THE UNIVERSITY OF
SYDNEY

The response of wheat genotypes to inoculation with *Azospirillum brasilense*.

Kazi Nayla Rashid

A thesis submitted for fulfilment of the requirements for the degree of Doctor of Philosophy

Faculty of Agriculture and Environment
The University of Sydney
New South Wales
Australia

August 2014

STATEMENT OF ORIGINALITY

I hereby certify that this thesis has not been submitted for any degree or diploma at any university and that to my best knowledge any help received in preparing this thesis and all reference materials used, have been acknowledged.

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Kazi Nayla Rashid

August 2014

ABSTRACT

It is well documented in many studies that plant growth promoting rhizobacteria (PGPR) are capable of increasing plant growth and productivity in a range of agricultural crops, reducing dependence on chemical amendments and maintaining a safe environment. Over the last two decades PGPR inoculants have been increasingly used in agriculture to improve crop productivity and farming system sustainability. Such eco-friendly technologies are needed to address sustainable food security and to avoid global dependence on hazardous agricultural chemicals which ultimately destabilize agro-ecosystems. The nitrogen fixing bacteria, *Azospirillum brasilense,* has been an important PGPB (plant growth promoting bacteria) used to enhance the growth and yield of many crops globally. This is attributed mainly to its ability to produce phytohormones. While much is known about *A. brasilense*, the promising effect of PGPBs in general in the field is limited by factors that influence their survival and activity in the rhizosphere. The attachment of bacteria to roots is an essential and necessary condition for the establishment of an effective association. This association is dependent upon the population density of active PGPB cells in the rhizosphere which are able to compete with indigenous bacteria.

However, how survival and persistence of inoculant bacteria in the rhizosphere, the effect of inoculum on the rhizosphere community, in particular the nitrogen fixing community, and the effect of plant genotype contributes to plant growth promotion by *Azospirillum* in the field have not been widely studied. Better understanding of the plant x inoculum

interaction requires determining if there is an effect of plant genotype and monitoring and estimation of the persistence of PGPB in the rhizosphere.

The overall aim of this project was to examine the effect of the wheat (*Triticum aestivum*) genotype x *Azospirillum* interaction on colonization of roots and plant growth promotion. These effects were studied under both controlled hydroponic conditions in the laboratory and in the field. Plant growth parameters and bacterial colonization of the rhizosphere were determined in both conditions.

Differences in root characteristics of twenty three diverse wheat genotypes were observed after growth in the hydroponic system; however responses to inoculation with *A. brasilense* Sp7 and Sp7-S were variable. In some cases growth parameters were increased and in others they were decreased. There was an apparent increase in responsiveness to inoculation with azospirilla by synthetically derived genotypes observed in root length measurements but otherwise there was no trend according to the genetic source of wheat. Microscopic observations confirmed the different root colonisation patterns by Sp7 and Sp7-S. However, colonisation pattern was not influenced by plant genotype. Relationships between shoot dry weight and root growth parameters were positive as expected but were strengthened with inoculation. There was a generally negative relationship between root growth parameters and bacterial number indicating that an optimum rather than a maximum number of azospirilla are required for plant growth promotion in this system.

A field experiment was conducted from May-November in 2010 and 2011 at the Plant Breeding Institute, Narrabri, Faculty of Agriculture and Environment, University of Sydney to evaluate bacterial inoculants on wheat genotypes in two different environments that varied for N. Five wheat genotypes were selected from among the 23 originally screened under controlled hydroponic conditions; 1. EGA GREGORY, 2. CBRD/KAUZ//KASO2, 3. CROC_1/AE.SQUARROSA (205)//KAUZ/3/2*METSO, 4. SOKOLL and 5. KRICHAUFF. The bacterial strains were applied to the seed as a liquid at a rate of 10^9 cfu/ml. Treatments were arranged in a split plot design with four replicates with inoculants as main plots and genotypes as sub-plots. N treatments (2011) and/or different environments (2010) were arranged in separate adjacent experiments.

Various morpho-physiological parameters such as plant height, root architecture, dry matter accumulation, relative chlorophyll, NDVI (Normalized Difference Vegetation Index), grain yield, biomass at maturity, harvest index and Thousand kernel weight (TKW) were assessed and analysed. Inoculation effects were observed for some traits, particularly in the early growth phase; however these effects diminished with time and no effect of PGPR on final biomass or grain yield was observed. Those traits most influenced by inoculation were root traits including total root length, surface area and volume in the early stages of growth. Some effect on early season plant height was observed and while TKW and chlorophyll were influenced by inoculation this did not translate into higher yield. There was no strong effect of plant genotype x bacterial strain interaction on grain yield or TKW; the important productivity traits, observed in any experiment.

The persistence of bacterial populations in the rhizospheric soil was also estimated three times during the season (active tillering, pre and post anthesis) using the most probable number (MPN) technique. A significant difference in the number of N_2 fixing isolates was observed at 43 days (active tillering) between inoculated and uninoculated plots in both years. However, these differences had disappeared by 103 (pre-anthesis) and 133 days (post-anthesis). Overall, bacterial counts, which included indigenous bacteria, were high in all treatments including control plots. Persistence of the inoculant strains was examined indirectly by measuring the effect on the nitrogen fixing community in the rhizosphere. This was done using terminal restriction fragment length polymorphism (T-RFLP). Little effect of plant genotype and inoculant strain was detected in the rhizosphere community. However, evidence of the persistence of inoculated azospirilla in the rhizosphere was indicated by the presence of the relevant T-RF fragments even in the post-anthesis period. Both the MPN and T-RFLP techniques indicated that the inoculated strains did survive throughout the growing season, although the total numbers in each treatment were difficult to estimate.

The cool growing season and adequate if not excessive moisture available throughout the anthesis and grain-filling periods in both years extended the growth cycle of the crop, thus diminishing the early season effects of inoculation. While the inoculated bacteria did persist throughout the growth period we can only conclude that growing conditions were too favourable to elicit an economic response to inoculum. The effect of plant genotype on PGPB inoculation response in the field was also not strong and future studies should examine a wider range of wheat genotypes and inoculum strains across a wider range of growing conditions.

ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
AMMI	Additive Main Effects and Multiplicative Interaction Model
BNF	Biological nitrogen fixation
cfu	Colony forming unit
DArT	Diversity Arrays technology
DAS	Days After Sowing
DAT	Days After Transfer
ET	Ethylene
GEI	genotype x environment interaction
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISR	Induced systemic resistance
MPN	Most probable number
NfB	Nitrogen Free Broth
nMDS	Non-metric multidimensional scaling
PCA	Principal component analysis
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
ONPG	o-nitrophenyl-β-D-galactoside
TRFLP	Terminal Restriction Fragment Length Polymorphism
TRFs	Terminal Restriction Fragments
VAM	Vesicular arbuscular mycorrhiza
YMA	Yeast Mannitol Agar
2,4-D	2,4- dichlorophenoxyacetic acid

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisor Professor Richard Trethowan for his outstanding guidance, suggestions, support and indefinite patience throughout the period of research work and in the completion of this thesis. I highly appreciate the substantial time and effort he has devoted in reviewing this thesis. I would like to extend my appreciation to my associate supervisor Dr Rosalind Deaker for her generous guidance, sharing her knowledge, expertise and friendly relationship, support and help throughout the research work. I also appreciate the enormous amount of time she invested to reviewing the thesis. I would like to thank Dr Mihaly Keskes and Professor Ivan Kennedy for their assistance and guidance in the establishment of the research project.

This was a huge project and without the support and help of many people it would not have been possible to conduct successfully such a large and intensive field experiment. I acknowledge and appreciated the support of the entire team at PBI Narrabri, particularly Graeme Rapp and Bill Wall, then station manager, for helping with sowing and harvesting. I would also like to thank Dr Tariq Chattha, Dr Babar Atta, Mr Tawanda Kapfuchia and Ms Grace Bolton for helping me take field measurements, particularly when I was unable to do the task in Narrabri.

I would also like to thank Ms. Apriwi Zulfitri, Mrs Nirmala, Ms Mirja, Associate Professor Michael Kertez, Dr Rosalind Deaker, Dr Mihaly Keskes, Mr.Kamal Muhammad, Ms Orsi,

Dr. Khanok-on Amprayn, Dr. Akitomo Kawasaki, Dr. Ganeshan Krishnen, Mrs. Humaira Yasmin , Ms Andrea, Ms Geraldine and Dr. Noor Hasniza for their practical assistance, fruitful discussion, help and friendship. My sincere appreciation goes to the faculty laboratory staff including Mr Iona Gyorgy, Ms Nishath Ganguli, Ms Kasia, and Ms Kathy Donohoe for their helpful assistance.

Special thanks are due to Dr.Neil Wilson for sharing his molecular expertise and his assistance and guidance throughout the project.

Finally, with love and respect, I would like to extend my deepest gratitude to my husband, Mr. Kamal Muhammad, for standing beside me and supporting me throughout the course of study, without his co-operation it would not have been possible to complete my PhD degree. I would like to especially thank my children Ms Noshin and Master Nafseen for their patience and sacrifice of quality time with their mum throughout the study. I would like to thank all my brothers and sisters for their encouragement to finish this work. I would like to extend my appreciation to my parents for their blessings to my entire education even though they are not here to witness its completion. With love and respect I would like to dedicate this work to my dear husband and my beloved parents (may The Almighty rest their souls in peace).

With love and respect

This work is dedicated

To

My dear Husband and Parents

1. Introduction

Wheat is the second major food staple for half the world's population and average global per capita consumption in 2013 was steady at around 67.7 kg (http://www.fao.org). However, per capita consumption is 60.4 kg in developing countries and 97.4 kg in the developed world. FAO's latest forecast for global wheat production is 702 million tonnes making it the third largest crop globally. Wheat was introduced to Australia after European settlement in 1788 and is now the most important agricultural crop nationally. While Australia produces only 4% of the world's wheat, the country is the third biggest exporter comprising 15% of global wheat trade. Australian wheat is exported to the Middle East including Egypt and Iraq and to Eastern Asia including Indonesia and Japan. Australian wheat has an excellent reputation in the global market for quality.

