undergoes a rapid expansion in cell size with exudation of nutrients and in the storage of carbohydrate reserves (11). The pot plants did not grow to this typical morphology or size, they may have been pot bound or the result of the consistent light/dark cycle encountered in plant growth rooms. The growth stages of sugar beet are responsive to the change in day length. The decrease in day length triggers the plant to lay down carbohydrate stores (11). The lack of stimulus may have caused the cessation of growth and resulted in this bacterial population increase. The reason the increase was only discovered in the WT bacterial population may result from the extra genetic load on the GM reducing its competitiveness under these environmental conditions although previously has been shown not to be a problem or there being growth of the introduced bacterium (45, 46, 48).

4.4.3. Impact of the inocula on the pea rhizosphere.

Total bacterial populations in the rhizosphere of pea demonstrated seasonal fluctuations between growth stages. GS1 had a slight but significantly larger population than the other growth stages for the water treatment. This was not observed in inoculated treatments. The WT and GM treatment populations were observed to maintain a higher population density compared to the water control for

subsequent growth stages. Data from the pseudomonad population densities indicate a gradual decline with growth stage for all treatments. This decrease is less pronounced in the inoculated treatments. It was shown that > 90 % of the cultured pseudomonads at GS1 were the inoculum and over time the proportion decline to be replaced by indigenous pseudomonads. The perturbation of the indigenous pseudomonad population has been previously described (3, 8, 30), although some reported no shift in carrying capacity (30) Over the course of the experiment the inocula persisted despite their gradual decline in numbers. This would account for the slightly higher pseudomonad populations in the inoculated treatments compared to the water control and the significant increase in the total bacterial population numbers for those treatments. Therefore the higher population numbers identified in the WT and GM compared to the water control could be attributed to the presence of the inocula. It has previously been reported that the introduction of GMMs had no effect on total culturable populations (2).

In the fungal population densities of pea rhizosphere it was identified that there were general seasonal fluctuation with no significant trends identified. The GM inoculated treatment had significantly larger fungal population at GS1 compared to the water control or the WT treatment. This increase declined over the subsequent growth stages but was not significantly different to the other treatments (Figure 4.4). This perturbation could be the result of several different possibilities. The GM biocontrol agent has been demonstrated to have increased antifungal activity to numerous fungal pathogens (45, 46). The inoculation of the BCA suppresses the fungal plant pathogens with the possible outgrowth of non pathogenic fungi in the plant rhizosphere resulting in a net increase in propagules. The out competition and suppression of other bacteria may cause a similar result. This may reduce bacteria that suppress certain fungi resulting in their growth or increased accessibility of carbon sources available to the fungal populations. Alternatively the bacteria in this instance may be protecting the plant from pathogen attack, allowing rapid and more vigorous growth. This may result in an increase in rhizodeposition of nutrients supporting higher populations of micro-organisms. This increase can also be caused by cell lysis as a result of pathogen attack (51).

Data from BIOLOG™ analysis of the pea rhizosphere confirms what has previously been demonstrated (Chapter 3) (30, 41). The bacteria supported in the rhizosphere confer different carbon utilisation profiles dependant on plant growth stage but this has been reported to not always be the case (9). It is reasonable to assume that in our study we are seeing a response to the exudates released from the plant during growth or to local nutrient level fluctuation in response to the plants needs during growth. The data analysis for treatment effects, demonstrates that the GM treatment is significantly different to the WT and water control treatments at the first growth stage on seedlings. This difference reduces over subsequent growth stages where populations are all similar in the final growth stage for all treatments. This demonstrates that the phenazine biosynthesis in the GM bacterium has a significant differential effect on the community carbon utilisation profiles compare to the WT and un-inoculated water control. Natsch *et al.*, (30) reported a similar result in numbers with plant growth stage dominating the treatment effects seen. This effect in pea was transient and could be the result of the high proportion of bacteria inoculum represented in the culturable populations. This alone could shift CLPP, in addition, the inoculum could impact fungal communities, resulting in a differential breakdown of organic compounds and thus nutrients available to the rhizosphere micro-organisms.

Analysis of average well colour on CLPP data demonstrated a significantly higher level of rhizosphere substrate utilisation at GS1 in comparison to the other growth stages, in the rhizosphere of pea plants. This would indicate high levels of microbial activity in young seedlings of pea. This differed to that identified in the field study where activity was low before a subsequent increase (Chapter 3). It is known at this stage in plant growth there is high competition in the rhizosphere colonisation of micro-organisms resulting in high activity of the bacteria. The level of activity also increases at the final growth stage of pea. This increase occurs in the field experiment and is likely to be a result of plant exudation changes at flowering. This would result in a potential change in nutrient requirements and therefore exudates released into the plant rhizosphere. This nutrient release would support greater microbial activity in the rhizosphere. At GS1 it was identified that the GM treatment had increased activity compared to the water control. The WT treatment was not significantly different to either treatment. This data reflects that shown in the principle component analysis of the CLPP data. Initial differential effect (increased activity) of the GM treatment to

the water and WT treatments, which returns to the water control by the final growth stage.

Assuming that in DGGE analysis each band represented an operational taxonomic unit, comparisons were made between community profiles by the presence and absence of bands. Data analysis of eubacterial communities indicated the same growth stage effects as previously has been described in bacterial populations (Chapter 3, (18, 25, 40, 41)). The effect on the bacterial populations community profile results in the separation of GS1 from GS2 and GS3. A level of significance cannot be put on this data, but its comparison to the data shown by CLPP can give us the confidence that the inferences made are substantiated. The plant growth stage has a greater impact on community structure than the release of the inocula. A shift in treatment effects is identifiable in the bacterial populations. The initial growth stage indicated no treatment effect. At GS2 the WT and GM treatments shift away from the water control. At GS3 the WT had returned a similar profile to that of the water control. The GM treatment was still different. This perturbation infers a longer term impact by the GM inoculum than the WT inoculum on the indigenous bacterial communities. This reinforces data confirming the transient shift in bacterial populations as a result of the inoculum (2, 3, 7, 31, 38).

The analysis of the α-proteobacteria communities were undertaken to identify if distinct group specific perturbations were identifiable in the community profiles. The effect of growth stage on community profiles continue to dominate over any treatment effects. Comparisons at individual growth stages identified that at GS1 there were clear treatment effects in the pea rhizosphere. This effect reduces with each subsequent growth stage with the effects of WT and GM inoculations moving together, and the subsequent regrouping with the water control in PCA analysis. This indicates that the GM inoculum has a small increased differential effect on the α-proteobacteria in the rhizopshere compared to the WT inoculum. This impact may effect the initial colonisation abilities of the α-proteobacteria, which include the rhizobia. This effect is transient with the diversity becoming more like the WT inoculum, and subsequently all treatments being similar to that of the water control.

No distinct growth stage effects were identifiable using the γ-proteobacterial primers in comparison with all treatments. Only under comparison for individual treatments were growth stage effects identifiable. This indicated there were significant treatment effects influenced by plant growth stage such that neither dominate under our comparisons for all data. The treatment effect identified consisted of a significant shift in the GM inoculated treatment away from the water and WT treatments when data was analysed and represented as a PCA scatter graph. This implies that the diversity seen in the GM is different to the WT and water control which had higher similarity. This shift occurs equally for all growth stages. It was identified that the inocula had replaced the majority of the pseudomonad population in plate counts which would account for the major shift. The WT inoculum had no effect on community profiles, which indicates that although replacement of a large number of the population there was no resulting impact in bacteria present, unlike the GM which had a large significant impact. The increased impact by the GM could then be attributed to the insertion and subsequent expression of the PCA operon. It has been previously shown that there was suppression of the enterobacteriacace (γ-proteobacteria) by the BCA (44).

The molecular analysis by 18S DGGE of the pea rhizosphere showed that on initial inspection of the DGGE it is possible to identify that there is low diversity in the water control at GS2 replicates two and three. This is also the case for the WT treatment at GS3 replicate three. This low diversity of bands will have naturally distorted the data with them lying as out groups in the analysis. This may have been the result of a high level of fungal disease infection dominating the community in these samples. These replicates were still included. Some might implicate an error in processing may have been made but these profiles represent true data and would cause user bias to the data if were removed. Analysis of the data identified a growth stage effect which confirmed the seasonal variance identified in Chapter 3 and by Gomes *et al.*, (17). GS1 was distinct from GS2 and GS3, which reflects the shifts previously identified in CLPP of bacteria and shifts in 16S DGGE profiles. This clustering of the data would follow the implications of high bacterial activity in the young seedlings of plants with dynamic populations as they compete to colonise the rhizosphere. This high bacterial activity would have greater impact on the fungal populations colonising

the rhizopshere. Subsequent growth stages become less dynamic as bacterial and fungal populations become established and stabilise. Specific growth stage analysis demonstrated that there were no immediate treatment effects. At GS2 (41 days after planting) a shift in community profile of the WT and GM treatment away from the water treatment was identified. A point to remember is the low diversity in two of the replicates of the water control at this growth stage, this may have caused the shift if any to be exaggerated. This shift declined on the subsequent growth stage. This indicates that the fungal populations in the rhizosphere may have a transient shift in the community profile in response to the inoculum. Previous studies have identified impacts on fungal communities as a result of biocontrol agents (16) but no one has addressed this by DGGE. The perturbation identified is slow in response, not evident ten days after inoculation. Fungi typically have slow growth in bulk soil with high resilience in response to detrimental perturbations. This would cause the delay in any detectable impact of the fungal community to the perturbation. The impact on the community profile is short lived and at GS3 is converging back to the water control community profile.

4.4.4. Impact of the inocula on the wheat rhizosphere.

Total bacterial populations in wheat rhizosphere demonstrated highly stable populations with only slight growth stage variance. There were no impacts detectable due to treatment effects on either population density or structure. These data are consistent with that identified in Chapter 3. Pseudomonad populations demonstrated similar growth stage variation to that identified for total bacterial populations in wheat. The inoculation of the WT bacteria demonstrated a significant increase in pseudomonad population numbers in comparison to the water control for the first growth stage. This impact was not identified at any other growth stage. This would be as a result of the high proportion of the pseudomonad population that the inoculum represented. The fungal populations also demonstrated slight growth stage effects but there were no treatments effects. This data implies that the wheat rhizosphere is a very stable and resilient environment to the external perturbations of bacterial biological control agents. The slight perturbation identified in the WT treatment at GS1 was just significant ($p = 0.04$). Looking at the data there were large error bars on that time point, and the raw data of triplicates had three plate counts of 7.77 $\log_{10}$ cfu g^{-1}, 7.76

log_{10} cfu g^{-1}, and 8.65 log_{10} cfu g^{-1}. Two of the replicates are very similar with one being an order of magnitude out. This appears to be unusual and not follow with the rest of the data. Therefore unless data from CLPP and DGGE indicate otherwise, no inference will be made on the significant difference.

CLPP data for the wheat rhizosphere demonstrated the same growth stage dependent effect as identified in the pea rhizosphere (section 4.4.3.2) and shown in Chapter 3. This would demonstrate that there are equivalent significant changes in the wheat plants rhizodeposition, influencing the carbon utilisation profiles of the rhizobacteria. There were significant treatment effects at the first growth stage, with the separation of WT and GM away from water control at GS2. The same effect as GS1 was observed at GS3, with the WT and GM clustering at GS4 significantly different to the water control. This implies the treatments do influence the carbon utilisation profiles in the rhizosphere, which are present until the final growth stage where their effect is less pronounced. This correlates with the high level of replacement of indigenous pseudomonads in the rhizosphere. As the population densities of the inoculum decrease so do the differences in CLPP. The genetic modification of SBW25 to produce 23:10 confirms the differential impact the community carbon utilisation profiles identified in the pea rhizosphere.

Analysis of AWC on CLPP data demonstrated a stable level of activity through all growth stages with only a slight decline in activity for the WT and GM treatments at later growth stages. There were marked treatment effects at GS1 between all treatments. The WT treatment had significantly higher activity in comparison to the water control. The GM treatments had a significantly higher activity than the water control and WT. This effect was transient as by the following growth stages there were no significant differences identifiable. This increase in bacterial activity probably resulted from the introduction of the inoculum with high pseudomonad replacement and competition, thus bacterial activity in the colonisation of the rhizosphere. This highlights again the differential effect being observed between the WT and GM inoculum.

The influence of growth stage is still a dominating factor in the 16S DGGE community profiles of the wheat rhizosphere with clear separation of each growth

stage. Treatment effects were not visible at this level but individual growth stages demonstrate the distinct separation of all treatments with the gradual reduction of separation, with the return to the water control as has previously been demonstrated (this chapter, (2, 3, 7, 31, 38). The differential impact of the WT and GM treatments reduce before they both return to the baseline of the water control. These data add support to the conclusions being made about the transient perturbations the inoculation of the BCAs have on the indigenous communities with the differential impact that the GM and WT bacteria have.

There was a distinct growth stage dependant effect on the DGGE community profiles of the α-proteobacterial populations. GS1 had a large level of separation from the other growth stages that demonstrated a more gradual shift in populations. In the α-proteobacteria there was not the very distinct treatment effect that was identified in the pea rhizosphere. For the first two growth stages there was no clear separation of treatments. At GS3 there was the possible separation of the WT treatment from the water control and GM treatment. This shift is most likely to be by chance as at the final growth stage the GM treatment separated from the WT treatment and the water control. The conclusion of this is it would appear there was no effect on the α-proteobacteria of the bacterial treatments. This is in contrast to the pea rhizosphere in which gave the clearest separation of treatments (section 4.3.4.1.) Wheat plants do not have any strong known symbiotic relationships with α-proteobacteria thus the lack of impact in comparison to pea plants in unsurprising.

The γ-proteobacterial DGGE communities of the wheat rhizosphere demonstrated no distinct growth stage dependant effect on the community structure. This would imply that there were significant effects of treatments. This inference would be correct as for the first two growth stages all treatments separated. The WT treatment then returned to cluster with the water control for the final two growth stages with the GM remaining distinct. This appears to demonstrate that there are differential effects on the sub-communities in the rhizosphere of the plant and that this effect also depends on the plant species involved. This effect was greater than in the pea rhizosphere with both the WT and GM affecting their indigenous bacterial group. This may be a result

of the slightly higher and longer survival rate of the inocula in the wheat rhizosphere when compared to the pea rhizosphere.

The impact on 18S DGGE communities was undertaken and the analysis of growth stages for all treatments confirms the dominating effect that growth stage has over the treatment that were imposed. There is a distinct shift over time in the profiles of the fungal communities. The effects of treatments demonstrated an immediate impact of the GM inoculation at GS1 with the separation from the WT and water control, this occurs again at GS2. The WT treatment separated from the water control to have a similar impact as the GM at GS3. At GS4 all treatments produce different community profiles. This would indicate that the impact on community profiles is fairly constant for the GM treatment with an increasing impact from the WT inoculation with subsequent growth stages. The alternative conclusion could be that the communities present in rhizosphere were initially perturbed by GM but as time progressed the original communities regained their position in the rhizosphere. Its initial impact could have been quite substantial and reduced the visible effects caused by the WT treatment in the principle component analysis. As its effect reduces the smaller impact of the WT treatments may have become visible.

4.4.5. Impact of the inocula on the sugar beet rhizosphere.

The culturable populations estimated on TSBA demonstrated a growth stage dependant effect on population numbers with a significant increase at GS3 of total populations. The populations were more stable than had been previously been identified in Chapter 3. There was no distinct treatment effect on the population number apart from GS2 where the GM inoculum had significantly higher populations of bacteria in comparison to the water control. When compared to the population densities of the pseudomonad populations general growth stage fluctuations were identifiable. It was identified that there was the same significant difference at GS2 as in total bacterial counts with the GM inoculation having higher population densities than the water control. Population densities of the inocula were not significantly different between the WT and GM in the monitoring of there persistence in the rhizosphere. However data indicate a slightly higher persistence (not significantly different) of the GM inoculum. This would imply the slight increased persistence of

the GM inoculum might have caused the significant increases of the pseudomonad and total population counts. It has been shown that a high proportion of pseudomonad counts are represented by the inoculum. Fungal populations demonstrated no effects of treatments or growth stage with the majority of data showing the high stability of population of fungi in the sugar beet rhizosphere.

CLPP data for the sugar beet rhizosphere does not show distinct growth stage effects. This has been shown to occur in some plant species (30) however this is different to what has been demonstrated with the other plant types (pea and wheat) and what was shown by data observed for plants grown under field conditions. Only analysis of individual treatments indicated that there might be treatment effects. Analysis of individual growth stages for the identification of treatments effects did not identify any separation of treatments. There were data sets at different time points that were statistically different but these were random and did not indicate any trends of divergence, convergence or oscillations of treatment effects. This could imply that the sugar beet rhizosphere is a highly dynamic environment as it was suggested by data under field conditions in Chapter 3.

Analysis of average well colour identified no differences between treatments or growth stages. This would imply that either the sugar beet rhizosphere community are very stable in CLPP so differences are minimal and cannot be identified, or the rhizosphere is highly dynamic with a rapidly changing environment causing the high variance within samples. The raw data (not shown) implies the later and would be consistent with data in Chapter 3.

Analysis of the eubacterial communities confirmed growth stage dependent shifts seen in the pea and wheat rhizosphere. At individual growth stages there were treatment effects identified with the separation of all treatments from one another at the first two growth stages. These effects were no longer visible at the subsequent growth stages, confirming the transient perturbations observed by the introduction of either of the BCAs. This is the first treatment effect identified in the analysis of the sugar beet rhizosphere implying that the inocula had no effect on the community function measured by CLPP or densities but only affected bacterial community structure indicated by inocula proportions shown in pseudomonad populations.

There were distinct growth stage dependant effects on the α-proteobacterial communities. This demonstrated a progressive shift of community profiles over time. Analysis of treatment effects at individual growth stages identified that at GS1 the WT and GM treatments shifted away from the water control. This separation of treatments had reduced and disappeared by the second growth stage. This has identified that part of the shift identified in community structure by 16S DGGE analysis is contributed by the α-proteobacterial communities.

There were distinct growth stage dependant effects on the γ-proteobacterial communities. This demonstrated the progressive shift of community profiles over time. At GS1 there were no treatment effect identified there was the clear separation of the three treatments at GS2. This effect had resolved again by GS3, although there was partial separation of the GM treatment at GS4. This would indicate that perturbations of the indigenous communities probably have resulted from the introduction of bacterial inocula. These perturbations may be fluctuating between bacterial populations as a result of the population dynamics of the sugar beet rhizosphere.

Analysis of fungal community dynamics did not identify any significant growth stage effects with total community analysis. In the analysis of specific treatments indications demonstrated some shifts in communities between growth stages but not to any identifiable pattern common for all treatments. In the identification of treatment effect there was also no apparent trend in the treatment separations. This appears to follow the same effects as in CLPP.

In conclusion the colonisation and survival of the two inocula in the rhizosphere of the three crop species has been successfully demonstrated with the subsequent decline in the population densities with successive growth stages. The differential carrying capacity of the three crop species, as previously been shown (Chapter 3) (46), is reinforced by the data in this chapter. This is reflected in the carrying capacity of the individual plant species of the bacterial inocula with highest densities demonstrated in pea and lowest in sugar beet. With regard to growth stage or treatment effects on the fungal and bacterial populations all plant species demonstrated high stability. The

only effect identified was the perturbation of the pea rhizosphere at GS1 with an increase in fungal, heterotroph, and pseudomonad populations in response to the GM inocula.

As previously demonstrated in field grown plants (Chapter 3), the dominating variable separating CLPP profiles in the rhziosphere of the three crop species grown in mesocosms was between growth stages, although this was very weak in the sugar beet. Transient effects of the inocula were detected in the pea and wheat rhizosphere as a result of the GM treatment that reduced over time. The same was observed in the WT treatment in the wheat rhizosphere. These treatment effects were reflected in the AWC of the pea and wheat rhizosphere. Growth stage effects were identifiable in the pea rhizosphere. In conclusion the analysis of culturable populations effects show fairly high stability with only small transient effects in some of the plant species.

The analysis of plant community profiles by DGGE allowed a greater understanding of the effects of the inocula. The effect of growth stage dominated community profiles in the pea rhizosphere with detectable shift in the fungal and eubacterial populations that reduced over time. The α-proteobacteria show very distinct separation of both bacterial treatments from the water control that reduced over time. The same additional effect was noted as a permanent perturbation in the pseudomonad populations as a result of the GM inoculum. The wheat communities had a distinct and different response to that of the pea rhizosphere. Growth stages cause the greatest separation of profiles again, but fungal populations were permanently perturbed in wheat following the GM inoculum, with a lasting effect on community diversity, which reduced with time. There was no impact on the α-proteobacteria unlike pea in which it was very marked it did demonstrate the same impact on the pseudomonad populations. In addition the WT treatment had a transient effect. Sugar beet communities were in general more stable with only transient effects at GS1 or GS2 in response the WT or GM inoculum for the different communities.

Therefore the following hypothesis have been confirmed:

1) The impact the on indigenous bacterial and fungal communities after the inoculation of the WT and GM-BCA bacteria will be small.

2) The impact on the indigenous bacterial and fungal communities as a result of the inoculation of the WT and GM-BCA will be transient.

3) Any impact observed on the indigenous fungal communities will be greater after inoculation of the microbial GM-BCA than that of the WT bacterium.

4) The impact on subsections of the indigenous bacterial communities as a result of the inocula will be most identifiable in the pseudomonad populations.

5) Plant species and growth stage effects will dominate any impact that the inocula have.

In summary any effects observed of the inocula on indigenous communities in general does not affect rhizosphere function for any significant period but does cause transient perturbations in the community structure above the normal background variance. The perturbations observed are different for each plant species as a result of the indigenous communities that are enriched for the plant species. Differences between the perturbations of the WT and GM inoculum can be identified; the only populations with substantial perturbations are pseudomonads as a result of the inocula. The data in this chapter demonstrates that microbial populations in the plant rhizosphere are very stable with strong plant specific enrichment by individual plant species. This enrichment is even resistant to the potential perturbation caused by the inoculation of 7.00 $\log_{10}$ g^{-1} soil of bacteria.

4.5. Figures.

Phenazine-1-carboxylic acid biosynthesis pathway.

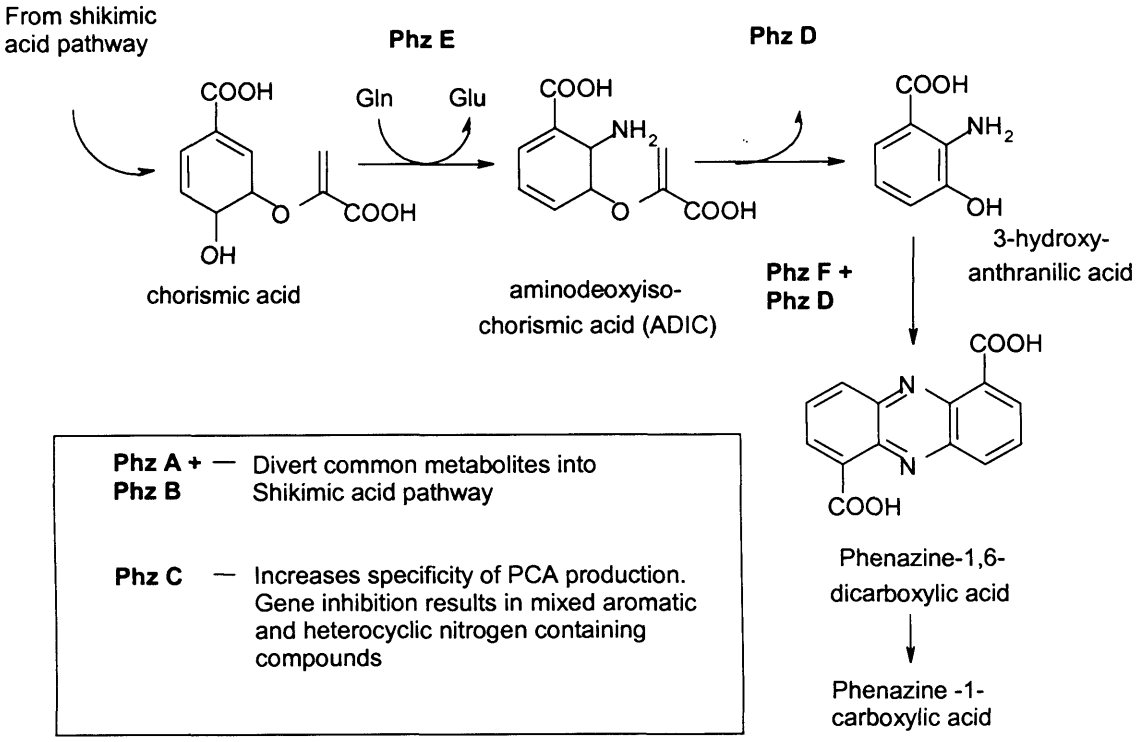

Figure 4.1. Schematic diagram demonstrating the proposed phenazine-1-carboxylic acid synthesis pathway (26).

Pea rhizosphere culturable population densities.

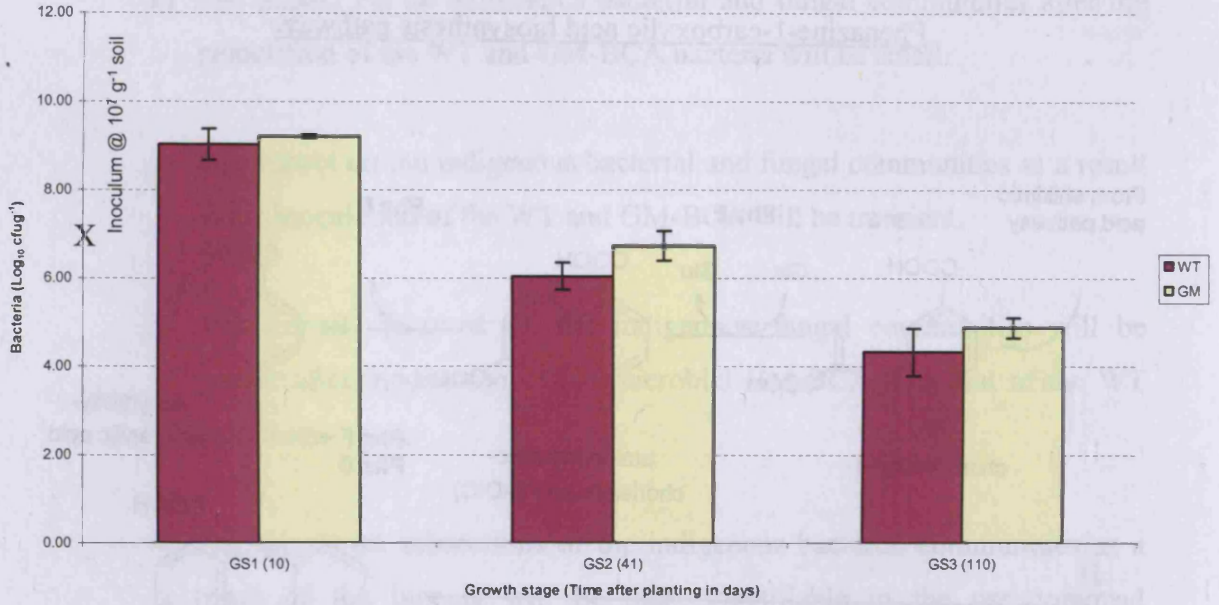

Figure 4.2. Pea rhizosphere; Mesocosm population densities of the bacterial inocula detected for WT (SBW25) and GM (23:10) bacterial treatment. No inocula were detected in the water control (data not shown). Bacteria were cultured using pseudomonad selective agar (PSA) with appropriate antibiotic selection for the inocula. Independent replicates were used (n = 3). I = standard error of samples.

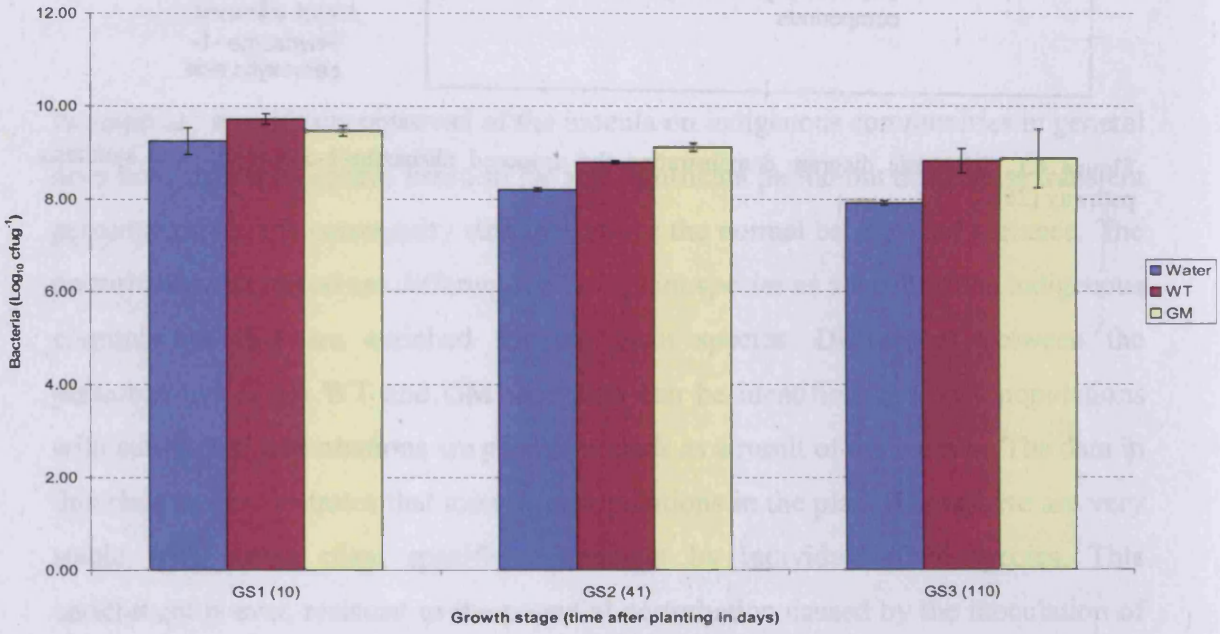

Figure 4.3. Pea rhizosphere; Mesocosm population densities of total culturable heterotrophic bacteria detected on TSBA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were cultured on tryptone soya broth agar with the appropriate antibiotics. Independent replicates were used (n = 3). I = standard error of samples.

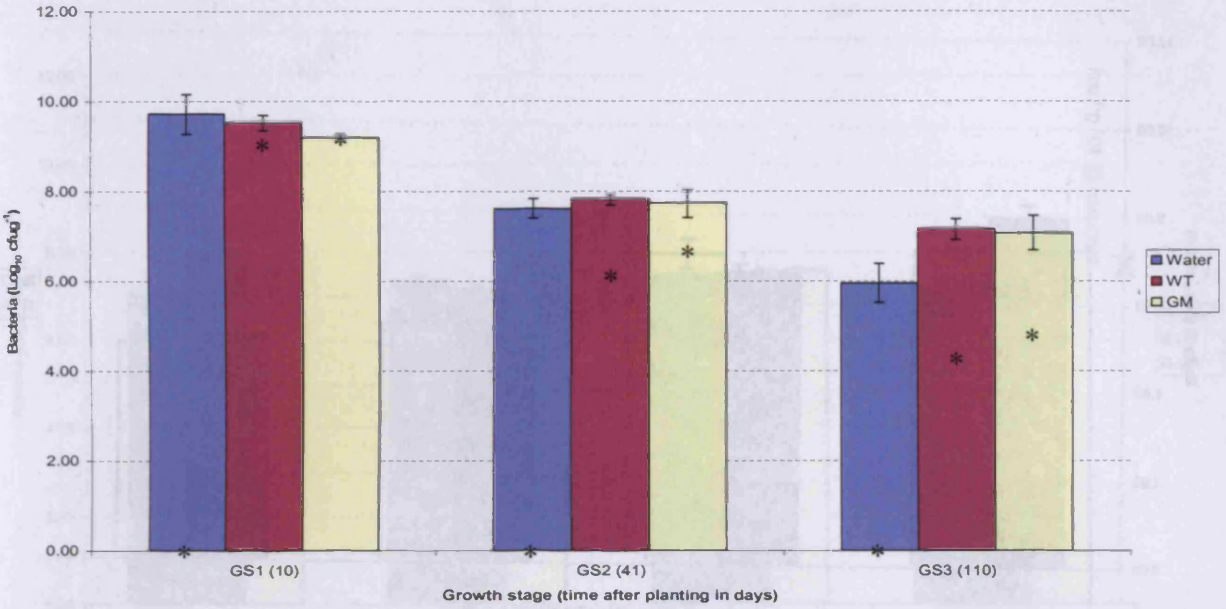

Figure 4.4. Pea rhizosphere; mesocosm population densities of pseudomonads detected on PSA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were grown on pseudomonad selective agar with the appropriate antibiotics. * = population densities of the inocula in each treatment. Independent replicates were used (n = 3). I = standard error of samples.

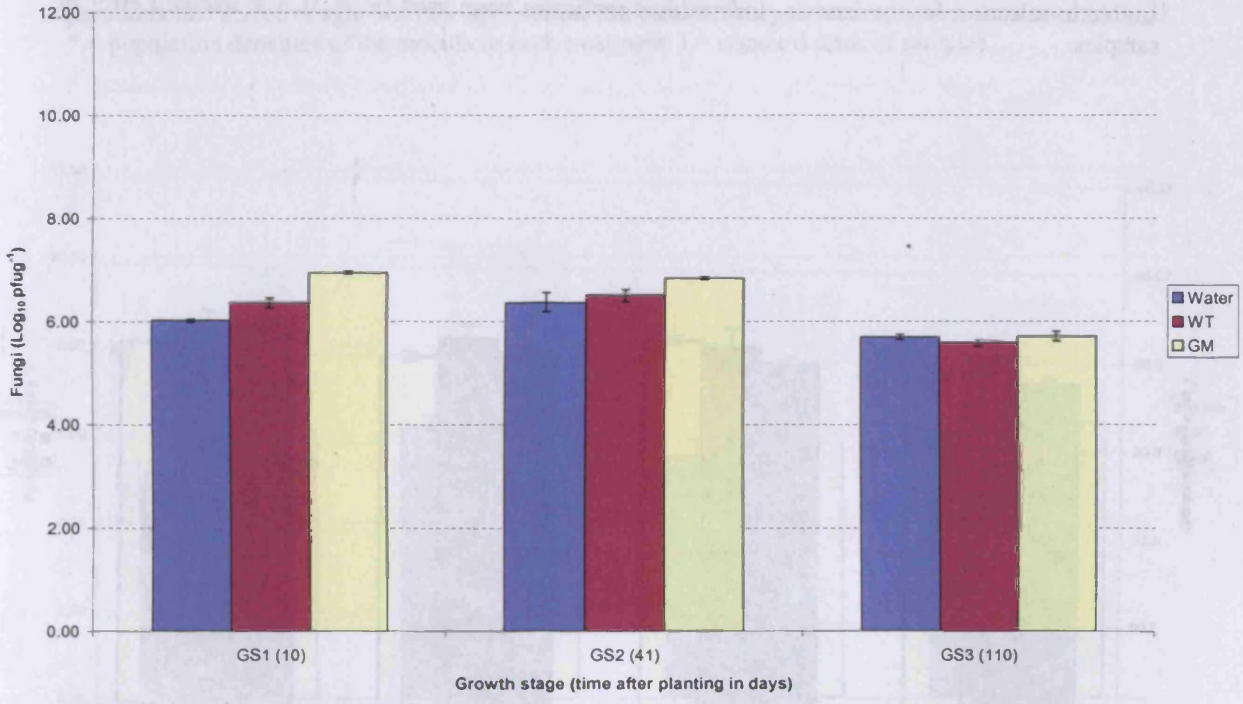

Figure 4.5. Pea rhizosphere; mesocosm population densities of fungi detected on PDA for the three treatments: water control, WT (SBW25) and GM (23:10). Populations were estimated by propapgule forming units (pfu) on potato dextrose agar with appropriate antibiotic selection. Independent replicates were used (n = 3). I = standard error of samples.

Wheat rhizosphere culturable population densities.

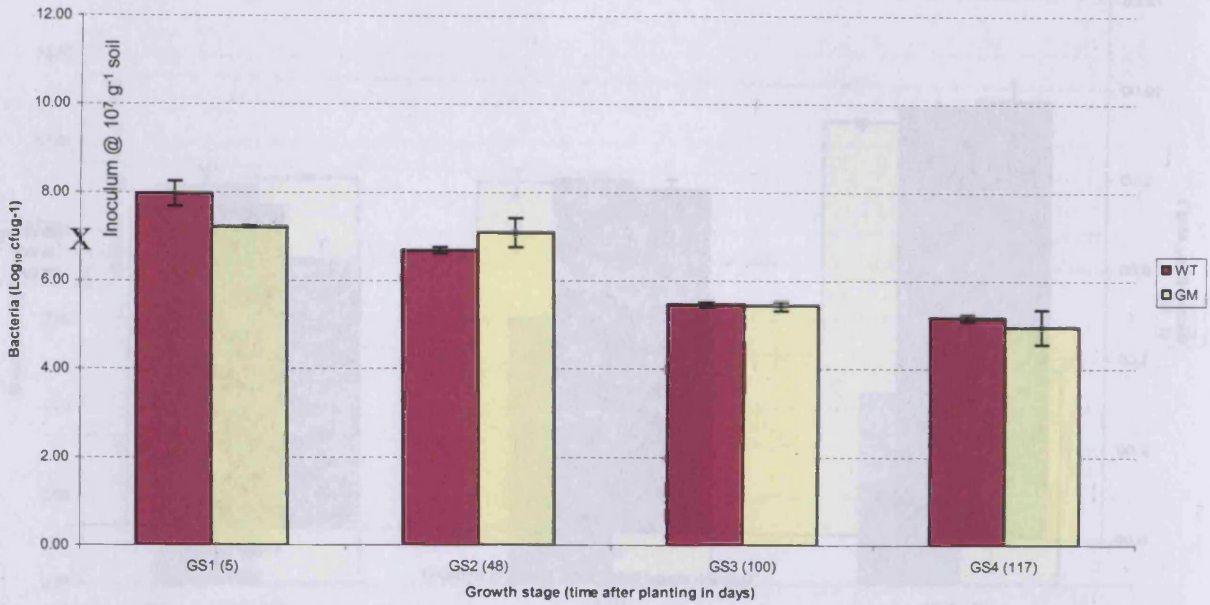

Figure 4.6. Wheat rhizosphere; Mesocosm population densities of the bacterial inocula detected for the WT (SBW25) and GM (23:10) bacterial treatment. No inocula were detected in the water control (data not shown). Bacteria were cultured using pseudomonad selective agar (PSA) with appropriate antibiotic selection for the inocula. Independent replicates were used (n = 3). I = standard error of samples.

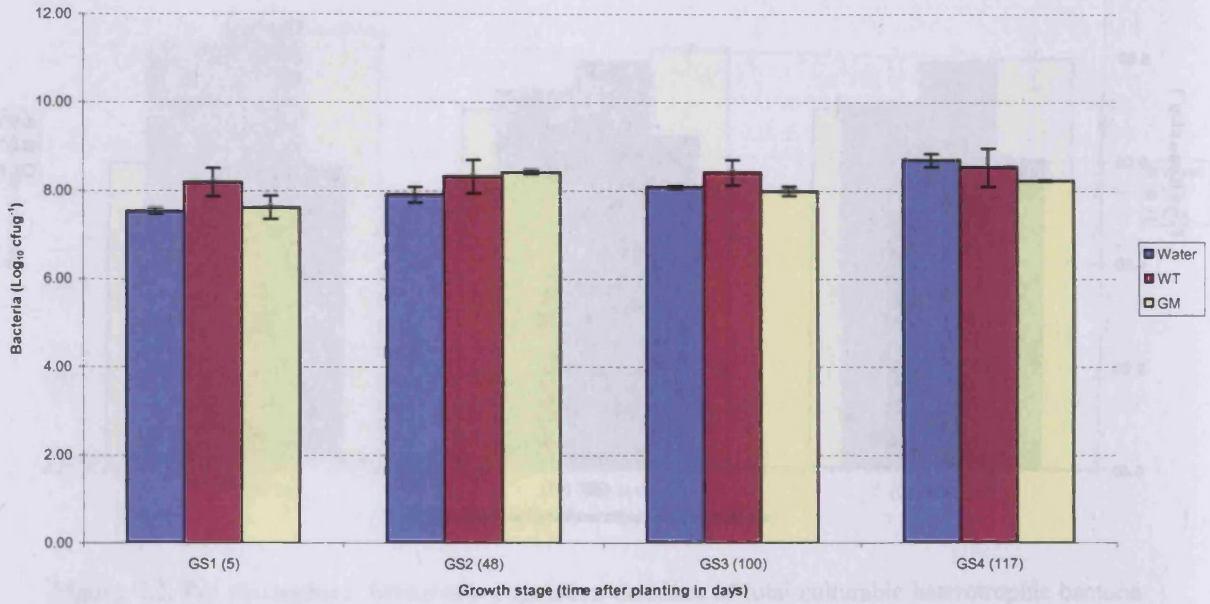

Figure 4.7. Wheat rhizosphere; mesocosm population densities of total culturable heterotrophic bacteria detected on TSBA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were cultured on tryptone soya broth agar with the appropriate antibiotics. Independent replicates were used (n = 3). I = standard error of samples.

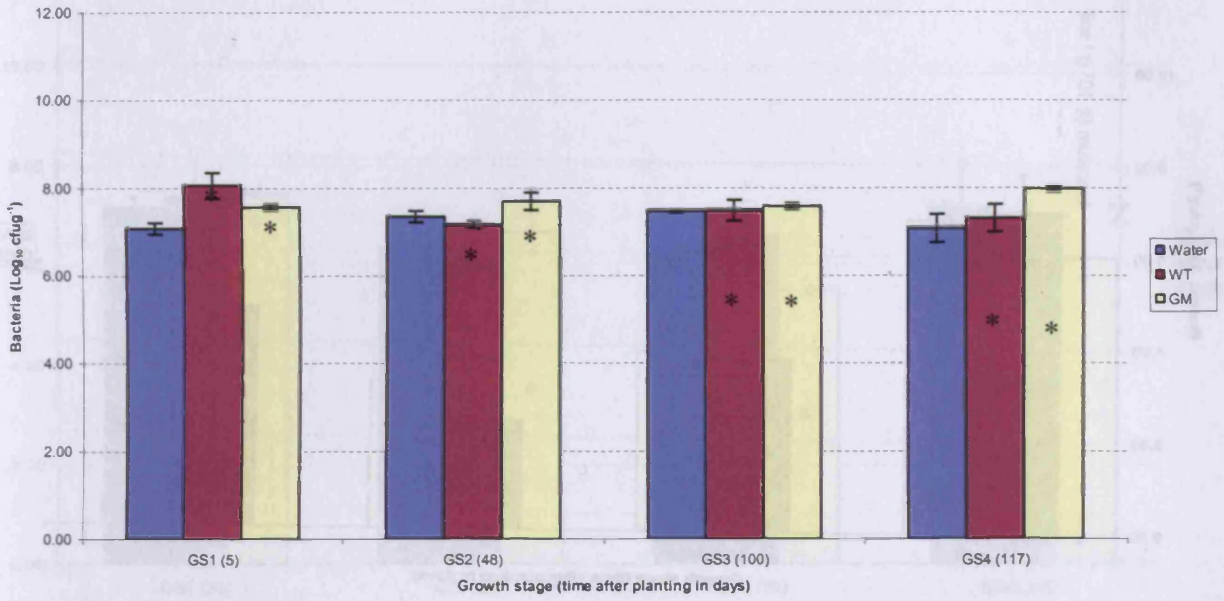

Figure 4.8. Wheat rhizosphere; mesocosm population densities of pseudomonads detected on PSA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were grown on pseudomonad selective agar with the appropriate antibiotics. Independent replicates were used (n = 3). * = population densities of the inocula in each treatment. I = standard error of samples.

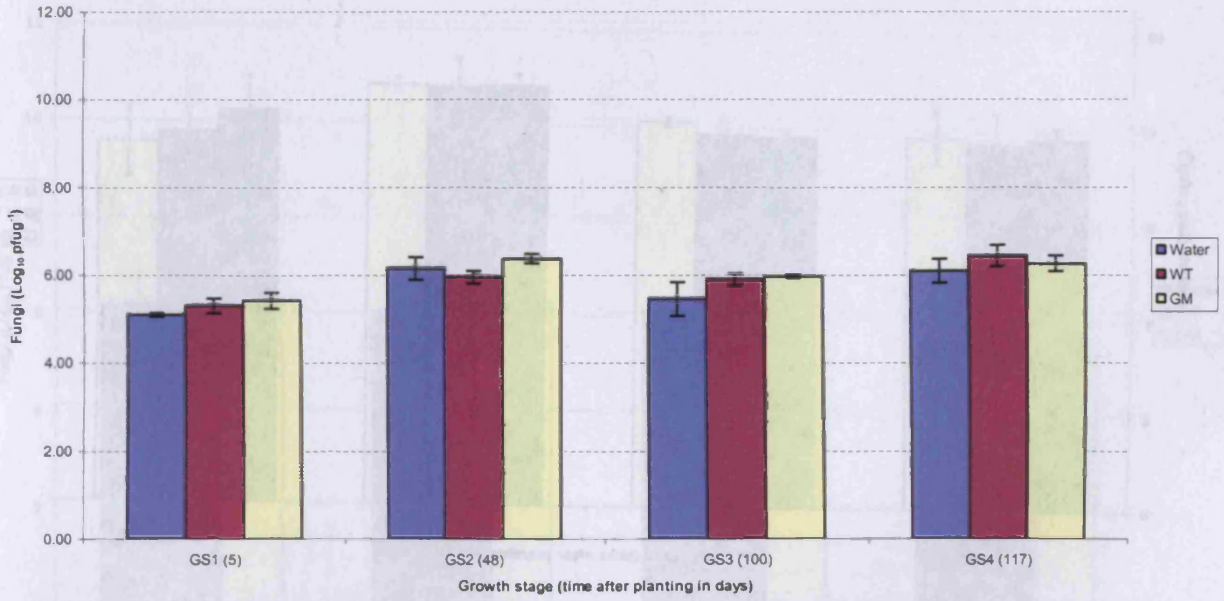

Figure 4.9. Wheat rhizosphere; mesocosm population densities of fungi on PDA for the three treatments: water control, WT (SBW25) and GM (23:10). Populations were estimated by propapgule forming units (pfu) on potato dextrose agar with appropriate antibiotic selection. Independent replicates were used (n = 3). I = standard error of samples.

Sugar beet rhizosphere culturable population densities.