The demand for food is growing steadily as the world population increases and chemical fertiliser is the most important input required for grain production. As a result, global fertiliser consumption is increasing steadily to meet the ever higher production targets. In a recent report, earth policy claimed that 181 million tonnes of fertiliser was used to produce 2438 million tonnes grain (data source fas.usda.gov/psdonline).

To meet the increasing demand, plant breeders must find ways to increase crop production on the same land area without substantially increasing resource use. High-yielding wheat varieties have increased wheat production; however they also require large amounts of chemical fertilizers and this has adverse effects on the environment (Kennedy and Cocking 1997).

Increased concern over the price of chemical fertilisers and environmental impacts has fostered research into alternatives to chemical fertilisers and pesticides. To reduce dependence on chemicals, a group of nonpathogenic free living bacteria, termed plant growth-promoting rhizobacteria (PGPR), have been introduced into agriculture to improve sustainability. PGPR offer an alternative that can promote plant growth and development by improving nutrient up-take and supressing diseases without any adverse effect on the environment (Kloepper et al. 1989; Vessey 2003). Thus, the number of bacterial species identified as PGPR has increased.

The use of PGPR is increasing worldwide because of their perceived effectiveness as growth promoting agents in many crops, including wheat, even though the biofertiliser effects in the field are often inconclusive (Diaz-Zorita and Fernandez-Canigia 2009).

There are several PGPR used to promote the growth of wheat including *Pseudomonas fluorescens,* (De-Freitas and Germida 1992; Shaharoona et al. 2008), *Rhizobium* (Afzal and Bano 2008)*, Azospirillum brasilense, (*Dobbelaere et al. 2001; Diaz-Zorita and Fernandez-

Canigia 2009), *Azotobacter chroococcum* and *Azomonas macrocytogenes* (Pati and Chandra 1995). These PGPR stimulate plant growth through various mechanisms including biological nitrogen fixation and the synthesis of phytohormones (Kennedy et al. 1997, Khalid and Zahir 2004).

Bashan and de-Bashan (2010) reviewed the possible plant growth promoting mechanisms of *Azospirillum* and grouped the possible biological processes. They concluded that the most effective process is production of phytohormones specifically IAA (Indole Acetic Acid), GA (Gibberellic Acid) and ABA (Abscisic Acid) which directly effect on the root architecture and ultimately water and mineral uptake, thus helping the plant manage environmental stresses. They also concluded that biological nitrogen fixation is an influential process; however the effect of this in *Azospirillum* is not well documented.

Numerous articles have reported that plant growth-promoting azospirilla isolated from the rhizosphere of cereal crops were able to fix nitrogen (Dobereiner and Day, 1976; Patriquin et al. 1983). These azospirilla can also produce phytohormones such as indoleacetic acid (IAA), gibberellins and/or cytokinin like substances (Okon and Labandera-Gonzalez, 1994). Azospirilla species and strains including *A. brasilense* Sp245, Sp7 and Sp7-S, were reported to fix nitrogen and produce phytohormone (IAA) (Katupitya et al. 1995; Roper and Ladha, 1995).

A. brasilense has been used during the past 40-years as an inoculant to improve the productivity of wheat worldwide (Okon and Labanderra-Gonzalez 1994). However, the persistence of inoculum strains in the field and the conditions that influence root colonization

including root morphology is still unknown, even though plant growth promotion has been widely reported (Bashan et al. 2004). Moreover, information on the impact of inoculation on the rhizospheric nitrogen fixing community, the response of wheat genotype and the persistence of the inoculum in the wheat rhizosphere is generally scarce (see the review of Trabelsi and Mhamdi 2013) and the plant-soil-biota interaction is still not well documented and should be the focus of future research. Thus, the responses of wheat to the PGP *A. brasilense* strains Sp7, Sp7-S and Sp245, must be determined to know exactly how plants behave after inoculation in the laboratory as well as under field conditions. Better understanding of the plant growth promoting effect requires monitoring of bacteria number in the rhizosphere over time; in other words, estimation of persistence in the soil. In addition, assessment of the impact of inoculum in the wheat rhizosphere at the molecular level would provide better understanding of this complex interaction.

Microbial (most probable number) and molecular (terminal restriction fragment length polymorphism) approaches are used in this study to quantify bacterial numbers in the field after inoculation and the effect of inoculation on the nitrogen fixing bacterial community in the rhizosphere. These interactions are explored using different wheat genotypes screened under both laboratory and field conditions. Therefore, this thesis addresses the general hypothesis that a plant genotype x bacterial strain interaction that improves the grain yield of wheat exists.

The aims of this research are as follows:

- To determine the responses of a range of genetically diverse wheat genotypes with different root architecture to inoculation with *Azospirillum brasilense* strains under both controlled and field conditions
- To assess the interaction between inoculant strain, wheat genotype and environment
- To assess persistence of the inoculum in the rhizosphere
- To determine the effect of inoculation on the diversity of the *nifH* gene pool in the rhizosphere

2. Literature Review

2.1 Wheat

2.1.1 Origins and nomenclature

Wheat belongs to the *Poaceae* family, subfamily *Pooideae*, Tribe *Triticeae* and genus *Triticum.* The most common form is hexaploid (AABBDD) while durum wheat, largely grown for pasta, is tetraploid (Hancock 2004). Hexaploid wheat is generally higher yielding and more adapted to a wide range of climates than either diploid or tetraploid forms. There are various forms of wheat and those species that have been cultivated include:

- Hexaploid common wheat or bread wheat (*T. aestivum*); the most widely cultivated wheat globally

- Tetraploid durum (*T. durum*); the second most widely cultivated wheat.
- Diploid einkorn (*T. monococcum*); a wild species and possible origin of the A genome in wheat
- Diploid *T uratu*; a wild species that is also a contender for origin of the A genome
- Tetraploid emmer (*T. dicoccum*); an ancient cultivated species.
- Hexaploid spelt (*T. spelta*); an ancient cultivated species reported to have health benefits, particularly for those with gluten allergies. (ref:http://en.wikipedia.org/wiki/Wheat#Genetics).

2.1.2 Wheat cultivation and production

Wheat is one of the most important food grains globally and has been cultivated since ancient times. Wheat has been a staple for mankind for more than 8,000 years in regions such as Europe, west Asia and North Africa. Today, wheat is grown on more land area than any other crop and provides the primary source of calories for many people in developing countries. Wheat is second only to maize and rice in the total volume produced globally. In 2013, global wheat production exceeded 700 million tons (FAOSTAT http://www.fao.org/docrep/018/al999e/al999e.pdf). Australia produces just 3% of the world's wheat but accounts for 10-15% of the world's 100 million tonne annual global wheat trade. Source; (http://www.pwc.com.au/industry/agribusiness/assets/Australian-Grains-Industry-

Nov11.pdf).

Wheat is usually sown from May through June and harvested between October and January. Production is almost entirely rainfed and Figure 2.1, reproduced from the ABARE Australian crop reports 2011, shows the wheat growing areas of Australia. These extend from central Queensland in the north to the Wimmera in Victoria with significant production in southern South Australia and the south-western regions of Western Australia. While sowing times are similar in the different regions, the length of the growing season varies and farmers in central Queensland may begin harvesting in October whereas those in Victoria will harvest in January.

Table 2. 1 Australian crop production (2009-2010)

source: (http://www.pwc.com.au/industry/agribusiness/assets/Australian-Grains-Industry-Nov11.pdf)

Crop	**'000 ha**	**Production (kt)**	**Yield (t/ha)**
Wheat	13,881	21,834	1.57
Barley	4,222	7,865	1.86
Sorghum	498	1,508	3.02

Canola	1,712	1,920	1.12
Oats	850	1,162	1.37
Rice	19	65	3.42
Pulses	1,406	1,666	1.19

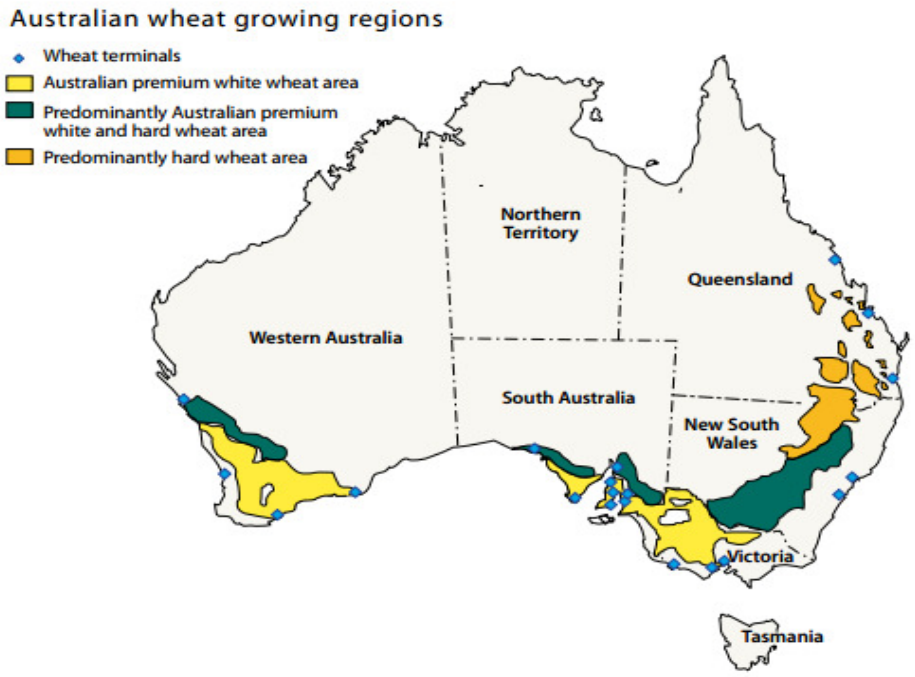


Figure 2.1 Wheat growing areas in Australia (source:

http://data.daff.gov.au/brs/data/warehouse/pe_abares99001787/ACR11.1_Feb_REPORT.pdf)

2.1.4 Uses of wheat

Wheat is mainly used as human food but can also be fed to livestock. It contains gluten protein which makes it suitable for the production of leavened breads. Wheat is a staple food for many countries and supplies 20% of daily global calorie consumption. It is a good source of carbohydrate, protein, minerals and vitamins and is primarily used for breads, noodles and pasta production based on the types of wheat produced. In Australia these include the classifications; Prime Hard, Hard, Premium White, Standard, Soft and Durum. (http://www.pwc.com.au/industry/agribusiness/assets/Australian-Grains-Industry-Nov11.pdf). Hexaploid bread wheat is mainly used to make leavened, flat and steamed breads, yellow alkaline and white salted noodles and soft-grained types processed to make biscuits, cookies and other processed and packaged foods. Tetraploid durum wheat (*T.durum*) is largely used to make macaroni, spaghetti, couscous and in some cases bread. Wheat is also used as livestock and poultry food and the straw used as forage or hay on farm. Wheat is also used to make alcohol, oil and gluten for industrial processes.