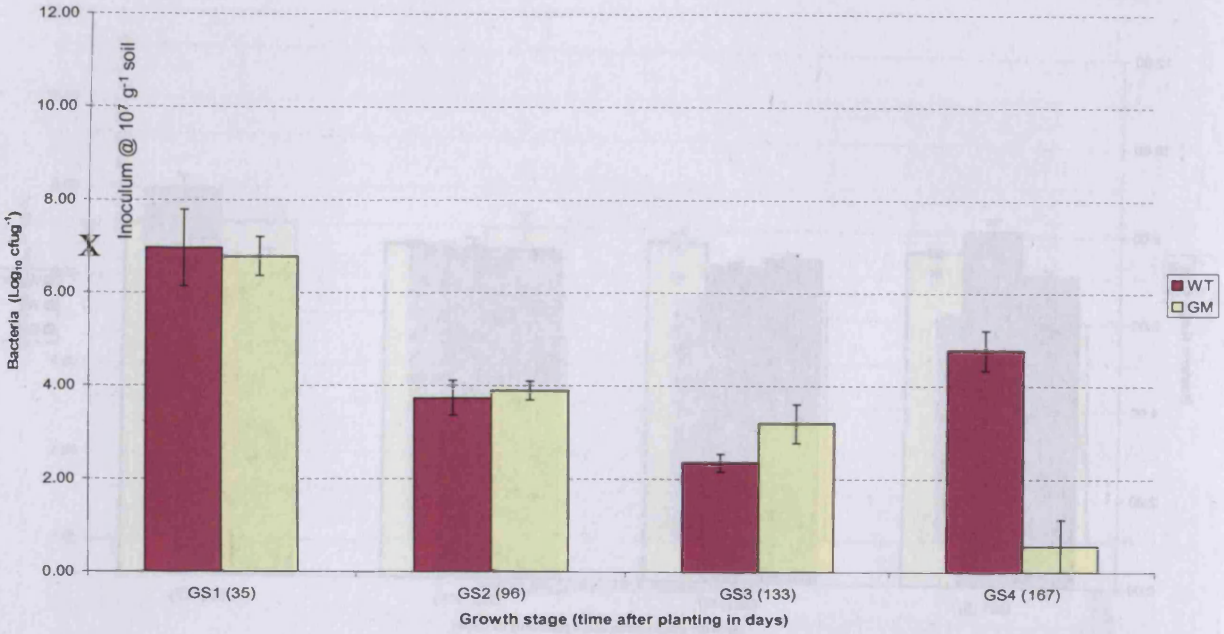

Figure 4.10. Sugar beet rhizosphere; mesocosm population densities of the bacterial inocula detected for the WT (SBW25) and GM (23:10) bacterial treatment. No inocula were detected in the water control (data no shown). Bacteria were cultured using pseudomonad selective agar (PSA) with appropriate antibiotic selection for the inocula. Independent replicates were used (n = 3). I = standard error of samples.

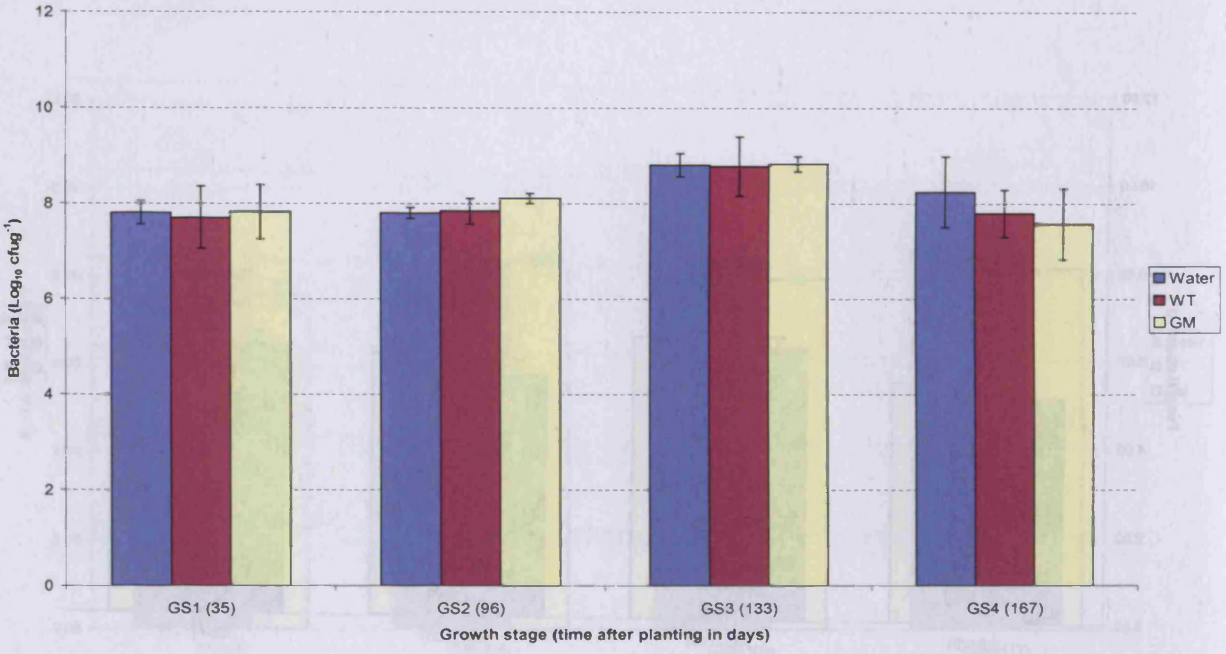

Figure 4.11. Sugar beet rhizosphere: mesocosm population densities of total culturable heterotrophic bacteria detected on TSBA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were cultured on tryptone soya broth agar with the appropriate antibiotics. Independent replicates were used (n = 3). I = standard error of samples.

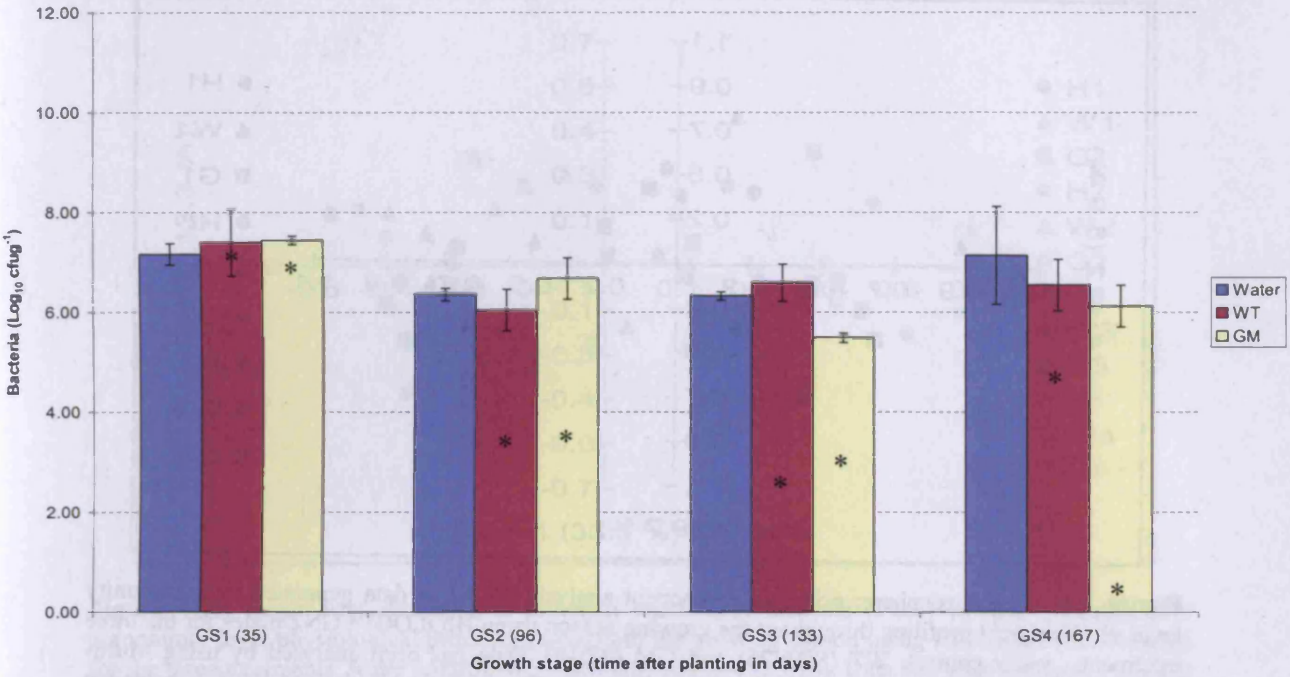

Figure 4.12. Sugar beet rhizosphere; mesocosm population densities of pseudomonads detected on PSA for the three treatments: water control, WT (SBW25) and GM (23:10). Bacteria were grown on pseudomonad selective agar with the appropriate antibiotics. * = population densities of the inocula in each treatment. Independent replicates were used (n = 3). I = standard error of samples.

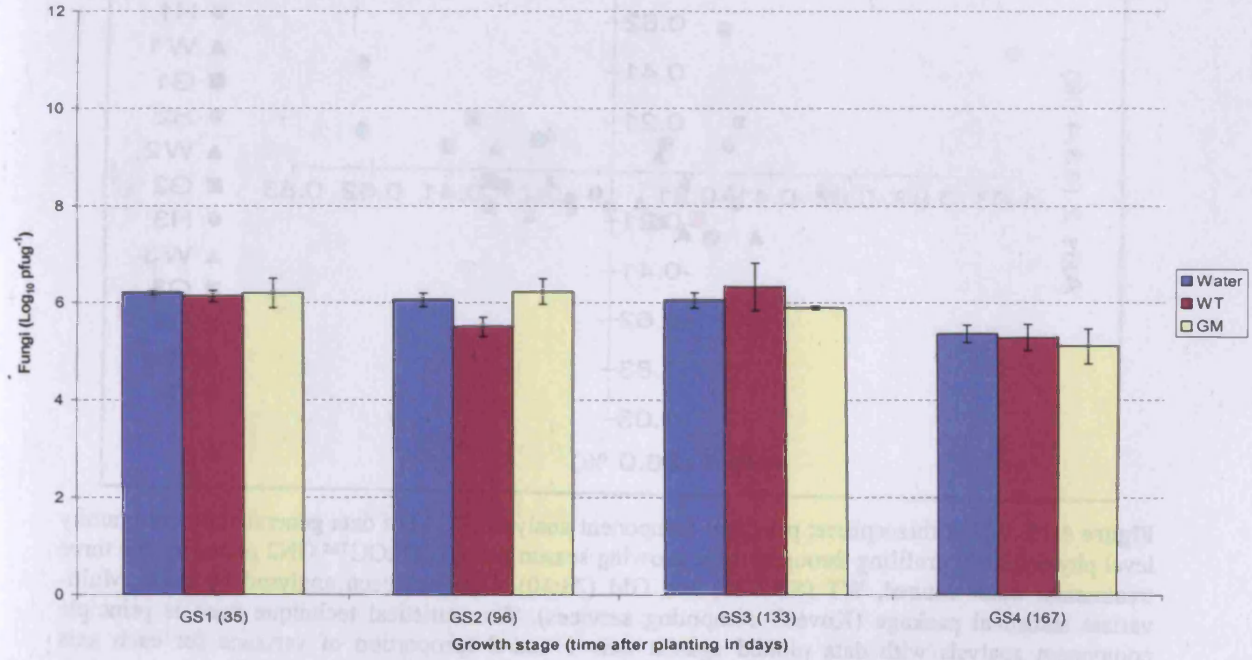

Figure 4.13. Sugar beet rhizosphere; mesocosm population densities of fungi on PDA for the three treatments: water control, WT (SBW25) and GM (23:10). Populations were estimated by propapgule forming units (pfu) on potato dextrose agar with appropriate antibiotic selection. Independent replicates were used (n = 3). I = standard error of samples.

CLPP of the rhizosphere of the three crop species.

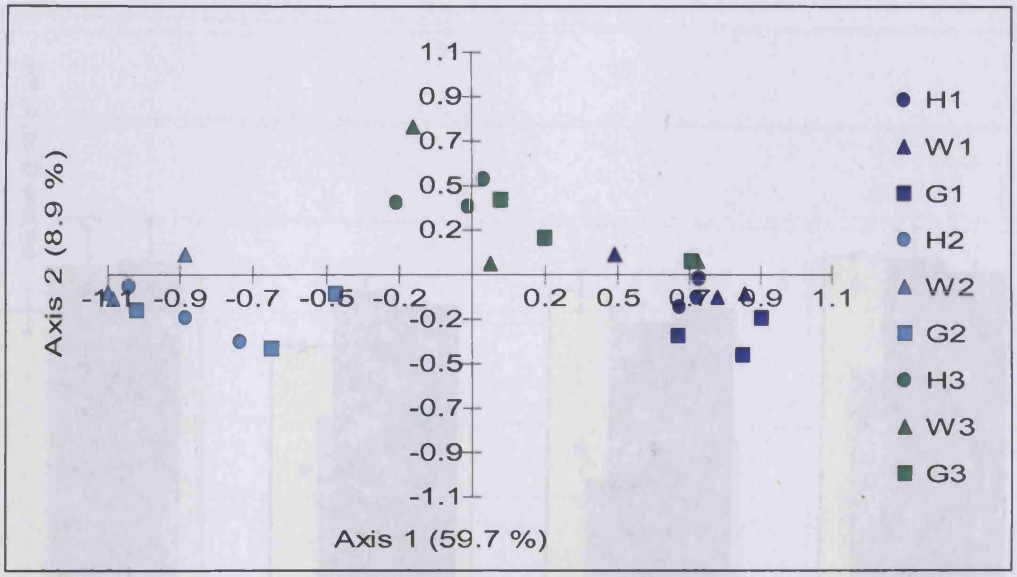

Figure 4.14. Pea rhizosphere; principal component analysis (PCA) of data generated by community level physiological profiling throughout the growing season using BIOLOG™ GN2 plates for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against Axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatments in legend. GS1 = 10 days after sowing, GS2 = 41 days and GS3 = 110 days. Independent replicates were used (n = 3).

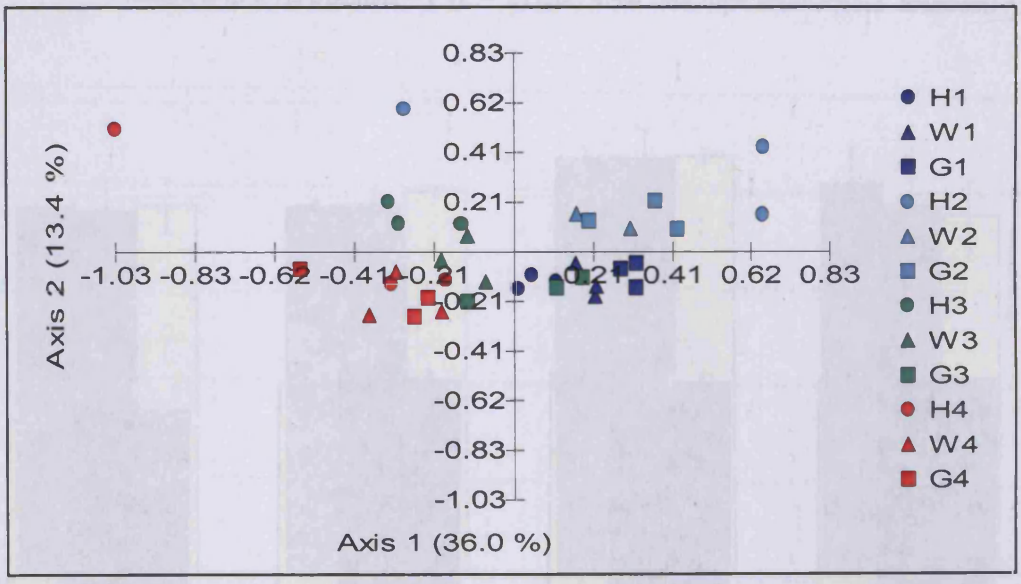

Figure 4.15. Wheat rhizosphere; principal component analysis (PCA) of data generated by community level physiological profiling throughout the growing season using BIOLOG™ GN2 plates for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatments in legend. GS1 = 5 days after sowing, GS2 = 48 days, GS3 = 100 days, and GS4 = 117 days. Independent replicates were used (n = 3).

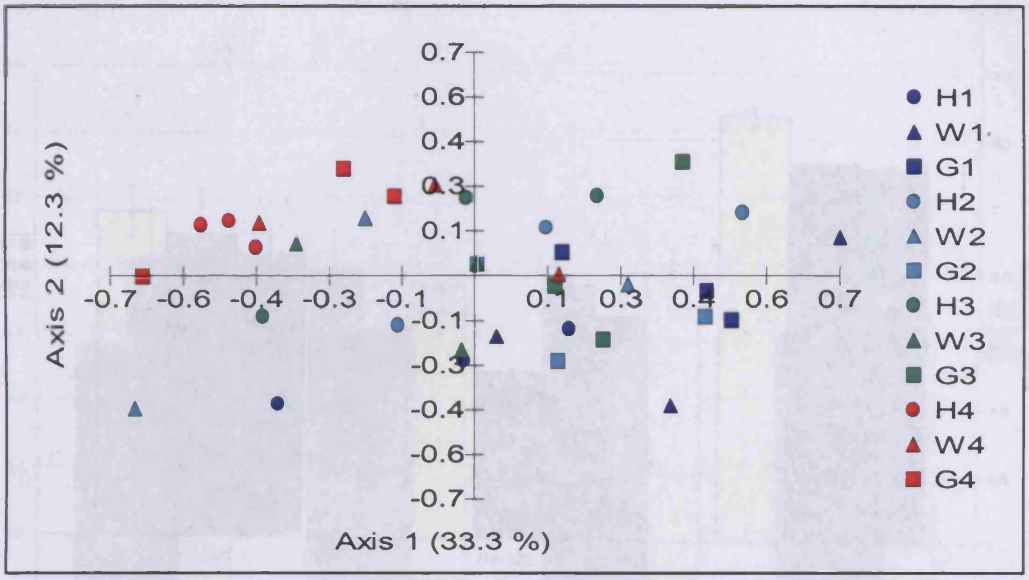

Figure 4.16. Sugar beet rhizosphere; principal component analysis (PCA) of data generated by community level physiological profiling throughout the growing season using BIOLOG™ GN2 plates for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using Multi-variate statistical package (Kovach computing services). The statistical technique used is principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data point for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatments in legend. GS1 = 35 days after sowing, GS2 = 96 days, GS3 = 133 days, and GS4 = 167 days. Independent replicates were used (n = 3).

AWC analysis of the rhizosphere of the three crop species.

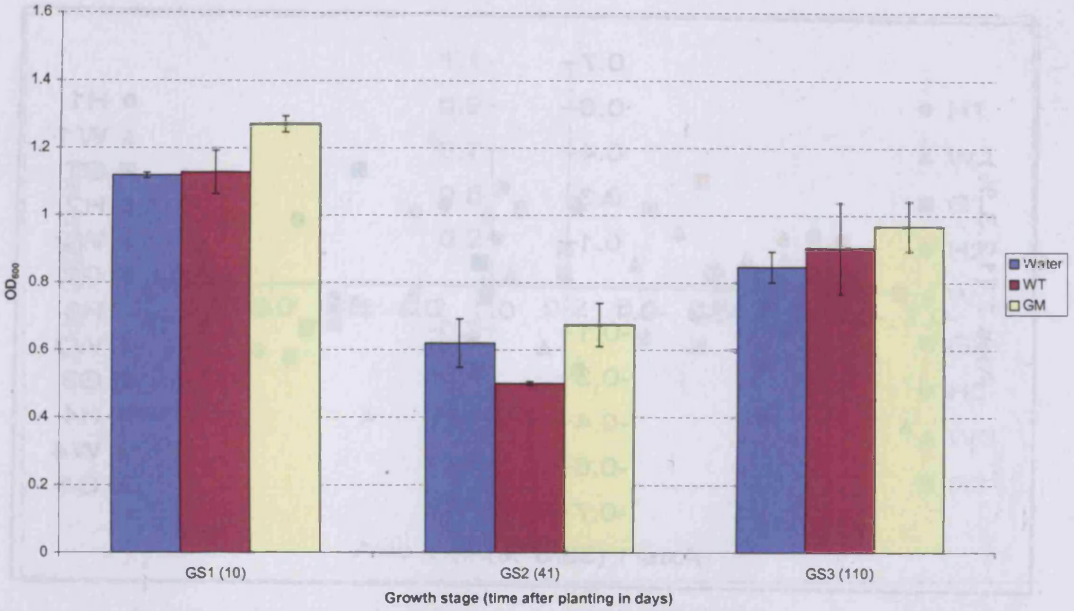

Figure 4.17. Pea rhizosphere; bar chart indicating average well colour difference throughout the growing season for the three treatments: water control, WT (SBW25) and GM (23:10).. using BIOLOG GN2 plates. Plate's optical densities were measured for each well and an average value was calculated for each plate. This value was used for average well colour difference comparisons. The three treatments were: water control, WT and GM. Independent replicates were used (n = 3). I = standard error of samples.

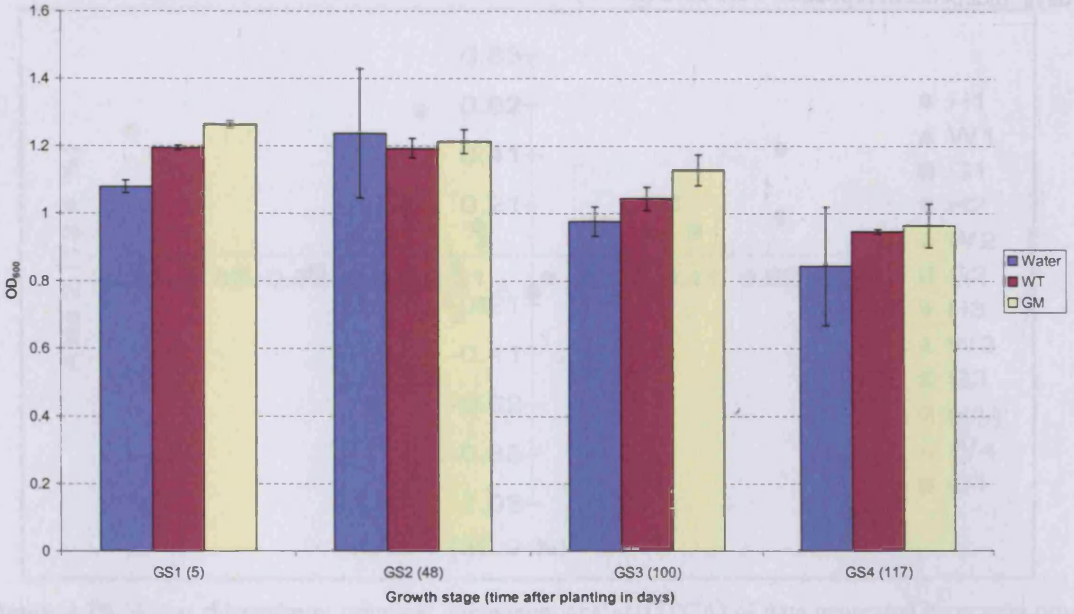

Figure 4.18. Wheat rhizosphere; bar chart indicating average well colour difference throughout the growing season using BIOLOG GN2 plates for the three treatments: water control, WT (SBW25) and GM (23:10).. Plate's optical densities were measured for each well and an average value was calculated for each plate. This value was used for average well colour difference comparisons. The three treatments were: water control, WT and GM. Independent replicates were used (n = 3). I = standard error of samples.

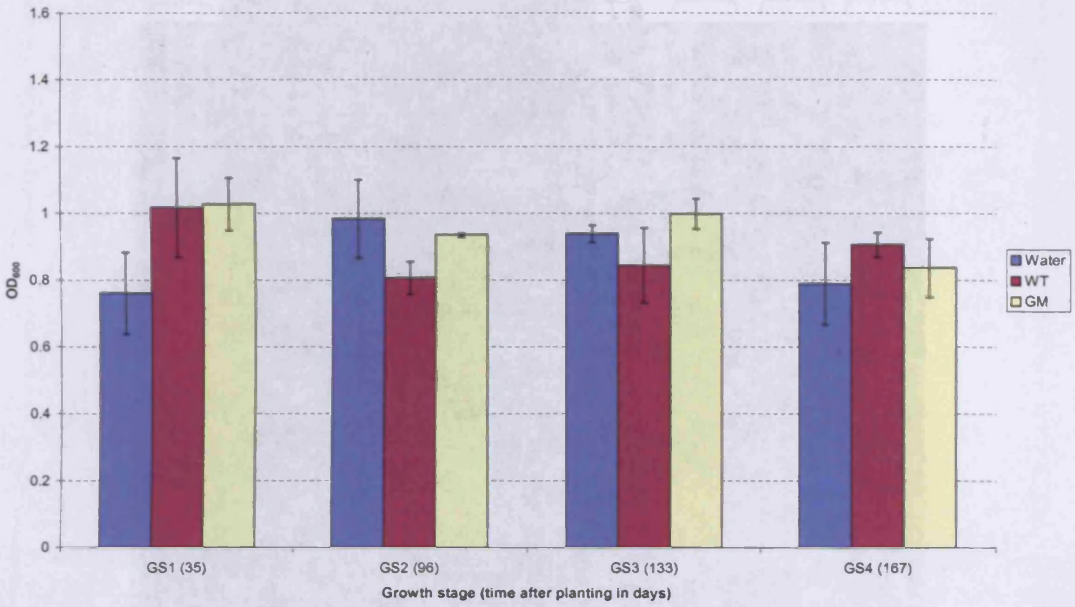

Figure 4.19. Sugar beet rhizosphere; bar chart indicating average well colour difference throughout the growing season for the three treatments: water control, WT (SBW25) and GM (23:10) using BIOLOG GN2 plates. Plate's optical densities were measured for each well and an average value was calculated for each plate. This value was used for average well colour difference comparisons. The three treatments were: water control, WT and GM. Independent replicates were used (n = 3). I = standard error of samples.