2.1.4 Root architecture of wheat

Roots play an important role in nutrient uptake and water absorption from the soil. The size and architecture of the root system determine the efficiency of the nutrient acquisition and a scholastic model of the plant root system architecture was formulated by Zhang et al. (2003). They described and modelled the continuous growth and development of the root system of winter wheat seedlings. In their experiments individual roots of wheat seedlings were scanned and images analysed with root image- analysing software; WinRhizo. The root anchors the

plant and is a store of carbohydrate. After germination, primary roots appear from the lower end of the embryo followed by lateral roots which steadily increase the absorption area while anchoring the plant. Most cereals normally have three seminal roots arising from the seed; one is the primary root and the other two are equally large lateral roots. These roots and their branches (sometimes supplemented by other primary roots) constitute the primary root system. The remaining portion of the root system arises from the nodes or joints of the stem in the soil. Roots not arising from the seed or as branches of seed roots but from stems or leaves are termed adventitious. In the case of the cereals and other grasses which have strong, threadlike or fibrous roots, the larger part of the root system is composed of the adventitious roots which collectively make up the secondary root system. It is noteworthy that the roots of the secondary system originate only about 3 cm below the soil surface, even if the grain is planted 6 – 7 cm deep.

2.2 Use of chemicals in agriculture and the impact on the economy and environment.

World population is projected to double by 2050 (Science daily April 16, 2001) and the need for food will also double. There is considerable pressure to not only increase productivity per unit area but to do this sustainably. During the first 35 years of the Green Revolution, global grain production doubled and this was a combination of superior genetics and the use of chemical fertilizer and pesticides (Trethowan et al. 2007). Fertilizer supplies the essential nutrients required by crops to maximise plant growth and grain yield. Chemical fertilizer has balanced levels of three important nutrients: potassium, nitrogen and phosphorus, which are essential for plant growth.

Crop production has increased worldwide through higher-yielding varieties, improved irrigation systems, chemical fertilisers and pesticide application (Rahim 2002). However, there are serious disadvantages when fertilizer is applied inefficiently (Kennedy and Tchan 1992). Chemical fertiliser is not only costly but also a potential source of environmental pollution. It impacts soil health, water quality and the climate through increased carbon emissions thus damaging the whole ecosystem (Camargo and Alonso 2006; Kennedy and Cocking 1997; Merrington et al. 2002). However, these chemicals increase yield despite the adverse effects on the environment (Kennedy and Cocking 1997). Consequently, various adverse effects such as soil deterioration, water contamination and global warming were reported to be indirectly influenced by excess use of agrochemicals including fertiliser and pesticide (Camargo and Alonso 2006; Kennedy and Cocking 1997; Merrington et al. 2002).

2.2.1 Water

Nitrogen rich chemical fertilisers leach through the soil and contaminate surface and sub-surface water and cause eutrophication. In surface water areas, algal blooms develop and use up the dissolved oxygen thus killing fish and other organisms resulting in dead zones in large lakes and oceans. A 2008 review by Diaz and Rosenberg reported that more than 400 dead zones exist all over the world and affect more than 245,000 square kilometers of the marine ecosystem. These dead zones are expected to increase worldwide from the uncontrolled use of fertilizers.

Blaha et al. (2009) reported that cyanotoxicity from cyanobacterial blooms in freshwater represent a major ecological and human health problem worldwide. Degradation of aquatic ecosystems by nutrient pollution resulting in massive cyanobacterial water blooms is a global problem representing serious health and ecosystem risks. It has been estimated that 25 to 75% of cyanobacterial blooms are toxic (Chorus 2001; Bláhová et al. 2007) and represent health hazards for humans and animals (Codd et al. 2005). Algal toxins can be accumulated, transferred and magnified throughout the food chain.

2.2.2 Leaching

Excessive fertilizer use can also impact sub-surface water. Leaching of nitrate from farmer's fields can raise these concentrations in ground water to an unacceptable level for drinking. These nitrates eventually find their way into the aquatic ecosystem promoting acidification and algal blooms including the production of toxic inorganic nitrogenous compounds (Camargo and Alonso 2006).

2.2.3 Soil

All nitrogen rich fertilisers (ammonium-based nitrogen) used on farm can accelerate acidification of soil through nitrification if significant leaching of nitrate occurs (Kennedy 1991). Under acidic conditions, soil nutrients can become less available while some other micro or macro nutrients could reach toxic levels. In many cases soil acidity is manifested through aluminium (Al) and manganese (Mn) toxicity.

Awareness has been growing of the negative impacts of excessive and imbalanced use of synthetic fertilizer and pesticide. Many in the scientific community are looking for alternatives. The rapid rise in the cost of fertilizers and pesticides has led farmers to try and find the ways of reducing fertilizer use. An alternative is biofertilizer and integrated pest management. Continuing agricultural research and education is needed if increased yield production is to be achieved without reducing the sustainability of the ecosystem and the long term productivity of soil.

2.3 Biofertilizer

Biofertiliser is comprised of living microbes that are added to the soil as inoculants. These microbes improve plant growth and help fight diseases in different ways. Most fertilizers provide nitrogen, phosphorus and potassium to the soil thus helping plant growth. Of these N is very important for plant growth and yield. Although present in the atmosphere (where 78% N is found) it is not in an available form. Some bacteria can fix the nitrogen from the air by changing nitrogen to ammonia; this process is called Biological Nitrogen Fixation (BNF) (Kim and Rees 1994). These nitrogen fixing micro-organisms are naturally present in the environment and BNF is an economically beneficial and environmentally friendly alternative to chemical fertiliser (Reymond et al. 2004; Peoples et al. 1995). Biological nitrogen fixation does not exceed the actual nitrogen requirements of plants so less pollution is generated (Kennedy and Tchan 1992). A number of bacteria are capable of fixing the nitrogen from the environment either symbiotically or asymbiotically. The area surrounding the root system is known as the rhizosphere (Walker et al. 2003) and this space contains rhizobacteria (Kloepper et al. 1991). In addition to mechanical support and water and mineral uptake, plant roots also

secret a number of chemical compounds into the soil which are generally known as root exudates (Walker et al. 2003). These exudates attract soil microbial communities to the roots. There are several different types of microbial communities which are commonly found in the soil including bacteria, fungi, actinomycetes, protozoa and algae (Paul and Clark, 1989). Of these, bacteria are the most common micro-organisms present. There are many species of bacteria that inhabit the rhizosphere and that benefit the plant in many different ways; these are called plant growth promoting rhizobacteria (PGPR) (Kloepler et al. 1980). The PGPR mainly belong to four groups of bacteria namely the Diazotroph, Bacili, Pseudomonads and Enterobacteriacea (Kloepper 1994).

The benefits of PGP bacteria are obvious. These include more sustainable agriculture, prevention of soil erosion and dust pollution and enhancement of ecosystems by reducing chemical fertilizer use (Bashan and Holguin 1995; De-Bashan et al. 2012). Despite limited understanding of PGPR-plant interactions a number of bacteria are commercially used in agriculture as biofertiliser (Lucy et al. 2004; Banerge et al. 2006). In a review by Glick (2012) a list of bacterial strains used as biofertiliser is presented and includes: *Agrobacterium radiobacter, A. brasilense, A. lipoferum, Azotobacter chroococcum, Bacillus fimus, B. licheniformis, B. megaterium, B. mucilaginous, B.pumilus, Bacillus* spp., *B. subtilis, B. subtilis* var. *amyloliquefaciens, Burkholderia cepacia, Delfitia acidovorans, Paenobacillus macerans, Pantoea agglomerans, Pseudomonas aureofaciens, P. chlororaphis, P. fluorescens, P. solanacearum, Pseudomonas* spp., *P. syringae, Serratia entomophilia, Streptomyces griseoviridis, Streptomyces* spp., *S. lydicus* and various *Rhizobia* spp. However, PGPR

inoculated crops represent only a small fraction of current worldwide agricultural practice (Glick 2012).

2.3.1 Mechanisms of growth promotion

Several mechanisms control the process of growth promotion either directly or indirectly. Direct growth promotion occurs when a rhizobacterium produces metabolites i.e. phytohormone that directly promotes plant growth by increasing the efficiency of nutrient and water uptake (phytohormones, ACC deaminase) or increasing the availability of nutrients (eg. N_2 fixation, P solubilisation, Fe chelation). On the other hand, decreased incidence of disease by antagonism, induced systemic resistance antibiotics, siderophores and hydrogen cyanide production can increase plant growth, and are thus considered indirect promoters of growth (Zahir et al. 2004).

Table 2.2 Some mechanisms used by plant growth-promoting bacteria to stimulate plant growth (Glick 2012)

Direct mechanisms	Nitrogen fixation
	Solubilisation of phosphorus
	Production of phytohormones (Auxin, Cytokinin, Gibberellin)
	Sequestering iron by siderophores
	Lowering ethylene concentration

Indirect mechanisms	Antibiotic production
	Depletion of iron from the rhizosphere
	Synthesis of antifungal metabolites
	Production of fungal cell wall lysing enzymes
	Competition for sites on roots
	Induced systematic resistance

2.3.1.1 Direct mechanisms that influence plant growth

2.3.1.1.1 Nitrogen fixation

Some bacteria fix nitrogen from the air and supply the plant's requirements; this is termed biological nitrogen fixation (BNF). BNF does not exceed the actual nitrogen requirements of the plant so it causes less pollution (Kennedy and Tchan 1992). BNF has been measured using different techniques including the acetylene-reduction assay, ^{15}N dilution, ^{15}N fixation, and Kjeldahl N-content measurements (Okon, 1985). The fixed nitrogen values for many grain and forage grass crops are generally in the order of 10 kg N ha per year (Spaepen et al. 2009). In most cases, the contribution of biologically fixed N is far too low to contribute significant amounts of N to field crops. For example, it is common practice to apply 250 kg N ha^{-1} per growth season to high yielding maize.