Examples of 16S DGGE images.

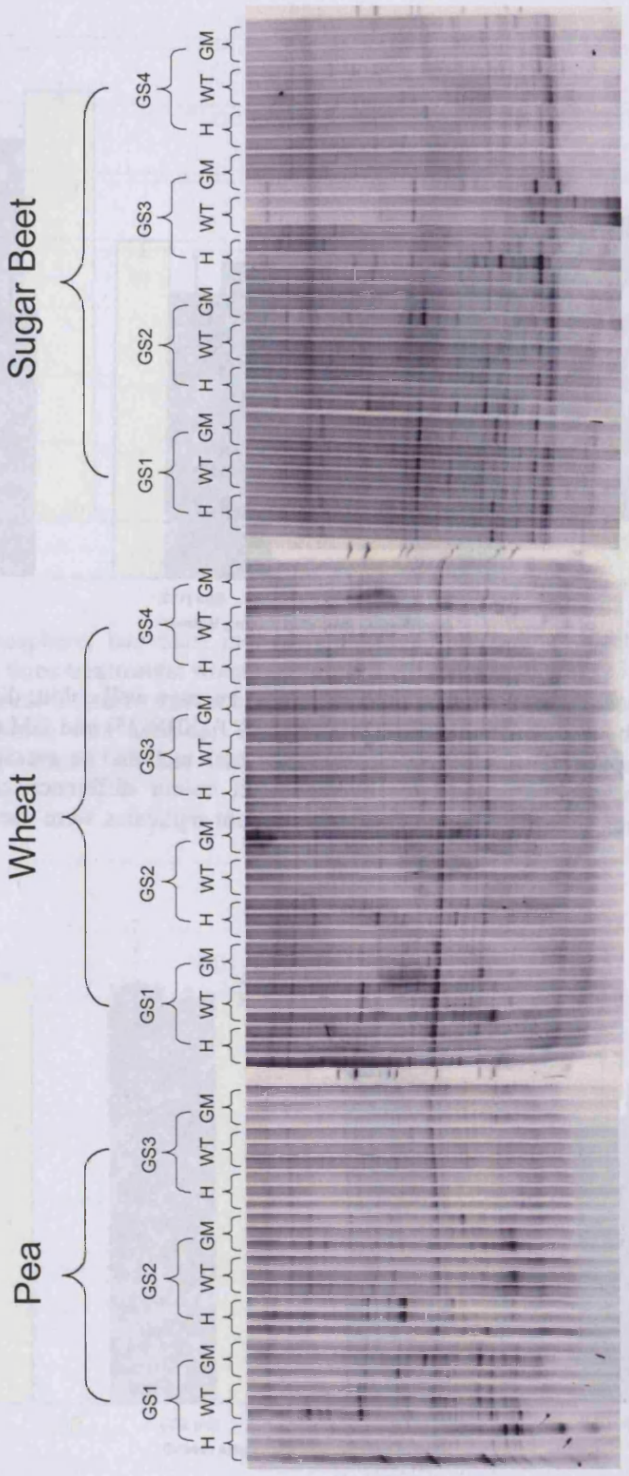

Figure 4.20. A composite image of the DGGE profiling of the rhizosphere of the three plant species using 16S eubacterial primers GC338F and 530R (52). Treatments are indicated by: H = water control, WT = SBW25 treatment, and GM = 23.10 treatment. Growth stages are indicated by the numbers 1, 2, 3, and 4. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; GS3 represented flowering mature plant; and GS4 represented fruiting plant, point of harvest. Independent replicates were used (n = 3).

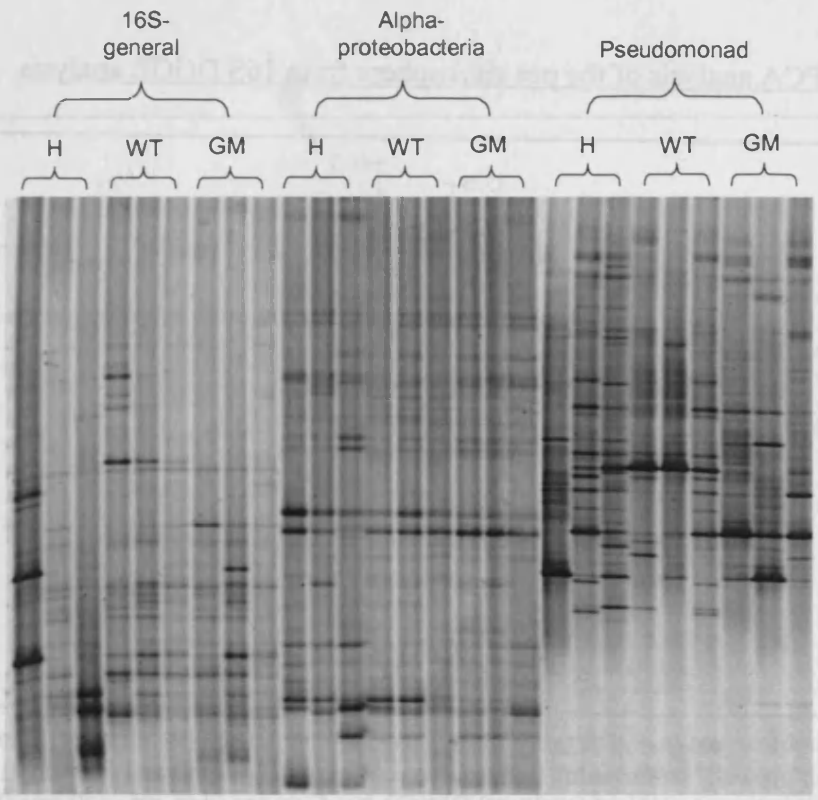

Figure 4.21. Pea rhizosphere; DGGE profiling using the 16S eubacterial primers (52), α-proteobacterial specific primers (18) and pseudomonad specific primers (53). DGGE show GS1 comparison of primer sets with the three treatments. Treatments are indicated by: H = water control, WT = SBW25 treatment, and GM = 23.10 treatment. Independent replicates were used (n = 3)

PCA analysis of the pea rhizosphere from 16S DGGE analysis.

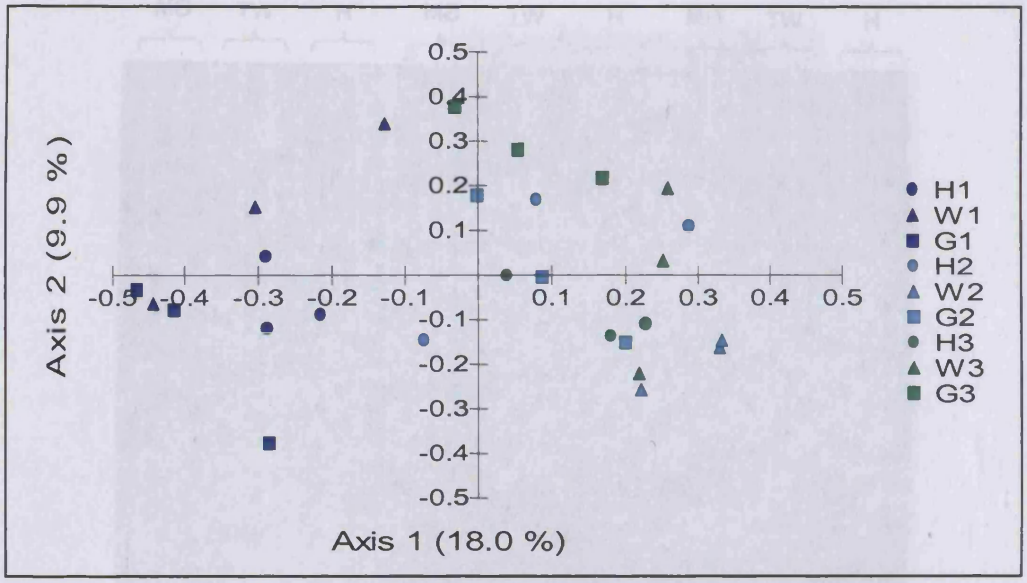

Figure 4.22. Pea rhizosphere; PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S eubacterial primers GC338F and 530R (52) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatment in legend. GS1 = 10 days after sowing, GS2 = 41 days and GS3 = 110 days. Independent replicates were used (n = 3).

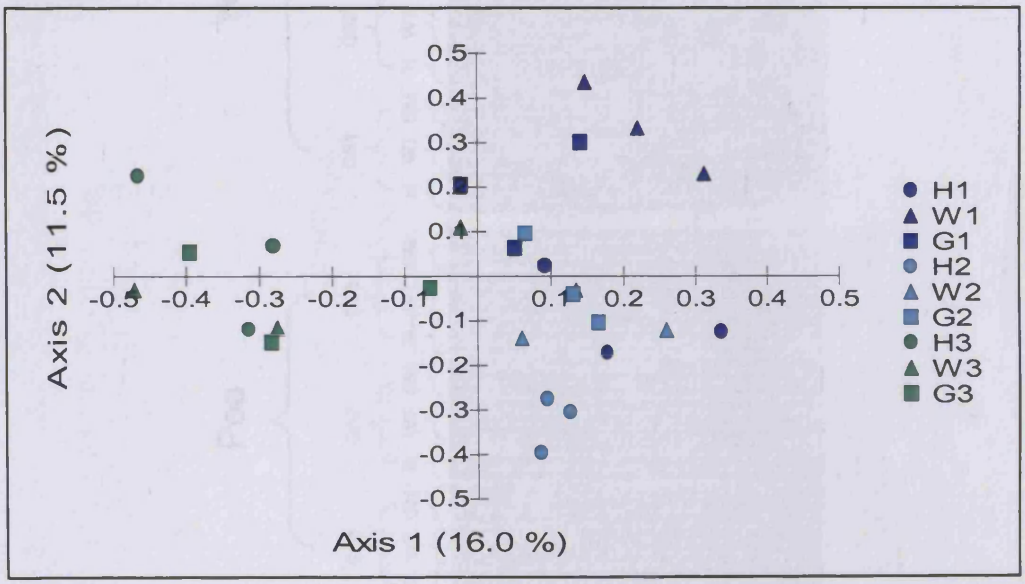

Figure 4.23. Pea rhizosphere; Seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S α-proteobacteria primers (18) for the three treatments: water control, WT (SBW25) and GM (23:10).. Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against Axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatments in legend. GS1 = 10 days after sowing, GS2 = 41 days and GS3 = 110 days. Independent replicates were used (n = 3).

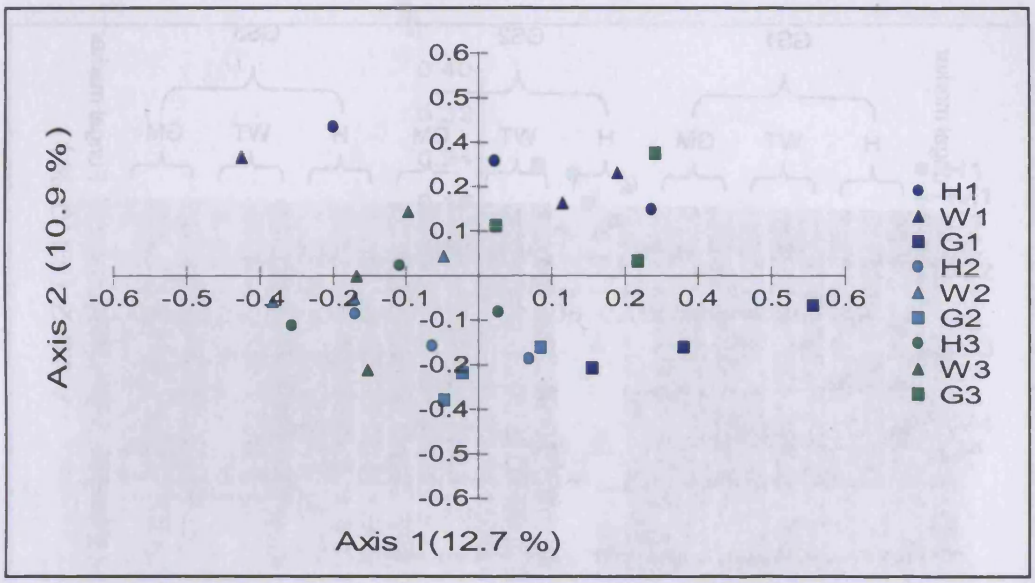

Figure 4.24. Pea rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S γ-proteobacteria (53) for the three treatments: water control, WT (SBW25) and GM (23:10).. Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted Axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatments in legend. GS1 = 10 days after sowing, GS2 = 41 days and GS3 = 110 days. Independent replicates were used (n = 3).

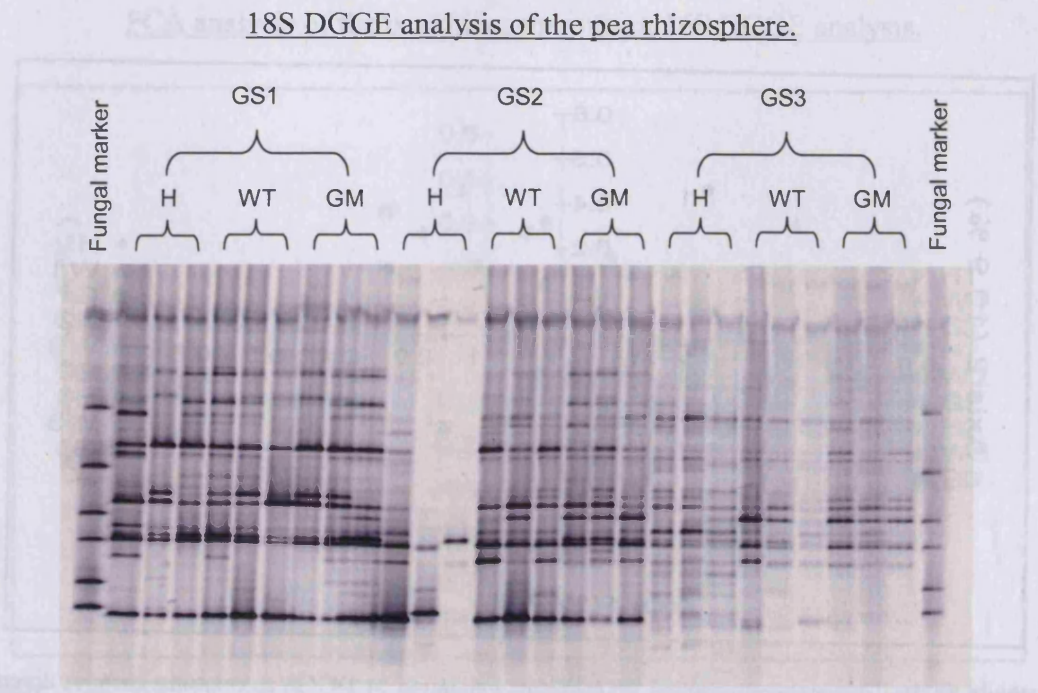

Figure 4.25. Pea rhizosphere; DGGE profiling using 18S primers NS1 and NS2-10GC (22). Treatments are indicated by: H = water control, WT = SBW25 treatment, and GM = 23.10 treatment. Growth stages are indicated by the numbers 1, 2, 3, 4, and 5. Growth stage one (GS1) represented young seedling; GS2 represented growing plant, approximate midpoint of rapid growth; and GS3 represented flowering mature plant. Independent replicates were used (n = 3)

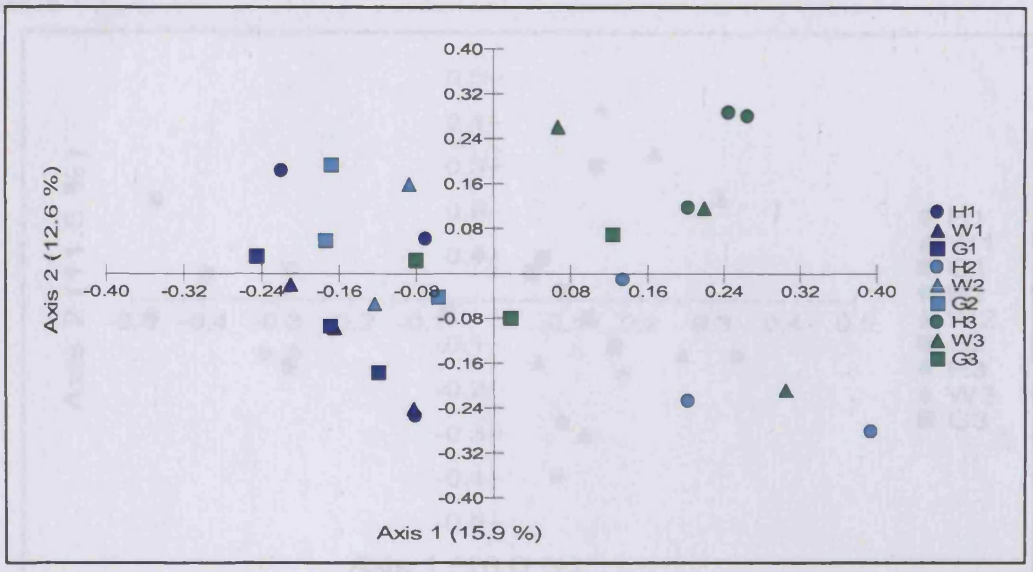

Figure 4.26. Pea rhizosphere; PCA of data generated by DGGE (Figure 4.21.) to measure fungal diversity throughout the growing season for the three treatments: water control, WT (SBW25) and GM (23:10). Profiles were generated using 18S primers NS1 and NS2-10GC (22). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against Axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2 and 3 to treatment in legend. GS1 = 10 days after sowing, GS2 = 41 days and GS3 = 110 days. Independent replicates were used (n = 3).

DGGE analysis of the wheat rhizosphere.

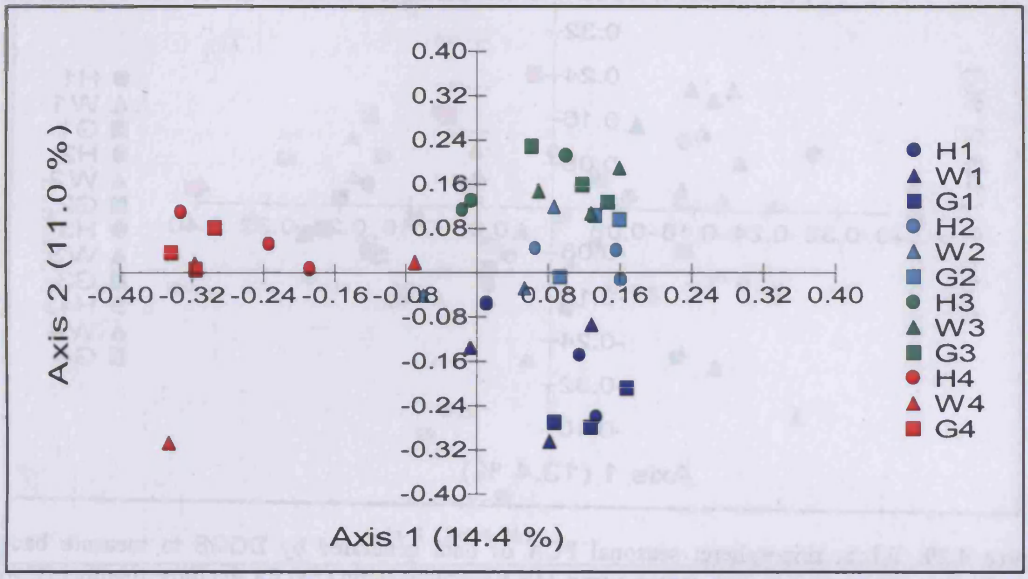

Figure 4.27. Wheat rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S eubacterial primers GC338F and 530R (52) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2, 3 and 4 to treatmentsin legend. GS1 = 5 days after sowing, GS2 = 48 days, GS3 = 100 days, and GS4 = 117. Independent replicates were used (n = 3).

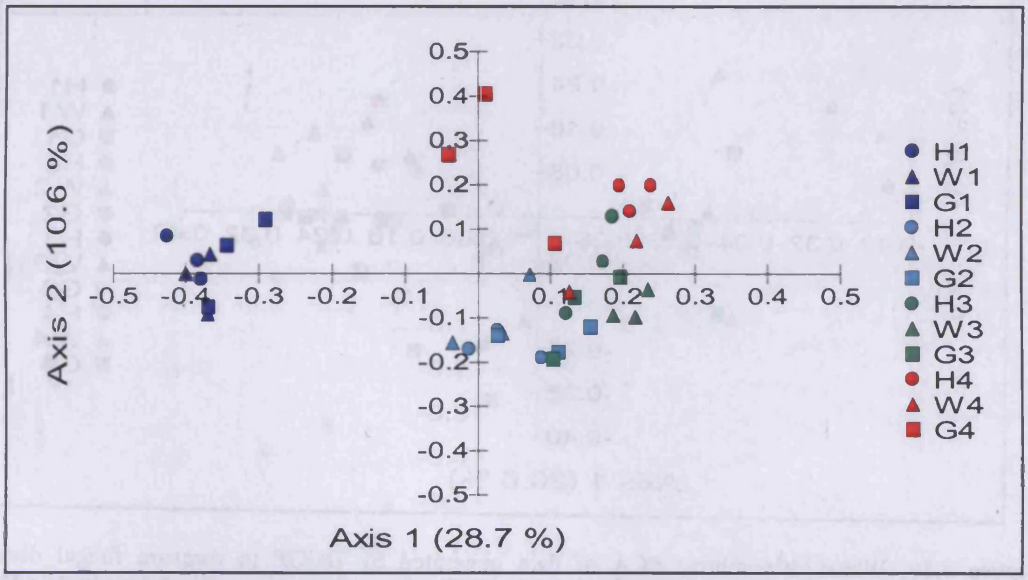

Figure 4.28. Wheat rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S α-proteobacteria (18) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2, 3 and 4 to treatments in legend. GS1 = 5 days after sowing, GS2 = 48 days, GS3 = 100 days, and GS4 = 117. Independent replicates were used (n = 3).

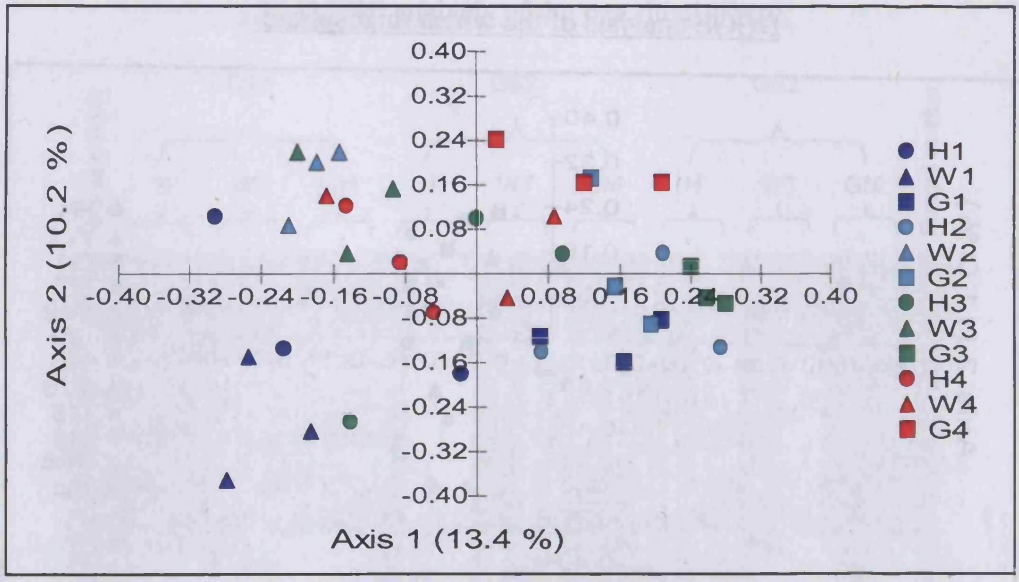

Figure 4.29. Wheat rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S γ-proteobacteria (53) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2, 3 and 4 to treatments in legend. GS1 = 5 days after sowing, GS2 = 48 days, GS3 = 100 days, and GS4 = 117. Independent replicates were used (n = 3).