A number of bacteria are capable of fixing nitrogen from the environment symbiotically. Symbiosis is an association between two different organisms where both benefit. The

relationship between the plant and microbes are often long-term and involves a special structure (eg. nodules on legumes) which houses the microbes (Graham 1998). A number of prokaryotes (eg. *Rhizobium, Klebsiella, Nostoc* or *Frankia*), a eukaryote (*azolla*) and leguminous and non-leguminous plants such as water ferns are involved in symbiotic nitrogen fixation. This kind of symbiosis improves the sustainability of the agricultural and natural ecosystems.

Legumes such as pea, soybean and alfalfa have a symbiotic association with specific bacteria such as *Rhizobium, Bradyrhizobium* and *Azorhizobium.* These species have the exceptional ability to produce root or stem nodules on the host plants. N is then fixed in these structures by the production of ammonia through nitrogenase activity which is then assimilated onto amino acids, ureides or amides for export to the host plant; whereas the host plant provides the carbon compounds required by bacteria for survival (Kennedy and Cocking 1997). Some symbiotic associations are host specific: for example, *Rhizobium meliloti* nodulates *Medicago, Melilotus* and *Trigonella*; whereas *Rhizobium leguminosarum* nodulate*s Pisum, Vicia , Lens, and lathyrus* (Schlutz*e* et al. 1994).

Some of the diazotrophic plant growth promoting bacteria can fix low amounts of N and others such as *Azospirillum* can fix high levels of nitrogen (Bhasan and Levanony 1990); however, as mentioned earlier, these microorganisms only provide small amounts of fixed nitrogen

(James and Olivares 1997). Thus, non-symbiotic nitrogen fixation is a minor benefit provided to the plant by bacteria (Glick 1995; Zahir et al. 2004).

2.3.1.1.2 Solubilisation of phosphorus

Phosphorus (P) is one of the major plant growth-limiting nutrients, although it is wide spread in soils in both inorganic and organic forms (Gyaneshwar et al. 2002). Glick (2012) reported that the amount of soil phosphorus is normally between 400-1200 mg/kg of soil; however most of this is insoluble and therefore not available for plant growth. In addition, the soluble phosphorus from chemical fertilizer is immobilized soon after application to the field and is consequently wasted. Inability to access P often limits plant growth (Feng et al. 2004). Some PGP bacteria and fungi (mychorrizae) are capable of phosphate solubilisation and mineralization and are often called phosphate-solubilizing bacteria (Rodríguez and Fraga 1999; Richardson 2001). Up to 40% of the culturable bacterial populations in the rhizosphere are capable of solubilizing P in culture (Spaepen et al. 2009). Some of the genera reported to be capable of P solubiliziation are: *Pseudomonas, Bacillus, Rhizobium, Burkholdaria, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* (Goldstein 1986; Rodriguez and Fraga 1999).

2.3.1.1.3 Production of phytohormones (Auxin, Cytokinin, Gibberellin)

Phytohormones or plant growth promoting substances are chemical compounds present in small amounts that are able to promote and influence one or more specific physiological

processes within the plant (Lemaux 1999; Spaepen et al. 2009). Plant hormones are important in plant growth and development and in controlling the response of plants to their environment (Davies 2004). Rhizosphere microorganisms may also produce phytohormones under in vitro conditions (Salamone et al. 2005), thus many PGP bacteria can modify phytohormone levels, subsequently altering the hormonal balance of the plant and its response to environment (Glick et al. 2007). Although this process has been studied for many years, the mode of action of some molecules in plants remains unclear. It is generally accepted that five major plant hormones: auxins, cytokinins, GAs, abscisic acid (ABA) and ethylene (ET), are produced. Yet, phytohormone production by PGPR is mainly focused on indole-3-acetic acid (IAA).

The best characterized and most produced auxin family is IAA. The biosynthesis of IAA by plants has been extensively studied and a diverse group of bacteria capable of producing IAA have been found. Around 80% of the rhizosphere bacteria are capable of producing IAA (Khalid et al. 2004; Patten and Glick 1996). *A. brasilense* cells are root colonizing bacteria that proliferate in root exudates. When root exudates become limited for bacterial growth, *A. brasilense* increases IAA production, thereby triggering lateral root and root hair formation which in turn simulates more root exudate production (Ona et al. 2003, 2005; Vande Broek et al. 2005). Many root associated bacteria, particularly fluorescent pseudomonads, *Bacillus, Rhizobium, Azotobactor* and *Azospirillum* produce IAA in pure culture (Ahmad et al. 2008; Suzuki et al. 2003; Donate-Correa et al. 2005: Tien et al. 1979; Datta and Basu 2000; Joseph et al. 2007). Other bacterial synthesized phytohormones that participate in growth promotion are the gibberellins. Joo et al. (2004) reported that some PGP species of the genus *Bacillus* can

produce great amounts of gibberellins in culture broth and that this promoted the growth of red pepper seedlings.

Table 2.3 Plant hormones, produced by plant growth-promoting Rhizobacteria (Spaepen et al. 2009)

Class	**Example**	**Effect on plants**
Auxin	IAA	Root and shoot architecture
		Apical dominance
		Tropistic responses
Cytokinins	Zeatin	Inhibition of root elongation
		Leaf expansion by cell enlargement
		Delay of senescence
Gibberellins	GA3	Seed germination
		Stem and leaf growth
		Floral induction and fruit growth

Ethylene	ET	Stress and ripening hormone
		Flower and leaf senescence and abscission
		Adaptation to abiotic and biotic stresses
Abscisic acid	ABA	Stomatal closure
		Bud dormancy
		Adaptation to abiotic and biotic stresses

Abbreviations: IAA, indole-3-acetic acid; GA3, gibberellic acid.

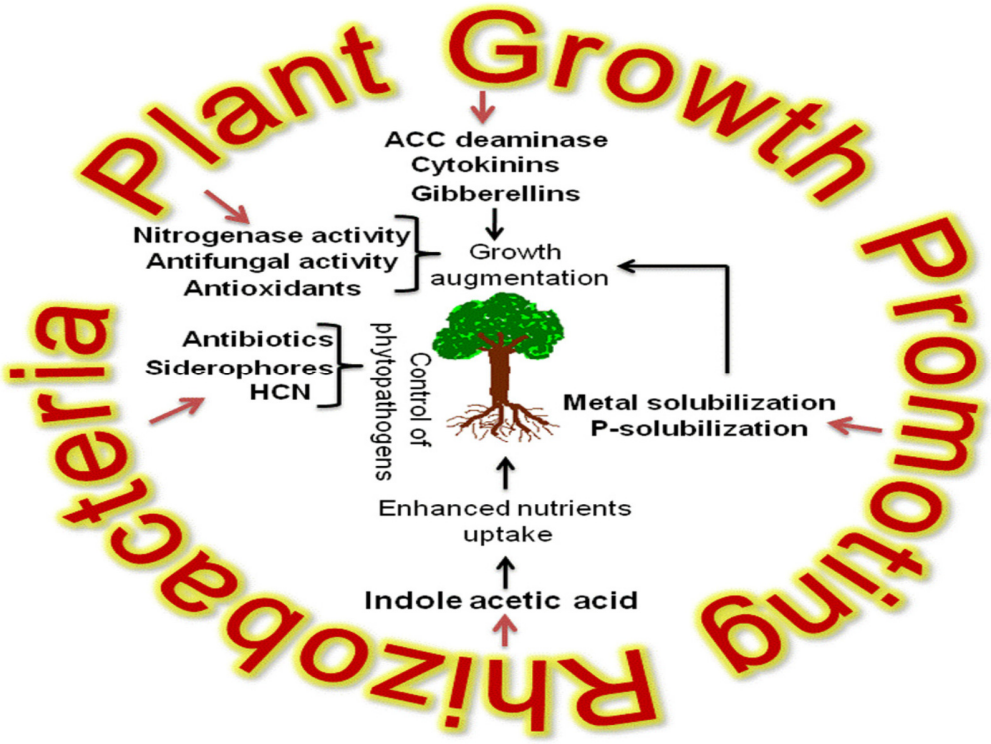


Figure 2.2 Mechanism of plant growth promotion by rhizobacteria (reproduced from Ahemad and Kibret 2014)

2.3.1.1.4 Sequestering iron by siderophores

Iron is very rich in soil but generally unavailable for assimilation by plants as it is present mainly as $Fe3^{+}$ oxides with a low solubility. Some PGP bacteria have developed a method for efficient uptake of iron by producing and secreting low-molecular-weight iron-chelating molecules, known as siderophores which bind to the iron molecules making them available plants and other rhizospere organisms (Raaijmakers et al. 1995; Ashrafuzzaman et al. 2009). Plant growth promotion by siderophore-producing bacteria is either the result of increased iron supply or the removal of iron from the environment so that phytopathogens become deficient thus reducing their competitiveness (Glick et al. 1999). Some siderophore producing fluorescent pseudomonads were reported to promote growth of potato (Kloepper et al. 1980; Zahir et al. 2003).

2.3.1.1.5 Reduction of ethylene concentration

Ethylene (ET) not only affects the process of fruit ripening but also plant growth and development. ET has a stimulatory effect on root initiation, but inhibits root elongation, promotes fruit ripening, reduces wilting, stimulates seed germination, promotes leaf abscission, activates the synthesis of other plant hormones and affects plant-pathogen interactions in response to biotic and abiotic stresses (Abeles et al. 1992). The inoculation of different plant species with PGP bacteria showed that ACC deaminase activity stimulates plant growth probably by reducing the stress hormone ET in the plant (Glick 2005; Glick et al. 2007; Saleem et al. 2007).

Transgenic expression of bacterial ACC deaminase genes in plants results in increased tolerance to stress, although to a lesser extent than bacterial inoculation, as bacteria possess other mechanisms for plant-growth promotion (Glick et al. 2007; Saleem et al. 2007). In addition, ACC deaminase-producing bacteria can also increase the rhizobial nodulation and mycorrhizal colonization in mung bean, chickpea and cucumber and subsequent plant growth promotion (Shahanora et al. 2006; Nascimento et al. 2012; Gamarelo et al. 2008).

2.3.1.2 Indirect mechanisms

PGPR also induce plant growth by protecting the host plant from soil borne plant pathogens; this is known as biocontrol (Bloemberg and Lugtenberg 2001; Mercado-Blanco and Bakker 2007; Schippers et al. 1987). Biocontrol by PGPR has mainly been described for Pseudomonas and Bacillus species, although a few reports indicate that diazotrophic bacteria are also involved. Biocontrol indirectly promotes plant growth and development by limiting the harmful effects of plant pathogenic organisms. This effect is achieved by: (i) production of siderophores that limit the availability of iron to the pathogen, (ii) production of antimicrobial compounds (antibiotics), (iii) induction of systematic resistance in plant, or (iv) competition for nutrients and binding sites on the root (Spaepen et al. 2009).

2.3.1.2.1 Antibiotic production

Some PGP bacteria (eg. Pseudomonads Spp.) are able to synthesize a range of antibiotics such as phenazines, 2,4-diacetylphloroglucinol pyrrole compounds (e.g., pyoluteorin and pyrrolnitrin), cyclic (lipo-) peptides and hydrogen cyanide (HCN). The production of phenazines has been investigated primarily in Pseudomonas, but has also been reported for Streptomyces, Brevibacterium, Burkholderia, and other bacterial species. The exact mode of action of phenazines is unknown; however, it is assumed they can act as reducing agents that decrease or prevent the growth of a wide range of microrganisms, including bacteria, fungi, and algae (Chin-A-Woeng et al. 2003; Dubuis et al. 2007; Mavrodi et al. 2006). Some PGP bacterial strains have been commercialised because of their biocontrol ability (primarily Pseudomonads and Bacilli). However, overuse of these strains may result in pathogen resistance (Whipps 2001; Raaijmakers et al. 2002; Haas and Keel 2003; Compant et al. 2005)

2.3.1.2.2 Depletion of iron from the rhizosphere

Some bacterial strains do not have a biocontrol mechanism but simply act as biocontrols by producing siderophores which bind most of the Fe^{3+} in the rhizosphere resulting in iron deficiency for plant pathogens (Kloepper et al. 1980). The host plant does not suffer from iron depletion as plants can grow at lower iron concentrations than microorganisms (Siddiqui 2005). This is an effective biocontrol technique as PGP bacteria are less affected than fungal pathogens which are unable to proliferate in the rhizosphere (Schippers et al. 1987; O'Sullivan and O'Gara 1992). Among the many PGPR pseudomonads studied, a siderophore producing strain produced a yellow-green fluorescent siderophore, called pseodobactin, and this was reported to suppress soil borne pathogens (Mullen 2005). By using this mechanism the

pseudomonad controls the growth of some fungal root pathogens by lowering the availability of Fe^{3+} to other microbes thus creating an iron-limited environment.

2.3.1.2.3 Competition for sites on roots

The rhizosphere is the nutrient rich zone of the plant where root exudates (organic acids, sugars and amino acids) are found and bacteria, including plant pathogens, are attracted. Bacteria colonise the roots using chemotaxis with flagellar motility. Therefore competition occurs between pathogens and PGPR for exudates and root colonisation sites (Miller et al. 2009).

2.3.1.2.4 Induced systemic resistance

Plants develop a defence phenomenon triggered by PGP bacteria; this is known as induced systemic resistance (ISR). ISR occurs when plants activate their defence mechanisms in response to infection by a pathogenic agent (van Loon et al. 1998; Pieterse et al. 2009). ISR-positive plants attack the pathogen faster following infection by inducing this defence mechanism. ISR is not pathogen specific but effective at controlling diseases caused by different pathogens. ISR is based on jasmonate and ethylene signaling within the plant in response to a range of pathogens. No direct interaction between the resistance-inducing PGP bacteria and the pathogen is required for ISR. Other bacterial molecules such as the *O*-antigenic side chain of the bacterial outer membrane protein lipopolysaccharide, flagellar proteins, pyoverdine, chitin, β-glucans, cyclic lipopeptide surfactants, and salicylic acid also

act as signals for the induction of systemic resistance (Verhagen et al. 2004; Bakker et al. 2007). Innerebner et al. (2011) demonstrated in *Arabidopsis thaliana* plants treated with the leaf bacterium *Sphingomonas* sp. that the bacterial pathogen *Pseudomonas syringae* was prevented from causing pathogenic symptoms and reducing plant growth. ISR has been confirmed in many plant species including bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis thaliana* (van Loon et al. 1998). PGPR induced ISR in plants was reported against fungal, bacterial and viral diseases (Liu et al. 1995; Maurhofer et al. 1998), insects (Zehnder et al. 1997) and nematode pests (Sikora 1988).

2.4 The use of PGP bacteria in agriculture

PGPR are defined by the following inherent distinct activities : (i) they must be able to colonize the root, (ii) they must survive, multiply and compete with other microbial communities in the rhizospere, iii) they need to express at least one of their plant growth promotion/protection activities, and (iv) they must promote plant growth (Kloepper 1994). PGPR often classified based on their functional activities such as: (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases by the production of antibiotics and antifungal metabolites) (Somers et al. 2004). Significant increases in the growth and yield of agronomically important crops such as potato, cotton, soybean and rice in response to inoculation with PGPR have been extensively reported (Kloepper et al. 1980; Chen et al. 1994; Zhang et al. 1996; Biswas et al. 2000; Vessey 2003). It has also been suggested that the growth-promoting ability of some bacteria may be

highly specific to certain plant species, cultivars and genotypes (Bashan 1998; Gupta et al. 2000; Lucy et al. 2004).

Much of the reported research has used growth chambers, green houses, pot and field experiments and most report significant improvements in plant growth following inoculation with PGP bacteria (Table 2.4).

Table 2.4 Examples of plant growth promoting rhizobacteria tested for various crop (Ahemad and Kibret 2014).

Bacteria	**Plants**	**Condition**	**Effect on plant**	**Reference**
Pseudomonas putida, *Azospirilium,* *Azotobacter*	Artichoke	In vitro	Significant increase in radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling vigority index, and significant decrease in mean time of germination	Jahanian et al. (2012)
Pseudomonas sp.	Greengram	Pots	Significantly increased plant dry weight, nodule nubers, total chlorophyll content, leghaemoglobin, root N, shoot N, root P, shoot P, seed yield and seed protein	Ahemad and Khan(2012) Ahemad and Khan (2011) Ahemad and Khan (2010)
Pseudomonas spp.	Soybean, Wheat	Field	Significantly increased soil enzyme activities, total productivity, and nutrient uptake	Sharma et al. 2011

Rhizobium phaseoli	*Vigna radiata* L.	Pots	Mitigated the adverse effects of salinity and increased the plant height, number of nodules per plant, plant biomass, grain yield, and grain nitrogen concentration significantly.	Zahir et al. 2010
Bacillus species PSB10	Chickpea	Pot	Significantly improved growth, nodulation, chlorophyll, leghaemoglobin, seed yield and grain protein; reduced the uptake of chromium in roots, shoots and grains	Wani and Khan 2010
Bradyrhizobium sp. *Pseudomonas* sp., *Ochrobactrum cytisi*	*Lupinus luteus*	Fields	Increased both biomass, nitrogen content, accumulation of metals (improved phytostabilisation potential)	Dary et al. 2010
Pseudomnas spp. *Azospirillum* spp. *Psychrobacter*	Maize	Fields	Plant height, seed weight, number of seed per ear and leaf area, shoot dry weight significantly increased	Gholami et al. (2009)

Bacillus spp.				
Azospirillum amazonense	Rice	Green house	Grain dry matter accumulation (7–11.6%), the number of panicles (3–18.6%) and nitrogen accumulation at grain maturation (3.5–18.5%) increased	Rodrigues et al. (2008)
Azotobacter chroococcum, Azospirillum lipoferum	Cotton	Fields	Seed yield (21%), plant height (5%) and microbial population in soil (41%) increased over their respective controls while boll weight and staple length remained statistically unaffected	Anjum et al. 2007

2.4.1 Use of *Azospirillum* as a PGP bacteria

The first species of *Azospirillum* was isolated from N-poor sandy soil in the Netherlands by Beijerinck (1925) and was originally named *Spirillum lipoferum*. Since the 1970's, *Azospirillum* strains have been isolated from cereals and most of the initial inoculation work was conducted on the main cereal crops and grasses. However, many non-cereal species have also been successfully inoculated with *Azospirillum* (Steenhoudt and Vanderleyden 2000). In a recent review (Saharan and Nehra 2011) it was reported that free-living rhizobacteria covers ten species, each one classified according to its particular biochemical and molecular characteristics. These are: *A. lipoferum* and *A. brasilense*; *A. amazonense* ; *A. halopraeferens* ; *A. irakense* ; *A. largimobile* ; *A. doebereinerae*; *A. oryzae* ; *A. melinis* and recently *A. canadensis* . *Azospirillum* spp. have been tested in the field and a positive effect on growth and yield of wheat in different soil and climatic conditions all over the world has been reported. *Azospirillum* is a gram –negative rod shaped motile bacteria with a single flagellum. *Azospirillum* are capable of fixing nitrogen from the air and binding it into amino acids and proteins (Katupitiya et al. 1995). They live in soil and in very close association with the roots of different grasses, cereals and tuber plants. They are also capable of producing high levels of IAA which plays an important role in root development (Steenhoudt and Vanderleyden 2000). Bashan and Holguin (1997) summarised twenty years of field experiments and concluded that 60-70% of all field experiments exhibited a PGP effect with significant yield increases ranging from 5 to 30% following inoculation with *Azospirillum.*