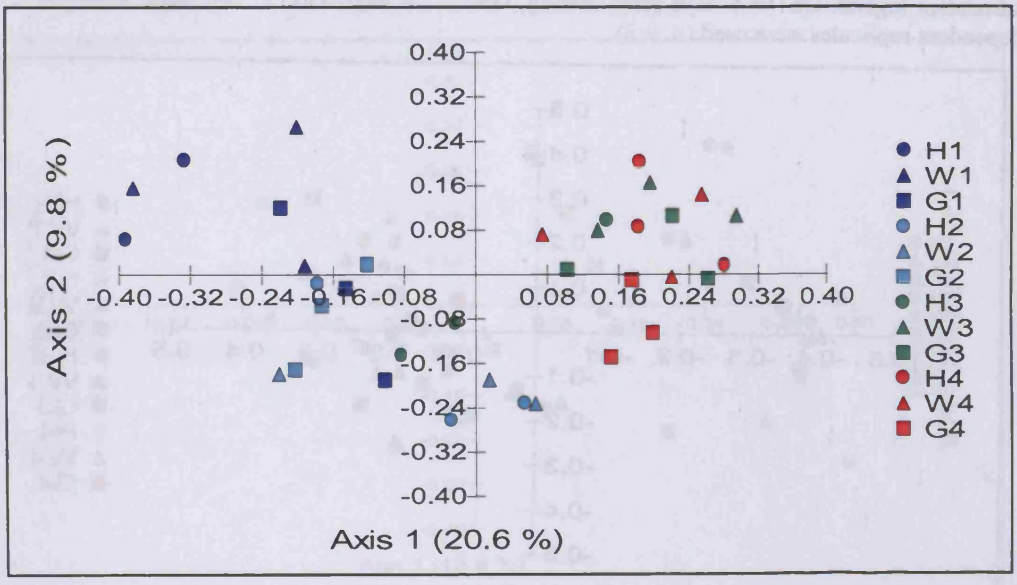

Figure 4.30. Wheat rhizosphere; PCA of data generated by DGGE to measure fungal diversity throughout the growing season for the three treatments: water control, WT (SBW25) and GM (23:10). Profiles were generated using 18S primers NS1 and NS2-10GC (22). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data points for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1 ,2, 3 and 4 to treatmentsin legend. GS1 = 5 days after sowing, GS2 = 48 days, GS3 = 100 days, and GS4 = 117. Independent replicates were used (n = 3).

DGGE analysis of the sugar beet rhizosphere.

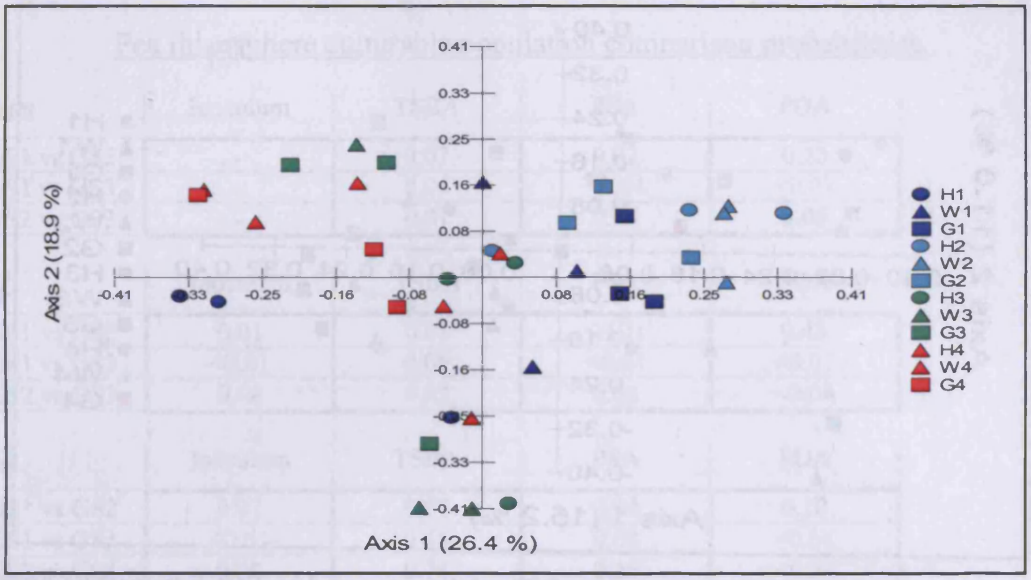

Figure 4.31. Sugar beet rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S eubacterial primers GC338F and 530R (52) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data point for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1, 2, 3 and 4 to treatments in legend. GS1 = 35 days after sowing, GS2 = 96 days, GS3 = 133 days, and GS4 = 167. Independent replicates were used (n = 3).

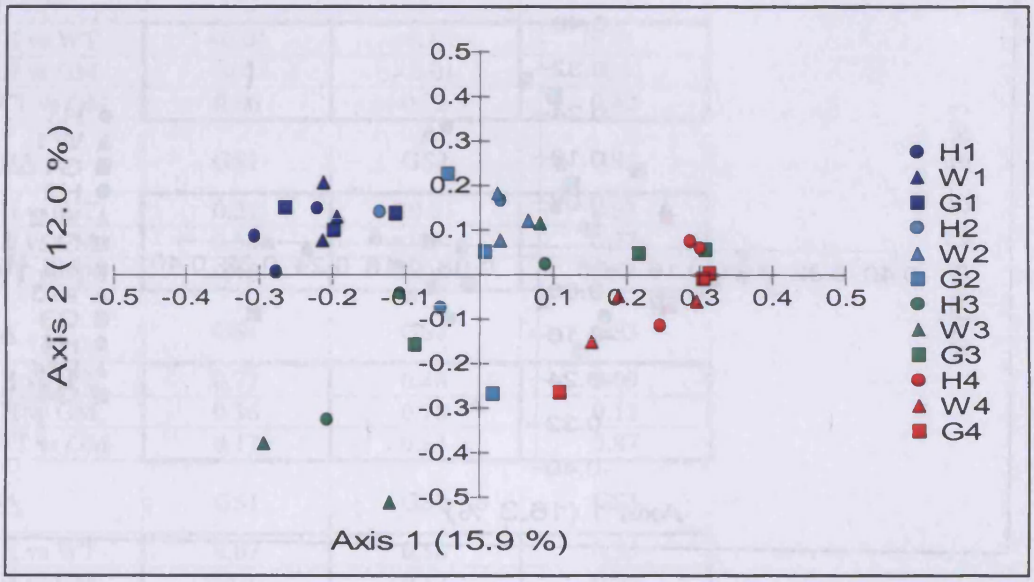

Figure 4.32. Sugar beet rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S α-proteobacteria (18) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data point for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1, 2, 3 and 4 to treatments in legend. GS1 = 35 days after sowing, GS2 = 96 days, GS3 = 133 days, and GS4 = 167. Independent replicates were used (n = 3).

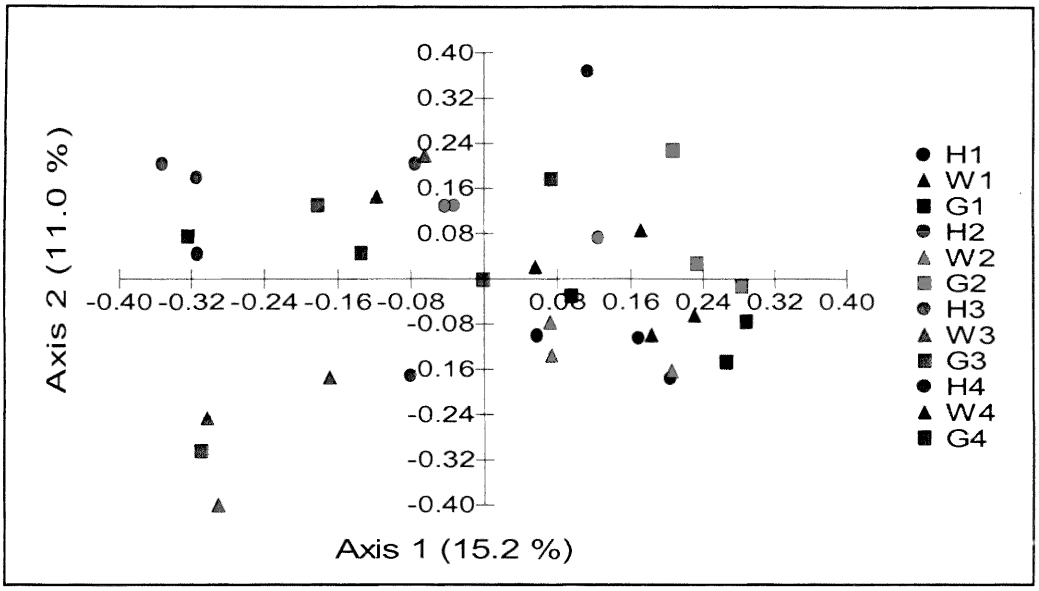

Figure 4.33. Sugar beet rhizosphere; seasonal PCA of data generated by DGGE to measure bacteria diversity throughout the growing season using 16S γ-proteobacteria (53) for the three treatments: water control, WT (SBW25) and GM (23:10). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data point for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1, 2, 3 and 4 to treatments in legend. GS1 = 35 days after sowing, GS2 = 96 days, GS3 = 133 days, and GS4 = 167. Independent replicates were used (n = 3).

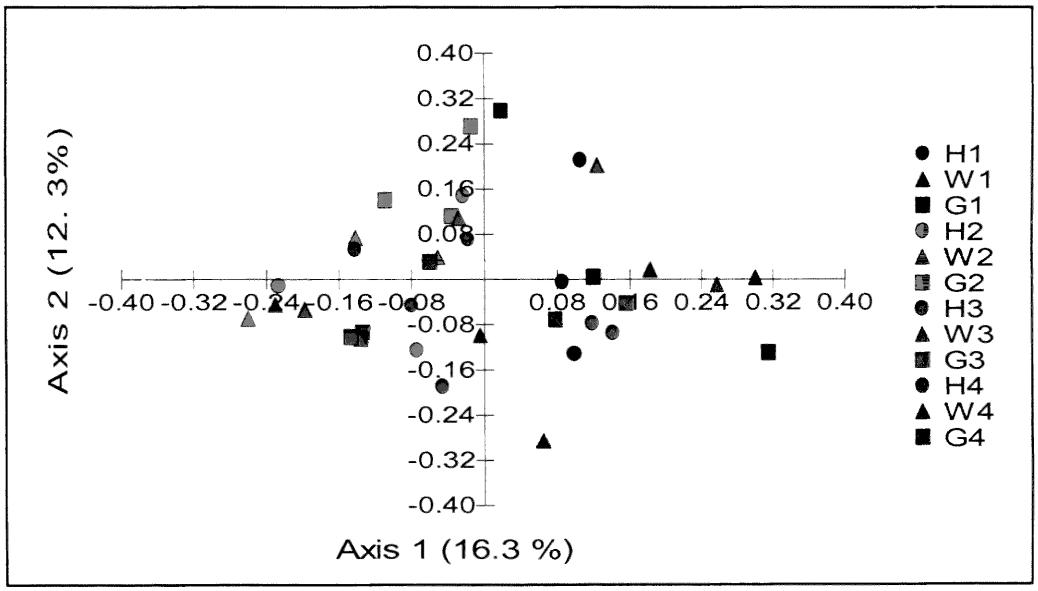

Figure 4.34. Sugar beet rhizosphere; PCA of data generated by DGGE to measure fungal diversity throughout the growing season for the three treatments: water control, WT (SBW25) and GM (23:10).. Profiles were generated using 18S primers NS1 and NS2-10GC (22). Data has been analysed by using the Multi-variate statistical package (Kovach computing services). The statistical technique used was principle component analysis with data plotted against axis 1 and 2 (proportion of variance for each axis represented in brackets). Data point for treatments are represented in the legend by H = water control, W = WT inoculum, G = GM inoculum. Growth stages are indicated as suffix 1, 2, 3 and 4 to treatments in legend. GS1 = 35 days after sowing, GS2 = 96 days, GS3 = 133 days, and GS4 = 167. Independent replicates were used (n = 3).

4.6 Tables.

Pea rhizosphere culturable population comparison probabilities.

Water	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	-	0.07	0.02	0.20
GS1 vs GS3	-	0.04	<0.01	<0.01
GS2 vs GS3	-	0.01	0.04	0.06
WT	**Inoculum**	**TSBA**	**PSA**	**PDA**
GS1 vs GS2	0.01	0.01	<0.01	0.43
GS1 vs GS3	<0.01	0.06	<0.01	<0.01
GS2 vs GS3	0.08	0.92	0.08	<0.01
GM	**Inoculum**	**TSBA**	**PSA**	**PDA**
GS1 vs GS2	0.02	0.07	0.04	0.10
GS1 vs GS3	<0.01	0.45	0.03	<0.01
GS2 vs GS3	0.02	0.74	0.26	<0.01

Table 4.1. Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the pea rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Tables are for each individual treatment; water control, WT (SBW25) treatment and GM (23.10) treatment. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Inoculum	GS1	GS2	GS3
H vs WT	<0.01	<0.01	0.01
H vs GM	<0.01	<0.01	<0.01
WT vs GM	0.66	0.22	0.42
TSBA	**GS1**	**GS2**	**GS3**
H vs WT	0.24	<0.01	0.07
H vs GM	0.54	<0.01	0.27
WT vs GM	0.18	0.06	0.95
PSA	**GS1**	**GS2**	**GS3**
H vs WT	0.72	0.48	0.09
H vs GM	0.36	0.78	0.13
WT vs GM	0.17	0.83	0.87
PDA	**GS1**	**GS2**	**GS3**
H vs WT	0.07	0.58	0.23
H vs GM	<0.01	0.12	0.91
WT vs GM	0.02	0.09	0.34

Table 4.2 Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the pea rhizosphere in comparisons between the water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK), fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

Wheat rhizosphere culturable population comparison probabilities.

Water	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	-	0.15	0.22	0.05
GS1 vs GS3	-	<0.01	0.08	0.44
GS1 vs GS4	-	<0.01	0.99	0.06
GS2 vs GS3	-	0.43	0.46	0.22
GS2 vs GS4	-	0.03	0.49	0.88
GS3 vs GS4	-	<0.05	0.34	0.25

WT	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	0.04	0.81	0.09	0.04
GS1 vs GS3	<0.01	0.64	0.21	0.05
GS1 vs GS4	<0.01	0.57	0.16	0.02
GS2 vs GS3	<0.01	0.86	0.31	0.81
GS2 vs GS4	<0.01	0.73	0.71	0.18
GS3 vs GS4	0.04	0.83	0.67	0.15

GM	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	0.74	0.08	0.59	0.02
GS1 vs GS3	<0.01	0.29	0.93	0.09
GS1 vs GS4	0.03	0.14	0.01	0.03
GS2 vs GS3	0.03	0.04	0.62	0.05
GS2 vs GS4	0.02	0.11	0.30	0.62
GS3 vs GS4	0.35	0.16	0.01	0.25

Table 4.3. Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the wheat rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Tables are for each individual treatment; water control, WT (SBW25) treatment and GM (23.10) treatment. Significance level, $p = <0.05$ indicated by values highlighted in red. Independent replicates were used ($n = 3$).

Inoculum	GS1	GS2	GS3	GS4
H vs WT	<0.01	<0.01	<0.01	<0.01
H vs GM	<0.01	<0.01	<0.01	<0.01
WT vs GM	0.12	0.35	0.81	0.66
TSBA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.17	0.40	0.38	0.76
H vs GM	0.77	0.09	0.50	0.09
WT vs GM	0.24	0.82	0.28	0.56
PSA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.06	0.33	0.95	0.63
H vs GM	0.04	0.23	0.27	0.10
WT vs GM	0.23	0.10	0.75	0.16
PDA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.37	0.55	0.36	0.40
H vs GM	0.22	0.51	0.31	0.65
WT vs GM	0.67	0.08	0.67	0.58

Table 4.4. Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the wheat rhizosphere in comparisons between the water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK), fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Significance level, $p = <0.05$ indicated by values highlighted in red. Independent replicates were used ($n = 3$).

Sugar beet rhizosphere culturable population comparison probabilities.

Water	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	-	0.98	0.04	0.41
GS1 vs GS3	-	0.04	0.04	0.43
GS1 vs GS4	-	0.62	0.98	0.03
GS2 vs GS3	-	0.04	0.87	0.98
GS2 vs GS4	-	0.61	0.51	0.04
GS3 vs GS4	-	0.53	0.50	0.04

WT	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	0.04	0.86	0.17	0.07
GS1 vs GS3	0.03	0.30	0.37	0.73
GS1 vs GS4	0.10	0.91	0.37	0.07
GS2 vs GS3	0.05	0.27	0.38	0.23
GS2 vs GS4	0.15	0.94	0.49	0.55
GS3 vs GS4	0.02	0.29	0.94	0.16

GM	Inoculum	TSBA	PSA	PDA
GS1 vs GS2	<0.01	0.67	0.20	0.95
GS1 vs GS3	<0.01	0.21	<0.01	0.42
GS1 vs GS4	<0.01	0.81	0.08	0.08
GS2 vs GS3	0.23	0.02	0.10	0.33
GS2 vs GS4	0.02	0.55	0.40	0.07
GS3 vs GS4	0.02	0.23	0.26	0.16

Table 4.5. Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the sugar beet rhizosphere. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK) fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Tables are for each individual treatment; water control (H), WT (SBW25) treatment and GM (23.10) treatment. Significance level, $p = <0.05$ indicated by values highlighted in red. Independent replicates were used ($n = 3$).

Inoculum	GS1	GS2	GS3	GS4
H vs WT	0.01	<0.01	<0.01	<0.01
H vs GM	<0.01	<0.01	0.02	0.42
WT vs GM	0.71	0.73	0.08	0.01
TSBA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.86	0.91	0.95	0.32
H vs GM	0.99	<0.05	0.88	0.14
WT vs GM	0.92	0.55	0.92	0.72
PSA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.67	0.52	0.62	0.34
H vs GM	0.28	0.53	0.04	0.32
WT vs GM	0.95	<0.01	0.09	0.32
PDA	**GS1**	**GS2**	**GS3**	**GS4**
H vs WT	0.48	0.03	0.56	0.61
H vs GM	1.00	0.68	0.44	0.51
WT vs GM	0.87	0.18	0.49	0.52

Table 4.6. Table of probabilities generated by two tailed *t*-test when comparing values enumerated for different bacterial population densities in the sugar beet rhizosphere in comparisons between the water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Total culturable heterotrophs were isolated on TSBA (Difco-oxoid, UK), pseudomonad population densities were estimated on PSA (Difco-oxoid, UK), fungal population densities were estimated on PDA (Difco-oxoid, UK) and the inoculum on PSA with appropriate antibiotic selection. Significance level, $p = <0.05$ indicated by values highlighted in red. Independent replicates were used ($n = 3$).

Rhizosphere BIOLOG™ and AWC comparison probabilities in the three crop species.

	GS1	GS2	GS3
H vs WT	<0.01	<0.01	0.22
H vs GM	<0.01	<0.01	<0.01
WT vs GM	<0.01	<0.01	0.03

Table 4.7. Pea rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified between the different treatments; Water control (H), WT (SBW25) and GM (23.10) at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	<0.01	<0.01	<0.01
GS1 vs GS3	<0.01	<0.01	<0.01
GS2 vs GS3	<0.01	<0.01	<0.01

Table 4.8. Pea rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified at different growth stages for the three treatments; water control, wt (SBW25 and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	GS1	GS2	GS3	GS4
H vs WT	<0.01	0.14	<0.01	<0.01
H vs GM	<0.01	0.43	<0.01	<0.01
WT vs GM	0.01	0.39	<0.01	0.46

Table 4.9. Wheat rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified between the different treatments; Water control (H), WT (SBW25) and GM (23.10) at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	<0.01	0.93	0.21
GS1 vs GS3	<0.01	<0.01	<0.01
GS1 vs GS4	<0.01	<0.01	<0.01
GS2 vs GS3	<0.01	<0.01	<0.01
GS2 vs GS4	<0.01	<0.01	<0.01
GS3 vs GS4	<0.01	<0.01	<0.01

Table 4.10. Wheat rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified at different growth stages for the three treatments; water control, wt (SBW25 and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	GS1	GS2	GS3	GS4
H vs WT	<0.01	<0.01	<0.01	<0.01
H vs GM	<0.01	0.09	0.13	0.01
WT vs GM	0.70	<0.01	<0.01	<0.01

Table 4.11. Sugar beet rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified between the different treatments; Water control (H), WT (SBW25) and GM (23.10) at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	<0.01	<0.01	<0.01
GS1 vs GS3	<0.01	<0.01	0.33
GS1 vs GS4	0.51	<0.01	<0.01
GS2 vs GS3	0.11	0.21	0.01
GS2 vs GS4	0.11	0.21	0.01
GS3 vs GS4	<0.01	<0.01	<0.01

Table 4.12. Sugar beet rhizosphere; Table showing probabilities of differences between BIOLOG™ data identified at different growth stages for the three treatments; water control, wt (SBW25 and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	<0.01	<0.01	<0.01
GS1 vs GS3	<0.01	0.21	0.01
GS2 vs GS3	0.06	0.04	0.04

Table 4.13. Pea rhizosphere: Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between identified at different growth stages for the three treatments; water control, WT (SBW25) and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	GS1	GS2	GS3
H vs WT	0.89	0.17	0.72
H vs GM	<0.01	0.60	0.24
WT vs GM	0.11	0.05	0.69

Table 4.14. Pea rhizosphere; Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between the different treatments, water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	0.46	0.94	0.24
GS1 vs GS3	0.10	0.01	<0.05
GS1 vs GS4	0.25	<0.01	0.01
GS2 vs GS3	0.26	0.03	0.22
GS2 vs GS4	0.20	<0.01	0.03
GS3 vs GS4	0.50	<0.05	0.11

Table 4.15. Wheat rhizosphere; Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between identified at different growth stages for the three treatments; water control, WT (SBW25) and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	GS1	GS2	GS3	GS4
H vs WT	<0.01	0.83	0.29	0.59
H vs GM	<0.01	0.91	0.07	0.56
WT vs GM	<0.01	0.68	0.21	0.80

Table 4.16. Wheat rhizosphere; Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between the different treatments, water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Significance level, p = <0.05 indicated by values highlighted in red. Independent replicates were used (n = 3).

	Water	WT	GM
GS1 vs GS2	0.27	0.33	0.17
GS1 vs GS3	0.34	0.23	0.74
GS1 vs GS4	0.83	0.42	0.10
GS2 vs GS3	0.80	0.82	0.53
GS2 vs GS4	0.19	0.56	0.38
GS3 vs GS4	0.25	0.35	0.24

Table 4.17. Sugar beet rhizosphere; Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between identified at different growth stages for the three treatments; water control, WT (SBW25) and GM (23.10). Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Independent replicates were used (n = 3).

	GS1	GS2	GS3	GS4
H vs WT	0.20	0.41	0.46	0.06
H vs GM	0.10	0.73	0.69	0.61
WT vs GM	0.94	0.46	0.15	0.56

Table 4.18. Sugar beet rhizosphere; Table showing probabilities of differences between average well colour for each BIOLOG™ plate used for comparison between the different treatments, water control (H), WT (SBW25) and GM (23.10) treatments at individual growth stages. Probability values (p) were calculated using paired two-tailed *t*-test on the average substrate well colour between growth stages. Independent replicates were used (n = 3).