Table2.5 Azospirillum application on wheat and observed plant responses

Reference	**Experimental design**	**Responses on plant**
Baldani et al. (1987)	Field experiments –with various strains of *Azospirillum* on wheat in Terra Roxa soil in Parana, Brazil	Grain yield increases 31% but were not significant.
Okon and Labandera-Gonzalez (1994)	Review (1974-1994) of trials in USA, India, Thailand, Israel, Egypt, Italy, France, Brazil, Mexico, Uruguay, Argentina	Significant yield increases 5 – 30 % Positive responses in 60 – 70 % of trials
Dobelaere et al. (2001)	Review of trials in Belgium, Uruguay, Mexico, Israel	Positive responses dependent on conditions (fertiliser and soil type)
Dalla Santa et al. (2004)	Green house Experiments on wheat inoculated with *Azospirillum* spp.	Significant increases were obtained for yield when the inoculation was with 100% of N level but only

		inoculation produced non significant7.4% above the control
Diaz-Zorita and Fernandez-Canigia (2009)	Field experiments with wheat at 297 sites in Argentina – liquid inoculation with *A.brasilense* INTA Az39	Increased grain yield 8% and positive responses 70% of the trials
Veresolglou and Menexes (2010)	Meta-analysis 1981-2008 of 59 articles on wheat inoculation with *Azospirillum*	Average of 8.9% increase in seed yield
Hungria et al. (2010)	Field experiments in Brazil with different strain of *A. brasilens*e and *A.lipoferum* on wheat	Increased wheat grain yields by 31%, general increases in uptake of several macro and micronutrients and not specifically to biological nitrogen fixation

2.5 Conclusion

Microbial inoculants for plant growth promotion in agriculture have developed rapidly over the past 40 years based on the need to reduce both fertilizer and pesticide use (Dobbelaere and Okon 2007). There is a good possibility that PGPR can be used extensively in agriculture; however more research is needed to understand the mechanisms and interactions of plant growth promotion by PGPR in field. Optimal application of PGPR could contribute to increased plant growth by improving nutrient availability, biocontol or bioremediation of soil contaminated with pollutants such as heavy metals and pesticides. Above all the use of PGPR can improve the long-term sustainability and productivity of the farming system. The beneficial effect of PGPR has been clearly established in the glasshouse but is much harder to assess in the field. This is likely due to the following constraints:

i) survival of the bacterial inoculum in the rhizosphere,

ii) selection of PGPB strains with appropriate biological activities

iii) the suppressive effect of indigenous microorganisms

iv) quality of PGPR inoculant,

v) lack of understanding of rhizospheric or endophytic bacteria

vi) selection of PGPB strains that function under specific soil conditions (e.g., those that work well in warm and sandy soils versus organisms better adapted to cool, wet and finer textured soil environments);

vii) application of PGPB to plants in various settings (e.g., in the field or in the greenhouse);

viii) better understanding of the interactions between PGPB and indigenous rhizospheric microorganism.

ix) lack of knowledge of crop genotype x bacterial strain interactions.

x) lack of knowledge of crop genotype x bacterial strain interactions.

Further research is required to better understand the above mentioned factors. Particular emphasis is required on the complex interactions between crop genotype x bacterial strain and the environment under field conditions.

Chapter 3: Response of wheat genotypes to inoculation with *Azospirillum* spp. under controlled conditions

3.1. Introduction

Azospirillum spp. are known for their plant growth promoting properties (Okon 1994; Okon and Vanderleyden 1997) and the growth and yield of many crop plant species can be increased under certain conditions (Boddey et al. 1986; Okon and Labandera-Gonzalez 1994). *Azospirillum* is the best characterized genus of rhizobacteria (Steenhoudt and Vanderleyden 2000) and grows extensively in the rhizosphere of gramineous plants (Kennedy and Tchan 1992). *Azospirillum* can be isolated from cereal and grass rhizospheres in most temperate and tropical regions globally (Dobereiner and Day 1976; Patriquin et al. 1983).). In a review of plant growth promoting rhizobacteria, Shaharan and Nehra (2011) identified ten species of Azospirilla and listed them according to their particular biochemical and molecular characteristics, these are: *Azospirillum lipoferum* and *A. brasilense,* (Tarrand et al. 1978); *A.amazonense* (Magalha-es et al. 1983); *A. halopraeferens* (Reinhold et al. 1987), *A. irakense* (Khammas et al. 1989) *A. largimobile* (Dekhil et al. 1997), *A. doebereinerae* (Eckert et al. 2001), *A. oryzae* (Xie and Yokota 2005) *A. melinis* (Peng et al .2006) and recently *A. canadensis* (Mehnaz et al. 2007). *Azospirillum* species are nitrogen fixing aerobic heterotrophs that are able to fix N_2 in microaerobic conditions (Roper and Ladha 1995) and produce IAA (indole acetic acid) which enhances plant growth and yield (Okon and Labandera-Gonzalez 1994) under both non-stressed and stressed conditions (Creus et al. 1997).

There is evidence of the beneficial effects of inoculation with *Azospirillum* on wheat in both green house and field experiments (Hegazi et al. 1998; El-Mohandes 1999; Ganguly et al. 1999). Okon and Labandera-Gonzalez (1994) reported a yield increase in wheat of 5-30% in 70% of field experiments following inoculation with *A. brasilense*. However, the effectiveness of inoculation is influenced by many factors including inoculation rate, inoculation procedure, the environment and N fertilizer application. Dobbelaere et al. (2001) reported that *A.brasilense* was more effective when N –fertiliser application rates were low (50-60 kg/ha) compared to higher rates (110-170 kg/ha). These findings indicate scope for *Azospirillum* inoculation to supplement a substantial amount of urea-N applied to wheat while maintaining grain yield (Kennedy et al. 2004).

Plant growth promoting rhizobacteria (PGPR) can improve root development and subsequent water and mineral uptake through the production of phytohormones. Dobbelaere et al. (2001) examined the physiological responses of plant roots to inoculation with *Azospirillum* in field and greenhouse experiments between 1994–2001 in Belgium, Uruguay, Mexico and Israel. Positive effects of *Azospirillum* were mainly due to morphological and physiological changes in roots which led to enhanced water and mineral uptake (Okon and Kapulnik 1986). Secretion of plant growth promoting substances such as auxins, gibberellins and cytokinins by bacteria were partially responsible for effects on roots (Dobbelaere et al. 1999). The genus *Azospirillum* produces plant growth promoting substances in culture, mainly auxins (indole-3-acetic acid, IAA) (Lambrecht et al. 2000), but also (in smaller amounts) cytokinins and gibberellins (GA3) (Bottini et al. 1989). It was demonstrated that auxin production by *Azospirillum* plays a major role in plant root development (Dobbelaere et al. 1999). When wheat seeds (*Triticum aestivum*

L.) were inoculated with *A.brasilense* Cd, Sp7, Cd-1, with 10^5 to 10^6 colony-forming units, root elongation and total root surface were maximized (Kapulnik et al. 1985); however, when 10^8 to 10^9 colony-forming units of *Azospirillum* were inoculated root development was inhibited. Similar effects were noted in 10 different wheat cultivars inoculated with *Azospirillum*; denser and longer root hairs were observed compared to the control which was inoculated with dead cells. The PGPR effects can increase N and P uptake in wheat in field trials (Galal et al. 2000; Panwar and Singh 2000), apparently by stimulating greater plant root growth. There are clear differences between strains of *Azospirillum* in their ability to promote wheat growth in greenhouse trials (Han and New 1998; Saubidet and Barneix 1998) and genotype x environment interaction (GEI) has been observed. Although *Azospirillum* promotes wheat growth and ultimately grain yield, it contributes little N as a direct result of BNF (Malik et al. 2002). Several investigators reported that the roots of wheat seedlings respond positively to *Azospirillum* inoculation and significant increases in root length, dry weight and lateral root hair development were found (Jain and Patriquin 1984; Glick 1995; Akbari et al. 2007). A number of authors reported that *A.brasilense* produces significant amounts of IAA and nitrate and these are possible factors causing enhancement of root growth in grasses (Zimmer et al. 1988; Tchan and Kennedy 1989; Akbari et al. 2007).

Bacterial colonization of roots is vital for the establishment of a successful association and depends on inoculum size, time in contact with roots and rhizosphere competency of the bacteria. Yegorenkova et al. (2001) observed that the colonization of wheat seedlings with *A.brasilense* 75, 80, and Sp 245 increased with higher inoculum potential and time. They also observed that azospirilla could be host specific and more readily attached to some wheat

genotypes than others. A possible influence could be the wheat germ agglutinin present on the roots, and early interaction of the extracellular polysaccharide of the *Azospirillum* with cereal root lectin (Skvortsov and Ignatov 1998; Fischer et al. 2003). The mechanism of *Azospillum* colonisation in the rhizosphere is not yet fully understood, but it is assumed that a number of bacterial characteristics like chemotaxis, which causes movement towards root exudates, are involved in initiating the plant bacterial interaction (Bashan and Holguin 1994). Nitrogen fixation, antagonism towards competing microorganisms and cyst formation allows survival in adverse conditions and may promote binding to plant roots (review by Okon 1985; Dobereiner and Perdosa 1987; Michiels et al. 1989). Michiels et al. (1991) described the attachment of *A.brasilense* Sp7 to wheat roots and concluded that the presence or absence of extracellular polysaccharides was responsible. Different patterns of *A.brasilense* Sp7 and Sp7-S colonization of wheat roots were revealed by Katupitiya et al. (1995) using a reporter gene (*nif*A-*lacZ*) and increased nitrogenase activity was observed with Sp7-S when the roots were modified with 2,4-D application. Using *nif*H-*lacZ* fusions, Deaker and Kennedy (2001) reported that *A. brasilense* Sp7-S, a mutant capable of more endophytic colonisation of wheat roots than the wild type *A. brasilense* Sp7, was able to fix more N_2 than the wild type Sp7. They also reported that the Sp7-S *nif*H gene was more strongly expressed in the wheat rhizosphere and that improved access to C compounds and a more favourable microaerobic O_2 concentration likely contributed to this effect. These results demonstrate the potential of BNF by *Azospirillum* to enhance the availability of N to wheat plants (review Kennedy et al. 2004). Wood et al. (2001) reported an increase in 15N transferred to the plant after inoculation with an ammonium excreting mutant of *A.brasilense* FP2 when a malate C source is added to the

system. These observations suggest that wheat genotypes with different root architecture could show differential responses to inoculation.

This chapter describes variation in bacterial colonization observed from associations between strains of *Azospirillum* and wheat genotypes that vary for root architecture/characteristics to see if colonization could be enhanced using *lacZ* reporter genes in the laboratory. A sub-set of diverse wheat genotypes were subsequently selected for field studies based on improved colonisation and better plant growth promotion under controlled conditions (see Chapter 4).