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Chapter Five: Discussion.

5.1. Discussion.

It is apparent that agricultural practices are highly dependent on agrochemicals in order to achieve maximum yields of crops by the suppression of disease and by plant growth promotion. Their use has become more regulated, with an increasing dislike of the use of broad spectrum pesticides as a preventative measure for disease suppression. This has resulted in the identification of residual toxic effects on the soil ecosystems by agrochemicals. These ecological concerns have resulted in investigations into the potential use of bacteria with natural suppressive abilities towards crop diseases as a potentially ecologically safer alternative (35). Numerous species of bacteria are considered to promote plant growth, they include *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Pseudomonas, Rhizobium and Serratia* (1, 5, 15, 16). The mode of action of these BCAs is multifactorial with a variety of mechanism identified as important in disease suppression. These include direct resource competition, induced resistance, siderophore production, niche habitation, and, perhaps the most important, the production of secondary metabolites (8, 36). Several secondary metabolites produced by pseudomonads and linked with disease control have been described. These include phenazines (23), phloroglucinols (9, 18), pyoluteorin (19), pyrrolnitrin (27), and hydrogen cyanide (HCN) (34). Phenazine biosynthesis has been demonstrated to be important in biocontrol ability, and the insertion as a novel trait greatly improves the host bacterium's ability for biocontrol (Figure 4.1.) (22-24, 32). *Pseudomonas fluorecens* SBW25 was chosen as a suitable bacterium for genetic modification because of the absence of any known secondary metabolites with anti fungal properties. It has been shown to be a plant growth promoting bacterium with excellent colonisation abilities, surviving and persisting well in the rhizosphere (29, 32). A great deal is known about its survival, persistence and the stability of gene (marker) insertions under environmental situations (2). In a field release (30), no genetic transfer was detected from the chromosomally marked bacteria, and therefore, transfer, if any had occurred, was below the levels of detection. This study also showed the bacteria could not over-winter in the absence of plant material. Although this bacterium was shown to have no impact on microbial community structure or dynamics in a field release, the genes used to mark the isolate were considered neutral. In this study we wished to identify

the impact a functionally active bacterium might have on the indigenous communities. If the inserted genetic element encoded a gene which increased the relative fitness or persistence of the bacterium there could be a selective advantage for this trait in the plant rhizosphere. Therefore, potentially having a very different impact when compared to a bacterium containing neutral markers.

To improve our understanding of microbial communities a suite of techniques was required which would be able to measure the diversity and relative abundance of components of communities and, in addition, possibly assess the functional ability of our rhizosphere samples. These measurements would be undertaken during a growing season to help improve our understanding of this highly complex rhizosphere environment. A standard sampling approach was adopted, which allowed sub-sampling of each replicate for all measures of community structure and function undertaken in this study. This study required the extraction of clean total DNA suitable for PCR amplification, clean soil-free rhizosphere samples for CLPP and associated tests, and samples for the enumeration of bacteria and fungi.

Once the identification of a suitable technique was identified for reproducible sampling from the rhizosphere of each plant, the methods to process the samples were standardised. It is important to note that in these studies the rhizosphere is often not clearly defined. In this study samples were made of the soil tightly adhering to the roots (< 1 mm from root surface) so that micro-organisms sampled were only those closely associated with the plant roots. The extraction of DNA from soils has been widely undertaken with many protocols developed and described in the literature. A highly successful protocol was developed in our laboratory (14), however, this method was developed on a high humic brown forest soil. The soil used in this study was from The University of Oxford field station, Wytham, and had significantly different characteristics, with a high clay content (53.5 %). It was, therefore, important for me to optimise extractions to yield high quality PCR amplifiable DNA from this soil. Successful extractions were achieved by the incorporation of a freezing lysis step into the protocol developed in our laboratory (14). Figure 2.2. clearly demonstrates the differences between extraction protocols and the efficiency of the protocol adopted in this study.

Much work has been undertaken by DGGE on bacterial communities since its first application to microbial communities (25), but very little has focused on fungal communities (12). This limited research has been undertaken using many different regions of the 18S rRNA gene that it does not allow for consistency of data or for comparisons between studies. For this thesis a number of 18S rRNA gene specific primer sets were considered before achieving a simple and reliable approach to monitor fungal communities.

The other techniques used for the sampling of the rhizopshere were the enumeration of culturable populations and CLPP analysis using BIOLOG™ GN2 plates. Plate counts were undertaken to identify population densities of total culturable heterotrophs, pseudomonads, fungi, and the recovery of the inocula. This data of total heterotrophs provided a valuable source of information for comparisons to the other techniques, allowing the conformation that shifts in community structure by DGGE or CLPP were not as a result of changes in population densities. The monitoring of total pseudomonads and the inocula were of great interest as it provided an opportunity to measure the colonisation ability and persistence of the inocula in the rhizosphere. It also provided a chance to observe the effect of the inoculation on pseudomonad populations by the identification of the level of replacement that occurs (Figures 4.4, 4.8, and 4.12). Studies on fungal populations gave an indication of whether the inocula suppressed fungal communities or resulted in a change of their composition.

Community level physiological profile (CLPP) analysis provided data of great importance complementing the community profiling data obtained by DGGE and culturable populations. This data provided information on the effect that plant species and the inocula had on the communities carbon source utilisation potentials, with the identification of shifts through growth stages and treatments. A new approach on BIOLOG™ GN2 plate data was used, with the calculation of the average well colour (AWC) over an entire plate. This data gave an indication of the metabolic activity of the bacterial communities in the rhizosphere. This proved to be very interesting, with the identification of major shifts in activity throughout the growth of plants and in response to the inocula.

Before the release of a functionally active GM-BCA (23:10) (32), a detailed understanding of the rhizosphere population dynamics would need to be understood. This was undertaken using the techniques optimised in my research to provide a baseline understanding of the rhizosphere populations in the chosen crop plants. The data from Chapter 3 addressed this, and demonstrated that there were large significant effects on the rhizosphere communities caused by the introduction of plants to the soil, in comparison to those seen in bacterial and fungal communities of the bulk soil. Three plants were studied and it has been shown that they all enriched the microbial populations when compared to the background soil communities. Marked growth stage dependent effects on community structure and metabolic activity have also been demonstrated (Figures 3.4.-3.5. and 3.7.-3.10.). These effects were a direct result of the plant presence, as bulk soil communities demonstrated high stability with no seasonal effects (Figure 3.6.). The use of plant growth stage comparisons allowed the identification of similar shifts in CLPPs between plant species that were distinct from the bulk soil, as the three plant types have quite different life cycles (Figure 3.4.). The crop species demonstrated plant specific influences in which the profiles identified were different to one another. It was also identified that there were marked differences in the activity of communities assessed by AWC, with early growth stages demonstrating lower activity in comparison to mature plants. The same enrichment by crop species was demonstrated for both bacterial and fungal communities when analysed by DGGE; a method based on total DNA extraction from rhizosphere samples, therefore culture independent. This gave a robust baseline for understanding the natural perturbations occurring in the rhizosphere of different crop species under field conditions.

As previously described, *Pseudomonas fluorescens* SBW25 was identified as a suitable candidate for genetic modification. The genetic modification of the bacterium to contain the *phz*ABCDEFG operon (encoding phenazine-1-carboxylic acid) was undertaken with expression of the gene under the control of the constitutive P*tac* promoter. Phenazine biosynthesis is normally controlled by a quorum sensing density dependant mechanism, so it was engineered for expression under a constitutive promoter. This allowed continued expression of phenazine-1-carboxylic acid with the

potential build up of the compound, making the strain ideal for impact assessment studies. The operon was introduced as a single chromosomal insertion into a rifampicin resistant mutant. This was acheived using a disarmed mini-Tn*5* suicide vector containing a kanamycin resistance gene to allow selection from a mixed background of micro-organisms. The resulting variant (23.10) was chosen after careful colonisation, fitness and survival checks in comparison to the WT. It was also shown to have improved biocontrol abilities when compared to the WT, SBW25, and increased survival and persistence in the rhizosphere/soil (32). The bacterium has also been demonstrated to persist well in the phyllosphere of several crop plants and can suppress *Pythium* infestations up to 100 times normal levels (33). There is tight legislation governing the release of GMMs into the environment to limit the potential ecological impact of such a release, and to reduce the likelihood of unforeseen situations arising (31). Research, therefore, takes the precautionary approach with environmental containment of the initial experiments to identify if there are major ecological impacts. This has lead to the design and development of mesocosm experiments using the GM-BCA (23:10) in unsterilised natural field soil in a growth chamber (Chapter 4). Mesocosm experiments were undertaken to simulate the conditions of an environmental release but in a contained environment, to allow for the possibility of larger unforeseen ecological impacts. The experiment was also undertaken on the same three crop species, with sampling to represent growth stages as previously described for the baseline study in the field. There were three treatments undertaken; a water control which allows laboratory based mesocosms to be compared to environmental field data, the inoculation of the WT bacterium (SBW25), and the inoculation of the GM bacterium (23:10). The inoculation of the parent strain allowed the identification of any differences as a result of the insertion of the PCA biosynthesis operon on survival and impact. The water control demonstrated the same plant effects and growth stage dependent shifts that were identified in Chapter three field data. These shifts were seen in CLPP and DGGE analysis with a high stability of the culturable populations of bacterial, pseudomonads and fungi. This confirmed that the data collected from the mesocosm experiments would give a very good indication of what would happen in a real environmental field release of the microbial BCAs.

The colonisation of the rhizosphere by the two different inocula was shown to be highly successful, confirming previous studies (32, 33). The population densities of the inocula colonising the rhizosphere reflected the differential carrying capacity of the individual crop species identified in Chapter 3 and 4. These populations densities are indicative of the Wytham field soil, with pea supporting the densest populations and sugar beet the least. One other study focusing on these three plant species in a different soil type identified a different pattern of rhizosphere colonisation abilities (33). It is, therefore, important to understand that soil type influences the rhizosphere communities as well the introduction of the plant (6, 10, 13, 21, 37). The survival of the introduced inocula demonstrated the characteristic traits that previous inoculations have, with initial high colonisation at an early stage and the decline of population densities over time (11, 17, 20, 30, 33).

The impact of the inocula on the indigenous culturable microbial community densities were identified as very minor and transient, based on the carrying capacity for all the three crop types. The only major perturbations identified were represented by the replacement of a large proportion of the indigenous pseudomonads by the inoculum, as seen on plate counts (Figures 4.4., 4.8. and 4.12.). The population densities in the pea rhizosphere displayed the greatest impact as a result of the inoculum (Figures 4.3.-4.5.), this may have been as a result of the high population densities, competition, and metabolic activity in the pea rhizosphere. CLPP analysis representing the metabolic potential of the rhizosphere communities for the three crop species, identified that there was also a transient impact on the communities in the pea and wheat rhizosphere. CLPP analysis also showed the separation of the two inocula at early growth stages, with a reduction of this effect with time. This impact was minor in comparison to the effects of plant growth stage on communities and any perturbations caused by the inoculum (Figures 4.14.-4.16.). In sugar beet plants, this effect was not identified, perhaps as a result of the high variation of data produced in this experiment. The impact seen in the CLPP data of the three crop species was also reflected in the AWC, demonstrating growth stage dependent changes with transient effects as a result of the inoculum.

The impact on bacterial community structure, identified by DGGE, showed the same impact in all crop plants. In summary, this effect was a transient impact as a result of the addition of the inocula to mesocosms, with a different effect caused by the WT and GM inocula. This transient effect on of the inocula community structure has been demonstrated by other studies in this area (3, 4, 7, 26, 28). It is important to note that these effects were minor in comparison to the growth stage shifts identified. Of interest, but not surprising, were the plant specific effects of the inocula on the α-proteobacteria and γ-proteobacteria; although these were still masked by growth stage effects. The pea rhizosphere showed a dominant shift in the α-proteobacteria community structure by DGGE, with a persistent shift in γ-proteobacteria for the GM inoculation. In comparison, the wheat rhizosphere displayed no distinct shift, as a result of the treatments, in α-proteobacteria profiles from the water control, whereas in the γ-proteobacteria there was an increased shift with an effect of the WT inocula as well. The sugar beet rhizosphere communities demonstrated a similar effect to the pea, with a dominant shift in the α-proteobacteria community structure by DGGE. There was, however, no real impact identified in the γ-proteobacteria. Fungal communities, profiled by DGGE, highlighted the dominating plant growth stage effects, with transient effects on fungal communities identified in both the pea and wheat rhizosphere. These effects reduced with subsequent growth stages with the two inocula having a different impact. This DGGE data highlights that the impact the inocula has is dependent on the plant species that it is being introduced to, and the inocula will effect different communities that are present in the rhizosphere to different extents depending on the plant species. These effects are disguised, however, by plant effects caused by growth stage, and are only identifiable by direct comparisons at individual growth stages. Fungal communities in the three crop species can equally be described in this way, with a greater impact identified by the plant growth stage than treatment.

This research has identified that, despite the inoculation of a large number of bacteria into the rhizosphere of the three crop species, the dominating effects on communities are the host plant species and growth stage. The impact identified by the enumeration of bacterial and fungal populations, measures of CLPP and AWC, and the impact on bacterial and fungal community structures, measured by DGGE, is transient. This data

was laboratory based and is thought to be indicative of the impact that the release of two bacterial strains into the environment may have.

Data from this study and other published and unpublished work in the laboratory was used in an application to the Secretary of State for Environment, Food, and Rural Affairs to apply for consent to under go a field trial with the WT bacterium SBW25 and its genetically modified derivative, 23:10. This was to measure the colonisation ability, survival and persistence and the impact on the indigenous microbial communities as a result of the inoculation. The application applied for the release to be undertaken on field grown spring wheat. This plant was chosen as it has a longer growing season than pea plants, and it is extensively grown commercially, thereby giving a good indication of length of survival and persistence of the inoculum. In addition to this, the wheat plants were shown to have stable populations and demonstrated small transient perturbations in communities in response to the inoculum. These perturbations, in comparison to sugar beet, were more marked and identifiable, so studies on the sugar beet would potentially have any impact masked by the high natural variance that has been identified in the sugar beet rhizosphere. Consent was granted on the 30th April 2004 (Ref 04/R39/1 – Strategies for risk assessment, evaluating the environmental impact and fungal disease suppressing GM bacteria on non-target species) for the release of 23:10 as the first UK trial of a genetically improved bacterial biological control agent. The release was undertaken two weeks later.

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Chapter Six: Production and conservation of hydrogen cyanide biosynthesis in bacterial biological control agents.

6.1. Reasoning for research.

In previous studies by Ellis *et al.* (12) the conserved traits in fluorescent pseudomonads that confer antifungal activity were described. The aim had been to identify if any correlation could be found between bacterial phenotypes and their biological control ability. HCN biosynthesis was identified as the only trait that rigorously predicts biological control activity in these pseudomonads. This work was undertaken because of the potential of *Pseudomonas fluorescens* SBW25 possibly containing the operon for HCN production. This could then be used and up-regulated in the bacterium to increase the efficacy of the bacterium for complimentary studies on the genetically modified SBW25 (23.10). Preliminary work indicated that *P. fluorescens* SBW25 possessed the trait by PCR amplification; a result confirmed by the primers produced by Ramette *et al.* (34). However, sequencing of the PCR products identified them as not representing the HCN biosynthesis operon, with the absence in SBW25 confirmed by its genome sequencing project. This work was then used for the identification of primers with specificity to the HCN biosynthesis operon, allowing conservation of the operon to be assessed among known biocontrol agents. This work was taken no further.

6.2. Introduction.

The potential use of bacteria as biological control agents has been well known for many years. This has resulted in increasing levels of research being undertaken to identify how bacteria express their biological abilities, determine how successful they are, and establish how these traits are conserved in bacterial species (12). Some important biocontrol traits that have been identified include: niche specialisation, rhizosphere colonisation, rapid growth and production of anti-microbial secondary metabolites. These metabolites include: phenazines (25), phloroglucinols (13, 17), pyoluteorin (19), pyrrolinitrin (31), and hydrogen cyanide (HCN) (41). All have been demonstrated in different bacterial strains to have important roles in plant growth promotion. Often biocontrol is not attributed to one of these traits but to several in combination, demonstrating the complex phenotypes expressed by plant colonising bacteria.

In previous studies by Ellis *et al.* (12) we described conserved traits in fluorescent pseudomonads that conferred antifungal activity. The aim had been to establish if any correlation could be found between the bacterial phenotype and genotype and their biological control abilities. This was done using a collection of 29 different biocontrol fluorescent pseudomonads isolated from across North America and around Europe, See Table 6.1. These isolates were tested for various measures of biocontrol and secondary metabolite production, including the level of inhibition of *Pythium ultimum in vitro*, an index of disease control on seed germination, HCN production, phenazine-1-carboxylic acid production, presence of the phloroglucinol operon, presence of the pyrrolinitrin operon, siderophore production, fatty acid methyl ester analysis and carbon utilisation profiles (12). Further comparisons were made between the biocontrol index of each strain, their ability to suppress *Pythium ultimum* infections of pea seeds in microcosms as a proportion of infected controls, and the other traits as described. Following detailed correlative analysis it was established that the levels of C17: cyclopropane fatty acids were higher in bacteria that confer biological control activity (12). The ability to produced HCN also rigorously predicted biological control activity. This was potentially interesting as until very recently (12 months after I began work for the thesis) little work has been undertaken on the comparison

between organisms and the relative roles of secondary metabolites (32, 34, 35). HCN was therefore shown to be an important factor in the control of disease. Several of the bacterial strains in the collection were shown to be good biocontrol agents but did not produce HCN in the *in vitro* studies. It was of interest to find out if they could potentially produce HCN but only *in vivo*.

6.2.1. Hydrogen cyanide production.

Hydrogen cyanide (HCN) is a highly toxic compound produced naturally in the environment. The intake of HCN into any organism causes the termination of the electron transport chain by inhibition of cytochrome oxidase a-a3. This prevents the production of ATP and reduces metabolic activity, thereby potentially causing death (18). The production of HCN occurs in a variety of organisms including plants (39), fungi (7), and bacteria (27, 41). It must, therefore, be concluded that HCN production must be beneficial to those organisms that produce and tolerate it.

The majority of plants produce HCN in small quantities as a result of ethylene production. There are, however, approximately 3,000-12,000 species of plants that have been identified to produce HCN in significantly greater levels than this (26). HCN is produced by plants as a defence mechanism against predation from other organisms. Cyanogenic glycosides are produced by plants via the shikimic acid pathway, these glycosides can then be broken down to produce HCN when required (36). This chemical conversion is facilitated by the enzymes β-glucosidase and hydroxynitrile lyase. The sugar is cleaved from the cyanogenic glycoside and the resulting cyanohydrin is broken down by the respective enzymes to release HCN. This cyanogenic glycoside breakdown occurs only in damaged plant cells. This is achieved by the partitioning of glycosides into the plant's vacuoles, while the enzymes remain in the cytoplasm and only under cell rupture can the substrate come into contact with the enzymes (36).

Cyanogenesis occurs in several bacterial species as a defence / survival mechanism to give them a competitive advantage in the environment. Bacteria that have been demonstrated to have this ability include *Chromobacterium violaceum*, some

Pseudomonas spp., Anarystis nidulans, Nostuc muscorum and *Piectonenia boryanum.* The pathway cyanogenesis in bacterial species has been identified, with the most detailed characterisation undertaken in *Pseudomonas fluorescens* CHA0. The operon responsible for HCN synthesis was first identified by Voisard *et al.* (41) and has been sequenced and characterised as the *hcn*ABC operon (23).

6.2.2. Importance of HCN production.

The importance of HCN production by *P. fluorescens* CHA0 in the rhizosphere was first established by Voisard *et al.* (41). In Switzerland, a clay soil was identified which had suppressive properties to the fungus *Thielavipis basicola*, the causal agent of black root rot in tobacco plants. The bacterium *P. fluorescens* CHA0 was isolated from this soil and confirmed to possess the ability to suppress *T. basicola.* CHA0 was characterised for its ability to produce antifungal secondary metabolites, which were identified as pyoverdine, HCN and several other antimicrobials (41).

These secondary metabolites were tested to identify their role in disease suppression, in particular the role of HCN production in the suppression of black root rot. A gnotobiotic system was developed to test the suppressive effects of CHA0 and mutants. Mutagenesis was undertaken and those lacking the ability to produce HCN were identified. Digested CHA0 genomic DNA was cloned into plasmids and complementation tests were undertaken, identifying clones that restored HCN production. Clones that conferred HCN production were used to create an insertion in the HCN biosynthesis operon to create a true HCN knockout mutant. In comparative studies on black root rot disease suppression, the inoculation of the wild type bacterium resulted in five-fold increase in plant weight when challenged with *T. basicola,* compared to no significant increase on inoculation by the knockout mutant (41). Thus inactivation of HCN biosynthesis abolished part of CHA0's suppressive ability on *T. basicola*, with no impact on the bacteria's root colonisation ability (41).

HCN biosynthesis has been demonstrated to be highly successful in plant protection. There are, however, cases in which the production of HCN has detrimental effects on its host. Several bacterial species have demonstrated this effect (1, 4). The effect caused by the bacteria has also been investigated for its potential use in the biocontrol

of weeds (20). The differences in the production of HCN in plant associated fluorescent pseudomonads that result in some strains being beneficial and others being detrimental it is not known. However this may be due to differential patterns in HCN production on plant surfaces and in plant tissues (34). Alternatively, it may be a result of the plant's own defence mechanism being activated as plant growth is reduced by the extensive colonisation of the HCN producing bacteria.

6.1.3. The *hcn*ABC operon.

HCN biosynthesis is a result of the *hcn*ABC operon identified by Voisard *et al.* (41). The operon consists of *hcn*A, encoding a protein homolog of formate dehydrogenase, and *hcn*B and *hcn*C encoding proteins with homologous motifs to the FAD / NAD(P) binding motif (see Figure 6.1) (23). The identification and characterisation of the *hcn*ABC operon has been entirely by nucleotide sequencing, as attempts to purify the proteins involved have been unsuccessful, highlighting their high instability (42). HCN biosynthesis from this operon is expressed in late exponential phase/early stationary phase of growth by the pseudomonad CHA0 (10). The production of HCN is dependent on the substrate glycine, and through the genetic characterisation of HCN biosynthesis the pathway proposed is the oxidation of glycine to iminoacetic acid and its cleavage by a dehydrogenase to produce HCN (10, 41).

The regulation of HCN synthesis in CHA0 is multi-factorial, with the combination of the *gac*S/*gac*A two component regulatory system (24), the anaerobic regulator ANR (FNR homologue) (23), and iron (6) (Figure 6.1). The ANR regulator is activated in microaerophilic conditions, promoting expression of *hcn*ABC from an ANR box in the promoter region (23). Under iron limiting conditions HCN synthesis is inactivated, and it has been suggested that iron depletion converts ANR into an inactive form (6), preventing transcription of the *hcn*ABC operon. *Gac*A regulation is required for HCN synthesis as deficient mutants will not express HCN (24), and it is hypothesised to be required for post-transcriptional regulation (6).

The *Pseudomonas aeruginosa* PA01 strain has the HCN biosynthesis genotype, but the regulation of gene expression is by a more complicated mechanism, see Figure 6.2. The promoter region of PA01 *hcn*ABC homologue contains two lux boxes

involved in quorum sensing regulated expression, in addition to the ANR box. Within the promoter there are two transcriptional start sites, T1 and T2 (30). Under aerobic conditions with high cell density the genes for quorum sensing transcriptional regulators, LasR and RhlR, are expressed and bind to the lux boxes in the promoter. This gives a low background level of expression from T1. In microaerophillic conditions, ANR binds to the –10 region of T1 inactivating it, promoting T2. The level of HCN production dramatically increases as a result (41). *Gac*A is also responsible for post transcription regulation (29). All components are required for high levels of HCN production as they act synergistically (30).