3.2. Materials and methods

Experiments were conducted to investigate the effect of 2 strains of the PGP bacteria *A.brasilense*, Sp7 and Sp7-S on 23 different genotypes of wheat to evaluate the genotype responses and genotype x bacterial strain interaction.

3.2.1 Wheat genotypes

Twenty three wheat genotypes were selected from a wide range of genetic diversity for response to inoculation with *A.brasilense* Sp7 and Sp7-S under controlled conditions (listed in Table 3.1). On the basis of the genotype x bacterial strain response and DNA fingerprints a subset was chosen for field testing (see Chapter 4). DNA fingerprints (based on Diversity Arrays Technology or DArT profiles) were used to ensure maximum genetic diversity among the selected genotypes. Figure 3.1 presents a dendrogram of the various genetic diversity groupings and care was taken to select materials that represented the breadth of genetic

diversity available in this gene pool. These genotypes differed for various morphological characteristics including plant height (although all genotypes were semi-dwarf and carried either the Rht1 or Rht2 dwarfing gene), flowering time (this varied by 7 days), root structure and seed size. The genotypes assessed represent a range of materials including Australian released cultivars (suitable for both northern and southern Australia), high yielding advanced lines from the International Maize and Wheat Improvement Centre (CIMMYT) and synthetic derived lines also from CIMMYT.

Table 3.1 Wheat genotypes evaluated for response to inoculum

Name/pedigree	**Released year and agency**	**Important character**
AUS 1. SUNCO	1986, University of Sydney	Bench mark for prime hard quality in NSW and Queensland. Mapping population parent
AUS 2. EGA GREGORY	2004, Enterprise Grains Australia (EGA)	High yield potential and the major variety grown by farmers at the time of this study
AUS 3 .VENTURA	2004, University of Sydney via Australian Grains Technology	High yield and disease resistance (at the time of release)
AUS 4.LANG	2000, Department of Primary Industries and Fisheries , QLD	High yield with prime hard quality

AUS 5. CROC_1/AE.SQUARROSA (224)//OPATA/3/PASTOR	CIMMYT advanced line	Synthetic derivative
AUS 6. QT6581/4/PASTOR//SITE/MO/3/CHEN/AEGILOPS SQUARROSA (TAUS)//BCN	CIMMYT advanced line	Synthetic derivative
AUS 7. mtc32	Advanced line from the Value Added Wheat CRC	High yield
AUS 8. QT9684/BORL95//ATTILA	CIMMYT advanced line	High yield
AUS 9. VEE/PJN//2*TUI/3/2*MILAN/KAUZ	CIMMYT advanced line	High yield
AUS 10. BJY/COC//PRL/BOW/3/SARA/THB//VEE/4/PIFED	CIMMYT advanced line	High yield

AUS 11. CHEN/AE.SQ//2*WEAVER/3/BABAX/4/JARU	CIMMYT advanced line	Synthetic derivative
AUS 12. CROC_1/AE.SQUARROSA (205)//KAUZ/3/BJY/COC//PRL/BOW/4/BJY/COC//PRL/BOW	CIMMYT advanced line	Synthetic derivative
AUS 13. LEICHHARDT//PASTOR*2/OPATA	CIMMYT advanced line	High yield
AUS 14. AUSGS50AT34/SUNCO//PASTOR	CIMMYT advanced line	High yield and crown rot & nematode resistance
AUS 15. BONASA//BAV92/RAYON	CIMMYT advanced line	High yield
AUS 16. KAUZ/PASTOR//PBW343	CIMMYT advanced line	High yield

AUS 17. CBRD/KAUZ//KASO2	CIMMYT advanced line	High yield
AUS 18. CROC_1/AE.SQUARROSA (205)//KAUZ/3/2*METSO	CIMMYT advanced line	Synthetic derivative
AUS 19. KRICHAUFF/2*PASTOR	CIMMYT advanced line	High yield
AUS 20. SOKOLL	CIMMYT advanced line	Synthetic derivative and mapping population parent
AUS 21. BERKUT	CIMMYT advanced line	High yield and mapping population parent
AUS 22. KRICHAUFF	1996, University of Adelaide	High yielding line released for drier areas in South Australia. Mapping population parent

AUS 23. TASMAN	1994, Department of Primary Industries and Fisheries , QLD	Mapping population parent

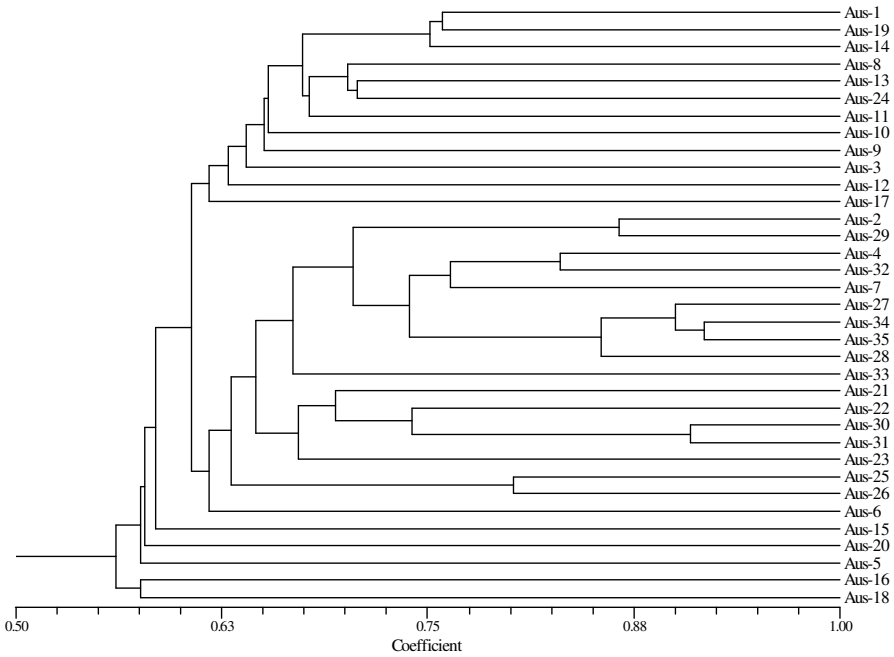


Figure 3.1 Dendrogram of relative genetic distances (based on DArT fingerprints) among hexaploid wheat genotypes used in inoculation studies. The pedigrees of genotypes Aus 1 – Aus 23 are listed in Table 3.1.

3.2.2 Bacterial strains

Azospirilla are Gram-negative free-living nitrogen-fixing rhizosphere bacteria. Bacterial strains for inoculation were collected from the bacterial culture collection of SUNFix, University of Sydney. A wild type of *A.brasilense* Sp7 and its spontaneous mutant *A. brasilense* Sp7-S were chosen as they were previously reported to improve plant growth (Zimmer et al. 1988) and they also represent different patterns of colonisation of wheat roots (Katupitiya et al. 1995). Both strains share similar morphological characteristics; they are motile with a curved-rod shape, however, Sp7-S lacks the *flcA* gene involved in exopolysaccharide production and flocculation which affects the way it colonises roots.

Table 3.2 List of the bacteria and plasmids used in inoculation experiments

Name	Origin	Gram +ve/-ve	Flocculation	IAA production	N fixation	Reference
***A.brasilense* Sp7**	Wild type strain, isolated from *Digitaria decumbens* rhizosphere soil,Brazil	-	+	+	+	Tarrand et al. 1978
***A.brasilense* Sp7-S**	Spontaneous mutant of Sp-7	-	-	+	+	Katupitiya et al. 1995
Echerichia coli S17-1		-	-	-	-	Simon et al. 1983

3.2.3 Construction of *A.brasilense* transconjugants

The donor strain, *E.coli* 17.1, was grown in LB (Luria Bertani) broth supplemented with tetracycline (Tc) 5µg/mL and *Azospirillum* strains were grown in Nfb broth. Plasmids carrying a pLA-*lacZ* (Arsene et al. 1994) fusion were transferred from the *E.coli* S17.1 donor into *Azospirillum* recipients using conjugation (Katupitya et al. 1995) on nutrient agar plates. Transconjugants were selected on minimal lactate medium containing Tc 5µg/mL. The transconjugants were then cultured on Nfb agar media with Congo red and Tc (5µg/mL). New transconjugants were tested for β-galactosidase enzyme activity in axenic culture according to

Miller (1972) using the substrate o-nitrophenyl-β-D-galactoside (ONPG) to ensure that conjugation was successful.

3.2.4 Experiments in controlled environments

Three experiments were conducted in controlled conditions to assess the root architecture: time course studies at different plant ages with and without bacterial inoculation and a colonisation study. Plants of all 23 wheat genotypes were grown with and without inoculation in test tubes using hydroponic media in a temperature controlled growth cabinet at the Faculty of Agriculture and Environment, University of Sydney. All 23 wheat genotypes were arranged in a completely randomized design with 4 replicates.

3.2.5 Seed sterilization

Wheat seeds were surface sterilized under vacuum using mercuric chloride ($HgCl_2$) as described in Zeman et al. (1992). Seeds were wrapped in muslin cloth and placed in an Erlenmeyer flask and washed with two drops of Tween 20 for one minute followed by several rinses with RO (Reverse Osmosis) water to remove detergent. The seeds were then surface sterilized with 0.05% $HgCl_2$ for 75 seconds and immediately washed seven times with sterile distilled water (approx. 2L).

3.2.6 Seed Transfer

Sterilised seeds were transferred aseptically on to yeast mannitol agar (YMA) plates for two days and placed in the dark until they germinated.

3.2.7 Growth in the test tube hydroponic system

Germinated seeds from plates without contamination were then transferred from the agar plate to the sterile hydroponic system. The system consisted of 15 mm test tubes containing 15 ml of N-free hydroponic solution (Zeman et al. 1992). Wheat seedlings were grown on a filter paper support and the roots submerged in the solution. A larger test tube (20mm) was placed over the smaller test tube (15mm) and supported by a test tube rack; the larger test tube maintained the sterility of the system. The chemical composition of the hydroponic solution is described in the appendix.