Many studies have been undertaken on the biosynthesis and enzymatic pathways of many of these secondary metabolites in order to improve the understanding of their function and role. In this study the biosynthesis of HCN was investigated and molecular analysis was undertaken to identify the level of conservation and if it could be identified in the natural soil environment as a mechanism of monitoring the persistence of organisms. The questions addressed were:

1) Is it possible to identify the levels of HCN production in the collection of bacteria under study?

2) Can universal primers be designed to amplify the *hcn*ABC operon from HCN producing bacteria?

3) Do some bacteria contain the *hcn*ABC operon and not express it *in vitro*?

4) Is the presence of the *hcn*ABC operon detectable in the natural environment?

5) What is the genetic conservation between the *hcn*ABC operons, and are there different subpopulations of genotypes?

6.3. Materials and methods.

6.3.1. Laboratory micro-organism growth and storage.

6.3.1.1. Laboratory strains.

The bacterial strains used are detailed in Table 6.1.

6.3.1.2. Micro-organisms growth conditions and storage.

Bacteria were grown as detailed in section 2.2.1.2., and stored as detailed in section 2.2.1.3.

6.3.2. Nucleic acid isolation techniques.

6.3.1.1. Total nucleic acid isolation.

DNA was extracted using the CTAB method described in section 2.2.2.1. and explained by Bailey *et al.* (3).

6.3.2.2. DNA fragment purification.

DNA fragments of less than 10 kb were purified from 1 % agarose gels using Qiagen QIAquick Gel Extraction Kit (28704), as per manufacturers protocol.

6.3.2.3. PCR product purification.

PCR products were purified using Sigma GenElute™ PCR Clean-up Kits (NA 1020), or by gel extraction (section 2.2.2.5.).

6.3.3. Nucleic acid modification techniques.

6.3.3.1. PCR fragment cloning.

PCR fragments were cloned as detailed in section 2.2.3.1.

6.3.4. DNA visualisation.

DNA was separated by agarose gel electrophoresis (10 V cm^{-1}) in 1 % (w/v) agarose, containing 0.1 μg ml^{-1} of ethidium bromide, made with 1x TBE (0.089 M Tris, 0.089 M boric acid, 0.002 EDTA, pH 8.0). Gels were visualised on a UV-transilluminator.

6.3.5. DNA amplification by PCR.

For PCR amplification of chromosomal DNA reactions were carried out, using the following protocol, on a Peltier thermal cycler, MJ research. Total reaction volumes were 50 μl.

The PCR Mix	
Target DNA	1-100 ng
Primers	1 p mole
dNTP's	0.2 μM
Buffer	1x (supplied with Taq)
Taq *	2.5 u

Reaction cycle		
95 °C	2 min	
95 °C	1 min denaturing	x 30
60 °C	45 s annealing	
72 °C	2.5 min extension	
72 °C	2 min	
10 °C	forever	

* Taq contained 10 % proof reading taq to maintain high fidelity for sequencing. The reaction cycle was modified as required.

6.3.5.1. Primer design.

Primers were designed using the web-based design programs, Primer Finder v 0.07 (http://eatworms.swmed.edu/~tim/primerfinder/) and Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

6.3.5.2. Primers used.

Primer Name	Sequence	Source
ACa	5'-actgccaggggcggatgtgc	(34)
ACb	5'-acgatgtgctcggcgtac	(34)
1804F	5'-actgccaggggcggatgtgc	This study
2381F	5'-gtacgccgagcacatcgt	This study
2379R	5'-acgatgtgctcggcgtac	This study
2519R	5'-cctggtggtcgcagaggaa	This study
2886R	5'-tcgaagcccttgtcctcggt	This study

6.3.6. DNA sequencing and alignment.

Sequencing and alignments were carried out as described in section 2.2.6.2.

6.3.6.1. Comparative sequence alignment.

Nucleotide and deduced protein sequences of bacterial strains were aligned using the BioEdit Sequence alignment editor (Version 5.0.9).

6.3.6.2. Phylogenetic analysis.

Phylogenetic analysis was undertaken with PAUP (Phylogenetic Analysis Using Parsimony) Version 4.0b10 (D. Swofford, Florida State University), while likelihood parameters were calculated using Modeltest, Version 3.6 by Posada *et al.* (33).

6.3.7. HCN assay.

Bacterial HCN production was assayed using a HCN sensitive indicator paper, as described by Castric and Castric (9). The indicator paper was placed over a microtitre plate containing a 1:20 dilution of a saturated culture in a volume of 200 µl per well. The plates were then incubated at 28 °C for 24 h without shaking. Results were scored by eye on a scale of 0-5 with increasing blue colour.

6.4. Results

6.4.1. Bacterial HCN biosynthesis.

The bacterial biocontrol agents, detailed in material and methods, were screened for HCN biosynthesis using the colourmetric paper indicator assay (9) which was then modified for use with microtitre plates (41). The additional bacterial strains to those screened by Ellis *et al.* (12) included: *Pseudomonas aeruginosa* PA01, *Pseudomonas fluorescens* PFO-1 and *Chromobacterium violaceum,* all known to possess *hcn*ABC homologues from genome sequencing projects ((11, 37) and http://genome.jgi-psf.org/draft_microbes /psefl/psefl.home.html), NCIMB11764 (a HCN degrading bacterium) (21), KT2442 and a *Bacillius pumulis* strain. From this point the bacteria will be referred to by their strain identifier only, species can be determined from Table 6.1. The resulting colour intensity from the paper assay was scored blind on a scale from 0-5, where 5 represented the richest colour intensity recorded. The bacterial strains identified with HCN production were assayed with 5 replicates in a randomised pattern on a 96 well microplate with blanks and non HCN producing bacteria controls and scored. The samples were independently scored by another researcher and the average of the 10 scores was made (x). bacterial strain ML5 was the highest producing bacterium with a score of x = 5, followed by PGSB8456 (x = 4), CHAO (x = 4), 76/10 (x = 3.6), PF5 (x = 3.4), PGS12 (x = 3), C7 (x = 2.9), F113 (x = 2.8), PA01 (x = 2) and M114 (x = 1.1). The remaining strains did not produce any detectable HCN by this method (Table 6.1.).

6.4.2. Design of universal HCN primers: Amplification of the *hcn*ABC operon.

To identify if the *hcn*ABC operon was present in other bacteria in the collection but not expressed under the conditions used in the HCN biosynthesis assay, and to identify if their was conservation between bacterial strains, primers were designed to the homologous regions in the *hcn*ABC operon from an alignment of CHA0 (23) and PA01 (30). These primers made a complement that could potentially amplify various size fragments from the majority of the *hcn*ABC operon. The primers were hcn 293, hcn 1404, hcn 1404R, hcn 2143, hcn 2143R and hcn 2867R which align with both

CHA0 and PA01. The numbers refer to binding position on the *Pseudomonas fluorescens* CHA0 sequenced operon. A positive control PCR was undertaken on CHA0 and demonstrated success with all primers, however amplification with other HCN producing and non-producing bacteria resulted in inconsistent fragment size amplification and non-specific binding. Following this initial screening of bacteria the complete genome of PF0-1 was released in 2003 (http://genome.jgi-psf.org/draft_microbes/psefl/psefl.home.html in draft form), which by blast analysis of this genome against the CHA0 *hcn*ABC operon identified another HCN biosynthesis operon homolog. In view of the previous inconsistencies in success of PCR with non-specific products and amplification of bands from *P. fluorescens* SBW25, a negative control bacterial strain known to have neither the HCN phenotype nor genotype (http://www.sanger.ac.uk/projects/ p_fluorescens). The primers were tested against the genomes of PA01, PF0-1, SBW25 and the HCN operon from CHA0 using "virtual PCR" a linux based software program (http://www.hgmp.mrc.ac.uk/software/EMBOSS/apps/primersearch.html). All primers were successful on CHA0, with the primer pair hcn 1404 and hcn 2867R amplifying a fragment from all known HCN producing bacteria sequences with no non-specific amplification with the negative control, SBW25. The use of these primers were therefore undertaken to screen the collection of bacteria. There was still, however, non-specific binding, with not all HCN producing strains amplifying products. The specificity of amplification did not improve despite optimisation of the annealing temperatures in PCR.

Whilst this research was being undertaken, a paper was published by Ramette *et al* (34) on the phylogeny of HCN synthase encoding *hcn*BC in biocontrol pseudomonads and relationship with plant host species in HCN synthesis ability. In this work they successfully managed to amplified a 590 bp fragment, spanning the gene junction between *hcn*B and *hcn*C. They found difficulties in producing clean amplification with non-specific gene amplification by primers aimed at *hcn*A or the start of *hcn*B. Utilising this information and the partial data release from the genome sequencing project of PF-5 (http://www.ars-grin.gov/ars/pacwest/corvallis/hcrl/pf5genome/index.htm), a new alignment was produced for PA01, CHA0, PF0-1, PF5 and F113 (short fragment from Ramette *et al.* (34)). Regions were identified that were ≥ 18 bp in length with < 1 bp difference for that length between all alignments. New sets of

primers were designed to homologous regions and were called hcn 1804F hcn 2381F, hcn 2397R, hcn 2519R, and hcn 2886R All combinations of primers were used and tested on PA01, CHA0, PF0-1, PF5, F113, PGS12 and SBW25 and there was successful amplification with some primer combinations (Figure 6.3). Hcn 1804F and 2397R partially amplified fragments and with some optimisation would be successful as these primers were designed to the same locations as the primers by Ramette *et al.* (34). Hcn 1804F and hcn 2886R produced a product of 1084 bp in all HCN strains, and was negative in SBW25. Hcn 2381F and hcn 2886R amplified products of 506 bp in size for CHA0, PF0-1, PA01, and PF-5 not F113 and PGS12.

The primers, hcn 1804F and hcn 2886R, were used to screen the entire bacterial collection to identify the specificity of the primers, and to identify if there were any other bacteria that could potentially produce HCN. These primers showed a high success rate despite some non-specific amplification. Products were obtained from the following bacteria in the collection: ML5, PGSB8456, CHA0, 76/10, PF5, F113, PAO1, and PF0-1. Despite not producing HCN in the *in vitro* assay, PF0-1, which is known to possess the operon from its genome sequencing poject, produced a fragment. For a comparison the primers designed by Ramette *et al.*, (34) were tested on the known HCN producing or gene containing bacteria in addition to two bacterial strains (SBW25 and 54/96) whose mode of biocontrol is not known (12). There was failure to amplify in some strains, with a false positive result with SBW25, see Table 6.1.

6.4.3. Detection of the HCN operon in environmental samples.

With the use of the designed HCN primers amplification of environmental DNA samples was undertaken. A calibration PCR was carried out to identify the level of detection using genomic DNA from the bacterium CHA0, mixed with DNA from SBW25. In each sample there was 100 ng DNA μl^{-1} from SBW25 with the addition of either, 100 ng, 10 ng, 1ng, 0.1ng, or 0.01 ng of CHA0 genomic DNA. The HCN operon in CHA0 could be amplified from the genomic DNA mix in which 0.05 % of DNA was from the strain CHA0. In terms of amount of amplifiable DNA (the operon)

it was assumed only one copy per genome was present, this translates to approximately 0.000025 % of total DNA.

6.4.4. HCN amplification from environmental DNA samples.

Total environmental DNA from the rhizosphere samples of mesocosms, described in Chapter four, were subjected to PCR using the primers hcn 1804F and hcn 2886R, as previously described in this study. The results show that, of the environmental DNA samples, PCR products were obtained of the correct size for the *hcn*ABC operon in 6 out of 27 samples over the three growth stages and treatments (see Figure 6.4).

6.4.5. Sequencing of amplified *hcn*ABC operon.

PCR products were successfully amplified from the bacterial strains 76/10, CHA0, PF-5, M114, F113, ML5, PGS12, PGSB 8456, PF0-1, and PAO1 using the primers hcn 1804F and hcn 2886R. Sequences of CHA0, PF0-1 and PAO1 are already known, however CHA0 was sequenced as a control to check the same sequence was produced, and PF0-1 was sequenced as its genome was in preliminary draft format only and so therefore may have contained errors. Successfully amplified products from these bacterial strains were purified by gel extraction as described in section 2.2.2.5., cloned as in section 2.2.3.1., and sequenced.

6.4.5.1. Sequence analysis.

Sequences were aligned using CLUSTAL W with adjustments of the alignment to conserve regions of high homology and allow polymorphism using the software, Bioedit sequence alignment editor, Version 5.0.9 (Department of Microbiology, North Carolina State University). In addition to the sequences produced, the published sequences of PA01 (37) and *C. violaceum* (11) were included. The aligned sequences were of a total length of 1057 bp (*hcn*BC long fragment), of which 601 (57 %) were non-polymorphic. There was high conservation of the Shine-Delgarno (SD) motif and the start codon in the strains, with polymorphism shown in the first base of the SD motif and PGS12 and PGSB 8456 having a transition event in fifth base of the SD motif. *C. violaceum* had a distinctly different SD sequence to the other bacterial

strains. In this alignment there was an insertion event in the sequence of *C. violaceum* at the 3′ end of *hcn*B of 9 bp encoding another 3 amino acids in the C-terminal domain. Another alignment was undertaken incorporating the sequences published by Ramette *et al.* (34), amplifying a shorter section of the *hcn*ABC operon (590 bp) (*hcn*BC short fragment). This consisted of another 35 sequences, added to the alignment of sequences produced by this study, flanking the *hcn*BC gene junction. Ramette *et al.* (34) had also produced sequences for F113, PF-5, and CHA0. F113 was included in the alignment as it differed to the sequence produced in this study. The alignment was 567 bp long of which 312 (55 %) were non-polymorphic. There was the similar high conservation of the Shine-Delgarno (SD) motif and the start codon in the strains as shown in the *hcn*BC long fragment. There was polymorphism in the first base of the SD motif with PGS12 and PGSB 8456 having a transition event in the fifth base of the SD motif. In addition to the 9 bp insertion in the *C. violaceum* operon, there was a 15 bp (5 amino acid) in frame insertion after the start codon of *hcn*C in *Pseudomonas corrugata*. The average GC content of the fragments was 64.7 %.

6.4.6. Phylogenetic analysis of sequenced *hcn*BC fragements.

6.4.6.1. Phylogenetic analysis of the *hcn*BC long fragment nucleotide sequence.

Phylogenetic analysis of the long fragment was undertaken with PAUP (Phylogenetic Analysis Using Parsimony) Version 4.0b10 (D. Swofford, Florida State University). For maximum likelihood analysis a substitution model was implemented, identified by Modeltest, Version 3.6. (33). The model that best represented the data was the TVM + G model. The substitution rate matrix was estimated using Modeltest. Two contrasting techniques were used; the optimality criterion method of maximum likelihood, and the clustering algorithm of Neighbour Joining (NJ) with likelihood settings. In both analyses *C. violaceum* was defined as the outgroup for tree drawing. Maximum likelihood was undertaken with the forced TVM + G model, with estimation of gamma distribution and invariable sites set to zero. A heuristic search was undertaken using default settings with tree bisection and reconnection (TBR) branch swapping algorithm. The nodal support of the resulting phylogram was

addressed by bootstrapping (100 replicates). This is shown in Figure 6.5. (a). The neighbour joining clustering algorithm was undertaken using the likelihood settings using ASIS stepwise addition and TBR branch swapping. The nodal support was addressed by boot strapping (100 replicates), and is shown in Figure 6.5. (b) The two resulting trees were of very high similarity, with identical branch lengths describing the data. There were two outgroup and 9 ingroup taxa which consisted of 4 clades. With regard to this tree two clades (iii and iv) had taxa of high similarity.

6.4.6.2. Phylogenetic analysis on the *hcn*BC short fragment nucleotide sequence.

For maximum likelihood analysis of the short fragment, a substitution model was implemented, identified by Modeltest, version 3.6. (33). The model that best represented the data was the TVM + G model, the same as identified for the *hcn*BC long fragments. The substitution rate matrix was estimated using Modeltest. The same techniques were used as in the *hcn*BC long fragment, the optimality criterion method of maximum likelihood, and the clustering algorithm of neighbour joining with likelihood settings. In both analyses, *C. violaceum* was defined as the outgroup for tree drawing. Maximum likelihood was undertaken with the forced TVM + G model with estimation of gamma distribution and invariable sites set to zero. A heuristic search was undertaken using default settings with TBR branch swapping algorithm. The resulting tree is shown in Figure 6.6. As the alignments were of a large data set, thus computationally highly demanding, bootstrap support was not calculated. To confirm the tree topology, likelihood settings were re-estimated from the tree produced and data reanalysed using the nearest neighbour interchange (NNI) branch swapping technique. This analysis resulted in a tree with identically branch lengths to the original tree (tree not shown). The neighbour joining clustering algorithm was undertaken using the likelihood settings estimated with ASIS stepwise addition and TBR branch swapping. The nodal support was addressed by boot strapping (100 replicates), and is shown in Figure 6.7. The two trees had 2 outgroup taxa (*C. violaceum* and PA01) and 44 ingroup taxa with six different clades. Four clades consisted of data from Ramette *et* al. (34) (named the same as in their research, Hcn-1 to Hcn-4) with some sequences from this study. The other clades were represented solely by sequences from this study. There were some sequences that were paraphletic (*P. corrugata,* and Q37 87). The two trees showed different topologies, but taxa

within clades were grouped with identical branch lengths in either tree, within clades four demonstrated high internal similarity and support. The bootstrap support of nodes between the clades arrangements, that differ between trees, showed low support (< 50 %). One point of interest is that the two versions of F113 clustered into different positions in the trees.

6.5. Discussion.

This study identified that there was a large range in the detectable levels of HCN produced in the bacterial strains screened. The values detected were similar to those previously found by Ellis *et al.* (12). In their study numerous traits were measured that were potentially responsible, or indicative of biocontrol. It was identified that HCN production was the only trait that had any significant correlation to biocontrol ability in strains. It was postulated that SBW25, which the mode of action for fungal antagonism has not been determined might contain the HCN biosynthesis operon but that is was not expressed *in vitro.* Universal primers were designed to the known *hcn*ABC operon for the detection of biosynthetic genes not expressed under laboratory conditions. Difficulties were encountered in the design of "true" universal primers that successfully amplified the operon, particularly in *hcn*A and the beginning of *hcn*B. These same problems were reported by Ramette *et al.* (34). Primers were successfully designed over the junction of the *hcn*B and *hcn*C genes that could amplify all known HCN biosynthesis operons. These primers spanned a greater proportion of the gene (> 1 kb) than those designed by Ramette *et al.* (< 600 bp) (34). Comparisons between the primer sets to identify which were the most highly conserved and therefore "universal" revealed that non-specific amplification occurred, neither primer set allowed 100% accurate and specific amplification in known HCN producing bacteria. The primers by Ramette *et al.* (34) successfully amplified 6 out of the 10 HCN producing bacteria, but produced a non specific amplification of identical size in SBW25; this bacteria is now known from the soon to be completed genome sequencing project not to contain *hcn*ABC (http://www.sanger.ac.uk/projects/p_fluorescens). The new set of primers designed for the investigations in this thesis (section 6.2.5.2) successfully amplified 9 of the 10 known HCN producing bacterial strains, without non-specific amplification. This illustrates their improved specificity to the *hcn*ABC operon. Interestingly, a PF0-1 *hcn*BC fragment was successfully amplified after sequence analysis its identity was confirmed; however it does not produce HCN in the *in vitro* assay. This suggests that some bacteria have the genetic potential to produce HCN and do not or that it is not produced in detectable levels *in vitro.*

Phylogenetic analysis of the collection was undertaken to assess diversity within the of HCN cluster. It was identified that the amount of non-polymorphic bases in the fragment in this study (57%) was not dissimilar to the fragment length amplified by Ramette *et al.* (34) (55%). This continued level of polymorphism would indicate the greater length fragments would yield more resolution for phylogenetic analysis. The *hcn*BC long gene sequence (Figure 6.5) demonstrates that the clades iii and iv cluster with high support and similarity. The sequences in clade ii are less similar with those in clade i highest divergence. Comparison of these clusters to data produced by Ellis *et al.* (12) using alternative similarity measures produced both comparative and dissimilar clustering (12). In comparison to data produced by RFLP analysis, identical clustering was observed, demonstrating similarity in relationships between bacterial strains.

The *hcn*BC sequences produced in this study were compared to other published *hcn*BC gene sequences (11, 23, 29, 34) to identify the phylogenetic relatedness of bacterial strains on the *hcn*BC fragment. The two different tree-building algorithms produced trees with different topologies, although the individual clades did not change. Differences occurred in the rooting of nodes between clades. These differences are a result of the different methods used to produce the tree. Neighbour joining is a clustering method which summarises data by a pair wise distance matrix, this loses information on how the sequences differ. Trees are then built to represent this data with the most parsimonious tree. Maximum likelihood takes into account the data represented by each sequence and tries to maximise the evolutionary similarity between sequences. This technique needs an explicit model of sequence evolution, this is estimated and re-estimated using Modeltest and PAUP. Likelihood analysis will produce the tree that is the most evolutionary likely from the data observed. The different topologies will be a result of the tree building algorithms, where the greatest confidence would be placed on the maximum likelihood tree. This is strengthened by the low bootstrap support in the neighbour joining tree of the conflicting nodes. The tree produced from maximum likelihood in comparison to the neighbour joining tree in the study by Ramette *et al.* (34) demonstrated the same clade structure of common data.

The two trees identify six distinct clades in comparison to the 4 identified by Ramette *et al.* (34). The additional two clades were created from some of the bacterial strains sequenced in this study, one represented by 76/10, M114, ML5 and PF0-1 (Hcn-5), the other by PGS12 and PGSB 8456 (Hcn-6). Of interest is the sequence of F113; the sequence of F113 from this study clusters in clade Hcn-1 as previously shown by Ramette *et al.* (34), But the sequence deposited in genbank (AJ418455) (http://www.ncbi.nlm.nih.gov/Genbank/index.html) on which the analysis was performed clusters in clade Hcn-3. This could only be explained by the mislabelling of the sequences when deposited on the genbank database. This data suggests that there is higher diversity of the *hcn*ABC operon than originally revealed. This is in agreement of the unsuccessful amplification of genes responsible for HCN biosynthesis in C7 additionally in respect my tree clade Hcn-5 has very diverse sequences in comparison to other clades and might represent individual isolates from further families of HCN biosynthetic operons.

In conclusion it has been found that there is high variability in the expression levels of HCN biosynthesis in *in vitro* studies from non-expression/undetected expression (PF0-1) to very high expression (ML5) This was not un expected but was of interest. The design of new primers to amplify all operons has been highly successful and data from this study indicates that there is a high level of diversity between sequences of bacterial strains. This variance identified subgroups suggesting that there are more HCN biosynthetic operons that would be successfully amplified if the appropriate primers could be designed. It may not be possible to truly identify universal HCN biosynthesis operon specific primers. The increase in length of products amplified in this study allowed a greater proportion of genes to be sequenced increasing the resolution of phylogenetic analysis.

It was identified that the HCN biosynthesis operon is amplifiable from environmental samples indicative that there may be high presence of the gene in the natural environment and its presence is of importance to the bacteria in the environment. These primers therefore could be applied to environmental samples to assess variation and distribution. This would suggest that the HCN biosyntheis operon would be a useful trait for the potential use in the genetic modification of bacterial involved in

biological control as it is already prevalent among different bacterial species and there would not be high section pressure for this trait.

6.6. Figures.

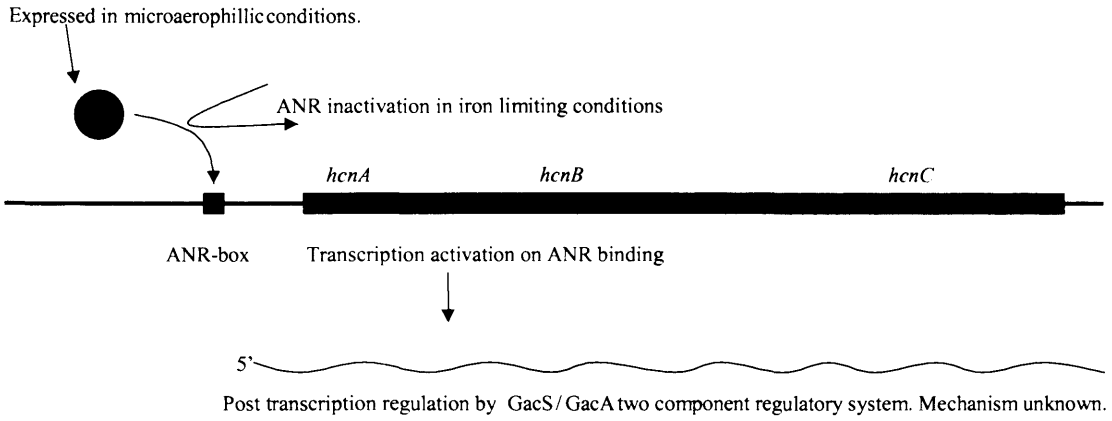

Figure 6.1. A schematic diagram showing the predicted regulation of *Pseudomonas fluorescens* CHA0 *hcn*ABC gene cluster (23).

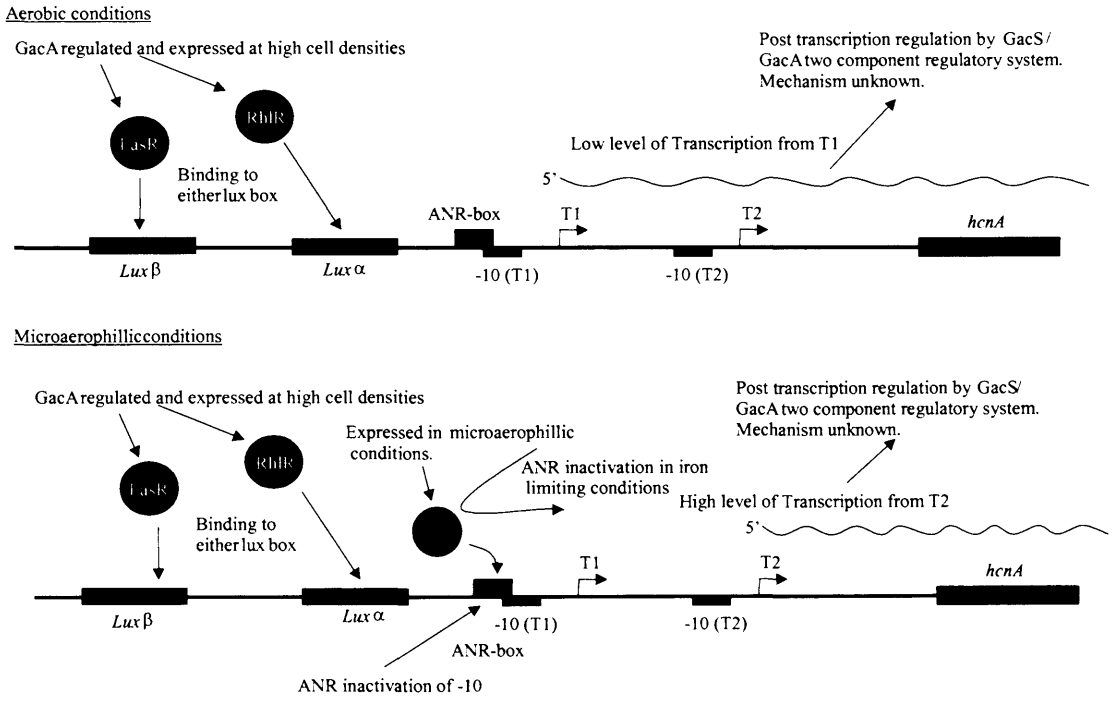

Figure 6.2. A schematic diagram showing the regulated expression of *Pseudomonas aeruginosa* PA01 in aerobic and microaerophillic conditions (30).

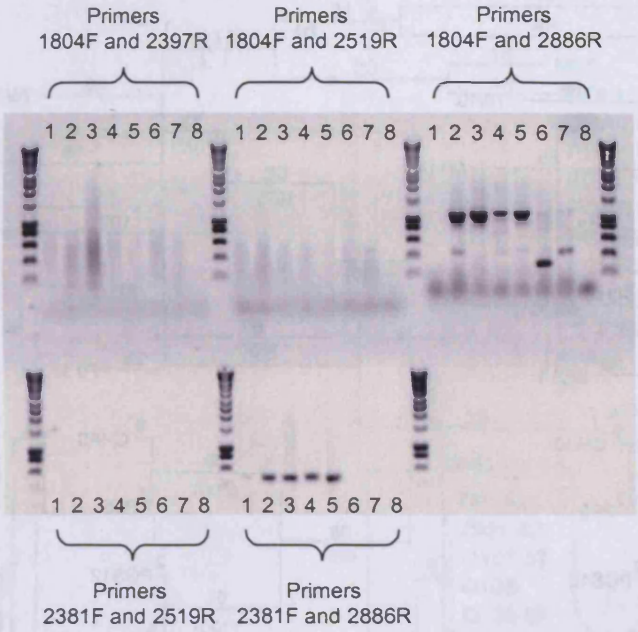

Figure 6.3. PCR amplification of the *hcn*ABC using 5 different primer combinations on the bacteria SBW25, PF0-1, PA01, PF5, F113, PGS12 and a water control represented by lane numbers 1-8 respectively. The primers were designed to alignments of the bacteria CHA0, PA01, PF0-1, PF5 and F113, with primer name representing start position of the primers on the CHA0 operon. Agarose gel electrophoresis was undertaken as described in section 2.2.4. using DNA Hyperladder I (Bioline, UK) as size marker between each set of PCR products.

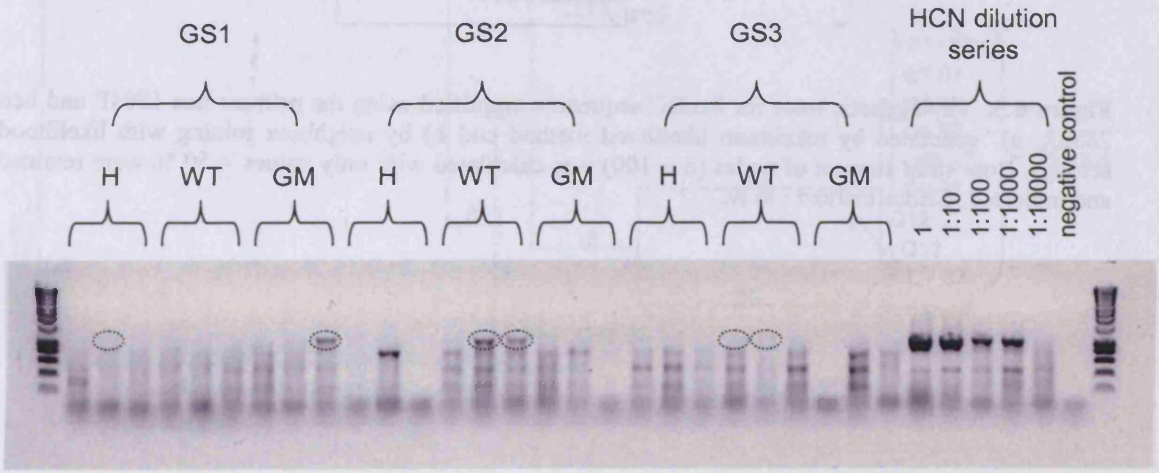

Figure 6.4. An agarose gel showing products of PCR amplification of environmental DNA samples using the primers 1804F and 2886R designed to the *hcn*ABC operon. In addition to this a dilution series of template was undertaken to identify the detection limit of the primers. A 1:1 mix was used of SBW25 genomic DNA and CHA0 DNA with serial 1:10 dilutions made to the CHA0 DNA before adding. The environmental DNA is from the rhizosphere of pea plants taken at three growth stages. There were three treatments: water control, WT-BCA inoculation and GM-BCA inoculation (n =3) (See Chapter 4). Bands corresponding to PCR products of HCN amplification representing the *hcn*ABC operon are circled in the environmental DNA samples.

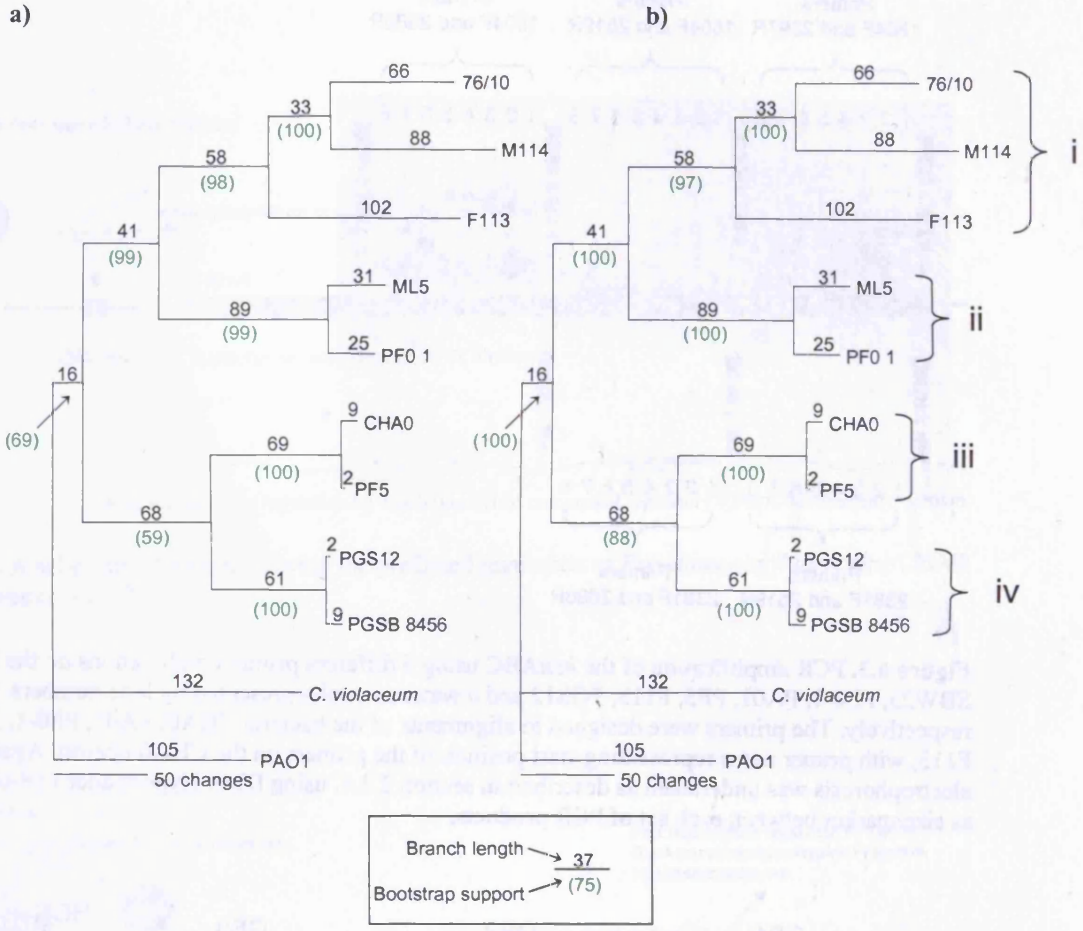

Figure 6.5. Phylogenetic trees for *hcn*BC sequences amplified using the primers hcn 1804F and hcn 2886R. **a)** generated by maximum likelihood method and **b)** by neighbour joining with likelihood settings. Boot strap support of nodes (n = 100) was calculated with only values > 50 % were retained and indicated. Clades marked i to iv.

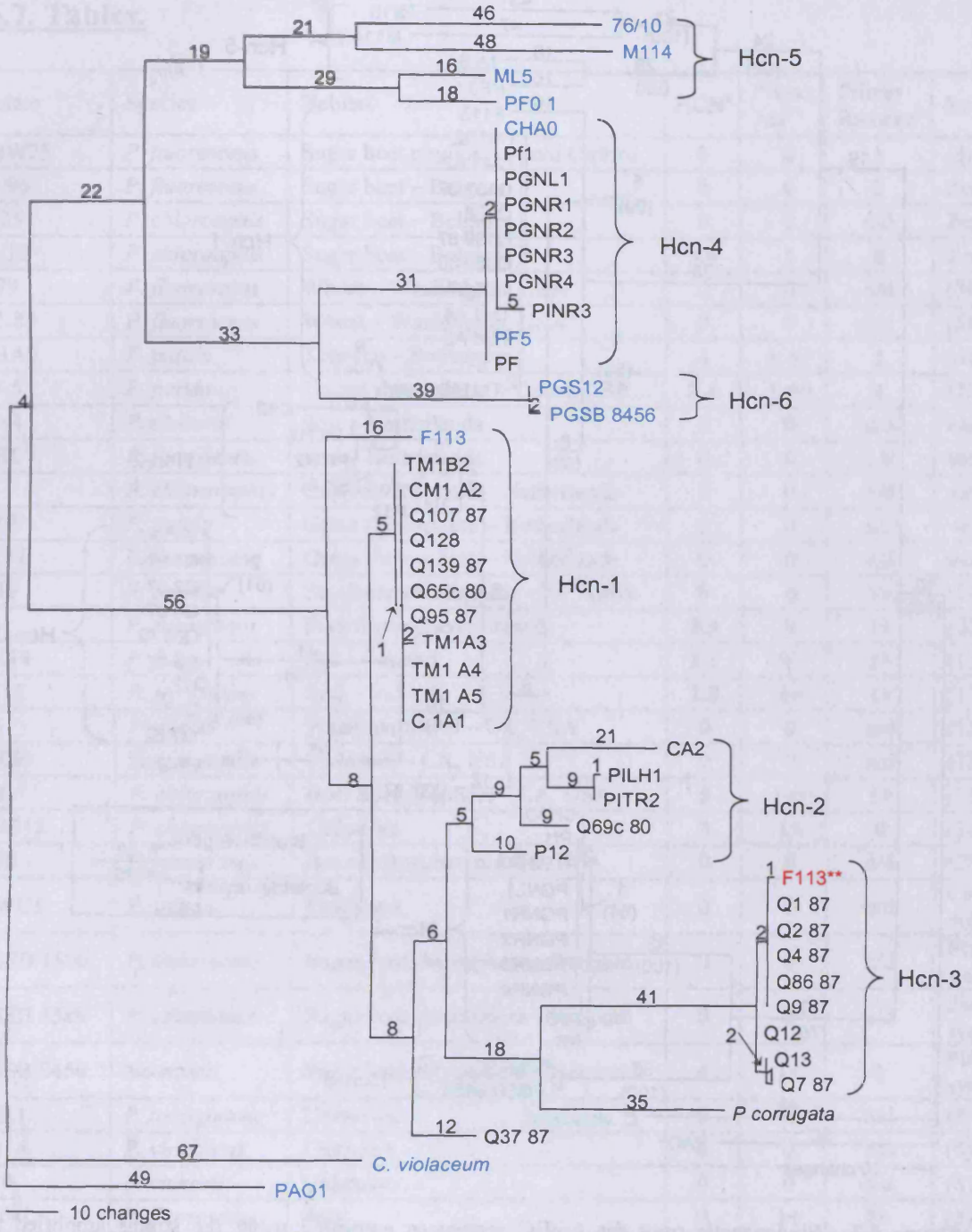

Figure 6.6. Phylogenetic tree for *hcn*BC sequences using the strains amplified in this study and sequenced in section 5.3.5 with trimming and alignment to sequences published by Ramette *et al.* (34). Tree was produced using maximum likelihood method with no boot strap support (computationally too demanding). Named strains in blue were sequenced in this study, remaining strains and strain F113** sequences were from Ramette *et al.* (34). Clades named as in Ramette *et al* (34) Hcn-1 to Hcn-4 with the addition of clades Hcn-5 and Hcn -6 found in this study.

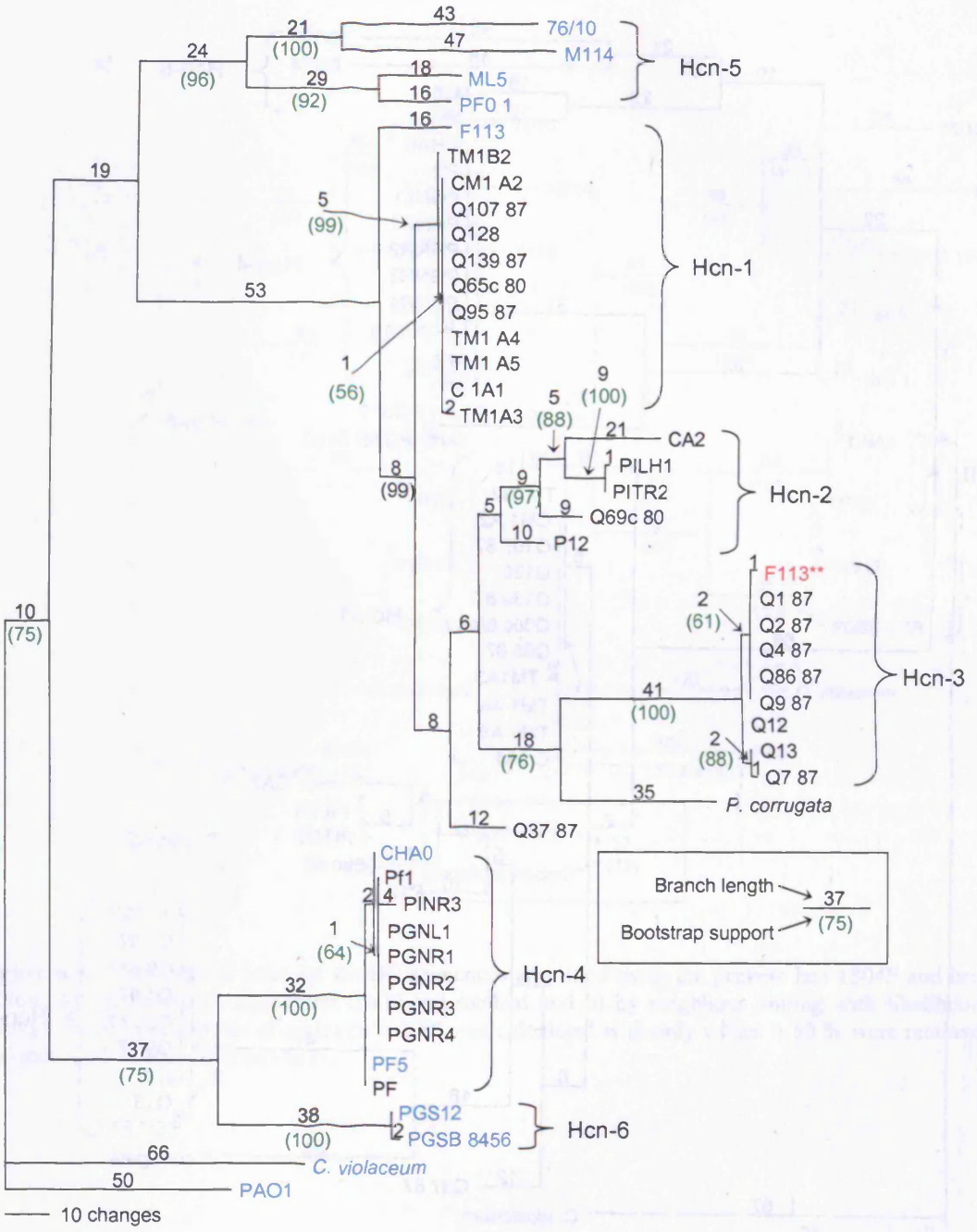

Figure 6.7. Phylogenetic trees for *hcn*BC sequences amplified using the strains amplified and sequenced in section 5.3.5 with trimming and alignment to sequences published by Ramette *et al.* (34). Tree was produced using neighbour joining with likelihood method settings and boot strap support of nodes (n = 100). Only values > 50 % were retained and indicated. Named strains in blue were sequenced in this study, remaining strains and strain F113** sequences were from Ramette *et al.* (34). Clades named as in Ramette *et al* (34) Hcn-1 to Hcn-4 with the addition of clades Hcn-5 and Hcn -6 found in this study. And correspond to those in Figure 6.6.

6.7. Tables.

Isolate	Species	Habitat	HCN[a]	Primer AH[b]	Primer Ramette[c]	Reference
SBW25	*P. fluorescens*	Sugar beet phylloplane n/d Oxford	0	0	1	(3)
54/96	*P. fluorescens*	Sugar beet – Belgium	0	0	0	Zeneca, (12)
1335	*P. chloroaphis*	Sugar beet – Belgium	0	0	n/d	Zeneca, (12)
76/10	*P. chloroaphis*	Sugar beet – Belgium	**3.6**	**1**	**0**	Zeneca, (12)
2-79	*P. fluorescens*	Wheat – Washington, USA	0	0	n/d	(38)
Q2-87	*P. fluorescens*	Wheat – Washington, USA	0	0	n/d	(5)
CHA0	*P. putida*	Tobacco – Switzerland	**4**	**1 ++**	**1**	(41)
PF-5	*P. putida*	Cotton rhizosphere – Texas, USA	**3.4**	**1 ++**	**1**	(19)
2Ps4	*P. cichorii*	Soil – Netherlands	0	0	n/d	van Elsas
GE1	*P. fluorescens*	Soil – Netherlands	0	0	n/d	van Elsas
P1	*P. chlororaphis*	Grass rhizosphere – Netherlands	0	0	n/d	van Elsas
R2F	*P. putida*	Grass rhizosphere – Netherlands	0	0	n/d	van Elsas
R12T	*P. fluorescens*	Grass rhizosphere – Netherlands	0	0	n/d	van Elsas
PH6	*P. putida*	Soyabean rhizosphere – NC, USA	0	0	n/d	(14)
C7	*P. savastanoi*	Flax rhizosphere, France	**2.9**	**0**	**1+**	(22)
M114	*P. fluorescens*	Soil – Ireland	**1.1**	**1+**	**1+**	(13)
F113	*P. savastanoi*	Soil – Ireland	**2.8**	**1+**	**1+**	(13)
A1	*P. putida*	Potato periderm – CA, USA	0	0	n/d	(15)
CR30	*P. marginalis*	Field soil – CA, USA	0	0	n/d	(15)
ML5	*P. chloroaphis*	Beet Spermosphere – CA, USA	**5**	**1++**	**1+**	(28)
PGS12	*P. chloroaphis*	Unknown	**3**	**1+**	**0**	(16)
R20	*P. savastanoi*	Bean rhizospheren/d CA, USA	0	0	n/d	(28)
UWC1	*P. putida*	Unknown	0	0	n/d	Cardiff collection
PGSB 1500	*P. fluorescens*	Sugar beet rhizosphere – Belgium	0	0	n/d	Plant genetic systems
PGSB 5589	*P. savastanoi*	Sugar beet rhizosphere – Belgium	0	0	n/d	Plant genetic systems
PGSB 8456	No match	Sugar beet rhizosphere – Belgium	**4**	**1+**	**0**	Plant genetic systems
7SR1	*P. aeruginosa*	Unknown	0	0	n/d	(8)
A214	*P. viridflava*	Unknown	0	0	n/d	(8)
B10	*P. cichorii*	Unknown	0	0	n/d	(8)
PF0-1	*P. fluorescens*	Soil	0	**1+**	**1+**	(2)
KT2442	*P. putida*	Soil	0	0	n/d	(40)
NCIMB 11764	*P. fluorescens*	Unknown	0	0	n/d	CEH collection
PAO1	*P. aeruginosa*	Patient wound infection	2	**1+**	**0**	(37)
-	*B. pumilis*	Unknown	0	0	n/d	CEH collection
-	*C. violaceum*	Unknown	0	0	0	CEH collection

Table 6.1. Bacterial strains used in this chapter, with measures of HCN production and amplification success using Primers designed to *hcn*ABC operon. **a.** HCN production determined as described by Voisard *et al.*, (41), **b.** Product by PCR using primers designed in this study, **c.** Product by PCR using primers by Ramette *et al.*, (34). n/d = not determined. In regard to PCR 0 = no amplification 1 = amplification with + indicating strength of amplification.

6.8. References.

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