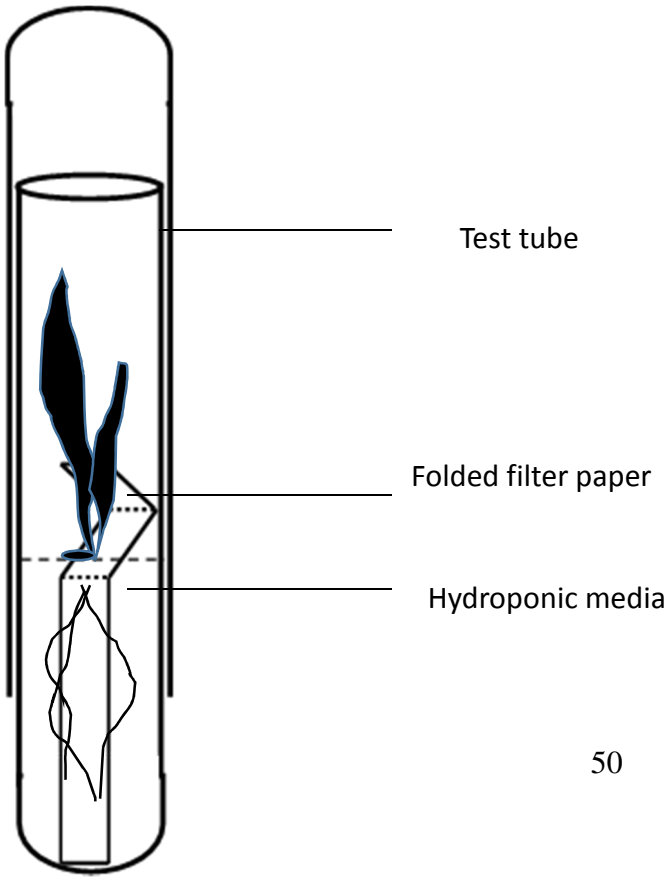


Figure 3.2 Description of the hydroponic system in the plant growth chamber

3.2.8 Temperature and light intensity in the growth chamber

Photoperiod

Seedlings were grown in test tubes with N –free hydroponic media in a controlled environment growth chamber at 20°C in long day (LD) conditions: 16 h light / 8 h dark cycle.

Light

The growth cabinet was fitted with fluorescent low-pressure 4000 K colour temperature mercury discharge lamps (Philips TLD 36W/840 cool white). Plants were grown using standard light condition at 120-150 $mol.m^2.s^{-1}$.

3.2.9 Inoculum preparation

Tranconjugants of *A.brasilense* Sp7 and Sp7-S were grown in Nfb liquid media with antibiotic (Tetracycline 5µg/mL) in an incubator with continuous shaking at 180 rpm at 30°C for 48 hours. The added antibiotic in the growth medium slowed bacterial growth and as a result cultures were grown for 48hours to maximise cell number.

3.2.10 Seedling Inoculation

Plants were inoculated in the test tubes after 6-7 days with 0.2 mL of *Azospillum* culture of approximately 0.9 OD (Optical Density) at Absorbance 600. Cell numbers in the inocula were counted using the viable plate counting technique and cultured on Nfb agar plates with

tetracycline to confirm the presence of transconjugants. Bacterial numbers were 9 x 10^9 cfu/ml and 8 x 10^9 cfu/ml for Sp-7 and Sp7-S, respectively.

3.2.11 Harvesting the plants

Plants were harvested 21 days after transfer to the hydroponic system to assess plant growth and bacterial colonisation on the root surface. Plant height was measured with a ruler and relative chlorophyll was measured immediately with a chlorophyll meter (Konika –Minolta). Plant material was then dried in an oven at 75°C for five days to determine dry weight. Roots were separated from shoots and four root systems of each genotype maintained in 50% ethanol for subsequent root architecture analysis followed by dry weight measurement, and another set of four roots were sampled for colonisation studies and bacterial enumeration.

3.2.12 Shoot height

Plant height from the base of the shoot to the tip of the leaf was measured with a meter ruler immediately after harvest. Four replicates per treatment were assessed and 276 plants (23 genotypes x 3 bacterial treatments x 4 replicates) were evaluated.

3.2.13 Relative chlorophyll measurements

Relative chlorophyll was measured immediately after harvesting tissue using a chlorophyll meter SPAD -502 plus (Konika –Minolta). The SPAD meter is hand held and can be used to assess chlorophyll content non-destructively. The meter determines the relative amount of

chlorophyll present by measuring the absorbance of the leaf in two wavelength (red and near–infrared) regions. A numerical SPAD value was then calculated proportional to the amount of the chlorophyll present in the leaves. The amount of chlorophyll is closely related to the nutritional condition (amount of the Nitrogen) of the plants; a higher SPAD value indicates a healthier plant.

3.2.14 Dry weight assessment of plant shoots and roots

The shoots and roots of four plants per treatment were dried separately at 75°C in an oven for four days. After drying, the shoot and root weights of every sample was determined and expressed in grams.

3.2.15 Assessment of root architecture

Plants were harvested 21 days after placement in the hydroponic system and roots and shoots were separated. Roots were then scanned and the images analysed using WinRhizo software (Regent Instruments Inc., Canada).

3.2.16 Quantification of bacterial numbers associated with plant roots

Roots were harvested 20 DAT and subsequently washed with Z buffer pH 7.0 to remove bacteria that were only loosely adhering to the roots. The roots were then transferred into a 25 ml falcon tube with 10 sterile glass beads (10mm diameter) and 20 mL of Z buffer, vortexed for 30 seconds and allowed to rest for 2-3 minutes. The solution was then serially diluted and

the number of the bacterial cells determined by viable plate count on Nfb with Congo red and antibiotics (Tc 5µg/mL). As the transconjugants were resistant to tetracycline they could be detected and counted. Root systems from three replicate plants were assessed to determine an average colonisation value.

3.2.17 Fixation of root segments in gluteraldehyde and staining for colonisation study.

The colonisation pattern of each bacterial treatment was observed by *in situ* staining of the roots with 5 bromo-4-chloro-3-indolyl-b-galactopyranoside (X-gal) as described by Arsene et al. (1994). Roots were cut into a few 2 - 3 cm segments in the elongation zone and placed in a McCartney bottle. Bacteria on root segments were then stained using X-gal as a substrate for β-galactosidase (Boivin, 1990). Root segments were covered with 2% gluteraldehyde solution in Z buffer (pH 7.4), placed under vacuum for 30 minutes and subsequently stored at room temperature and pressure for 60 minutes. The gluteraldehyde solution was then discarded and the roots were washed twice with Z buffer. The washed roots were submerged with X-gal solution and left overnight at room temperature under a cover of aluminium foil.

3.2.18 Light microscope examination

The next day roots were washed three times in Z buffer and twice in sterile distilled water for 5 min each. They were finally washed in 12% bleach for less than one minute. The root segments were examined and photographed under a light microscope (Olympus DP70) and images captured and analysed using Leica software (Leica Application Suite Version 4.0.0 [Build: 877], Leica DM 2500 M) to determine colonisation patterns.

3.2.19 Statistical Analysis

Data were analysed using the REML algorithm function of Genstat version 14. Mixed model analyses of the genotype x inoculant data were performed and the resultant means used in subsequent regression analyses and to construct comparative figures.

3.3. Results

3.3.1. Effects on root characteristics

3.3.1.1. Time course study of root architecture of 23 wheat genotypes

An experiment was conducted to examine differences in root architecture of 23 wheat genotypes at different stages of development in the hydroponic system and to determine the best growth stage to analyse plant growth promotion effects from inoculation in later experiments. Plants were grown in growth chambers without bacterial inoculum and harvested 10, 20 and 30 days after transfer (DAT) to the hydroponic system. Highly significant differences were found among genotypes for root length, surface area, average diameter and volume at 30 DAT (Table 3.3). However, only root diameter was significantly different at all stages of development. Variety, plant age and sampling time were significant factors affecting each root trait (Table 3.4). However, only root volume and average root diameter showed a significant genotype x sampling time interaction. While total root length and root surface area increased with time, root volume and average diameter decreased from 20 to 30 DAT in several genotypes potentially indicating nutrient limitation (Fig 3.3 A - D). Thus, effects of inoculation on plant growth in the hydroponic system were assessed at 20 DAT to reduce this variation.

Table 3.3 Wald statistics for different root traits of 23 genotypes grown without inoculation and sampled 10, 20 and 30 days after transfer to hydroponic media (DAT)

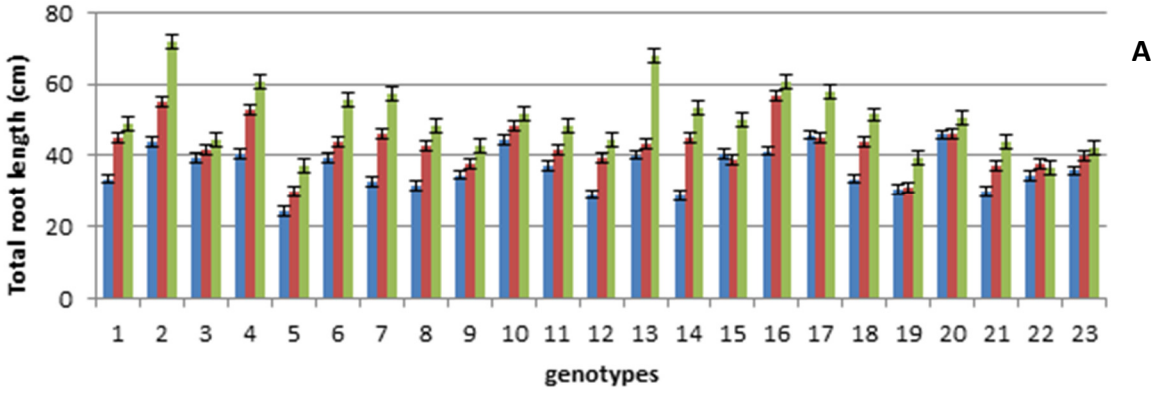
Root analysis	**10DAT**	**20DAT**	**30DAT**
Total root length	35.32	34.72	50.92**
Root surface area	31.33	29.69	75.14***
Root volume	32.11	30.69	81.41***
Root diameter	65.47***	62.45**	45.64*

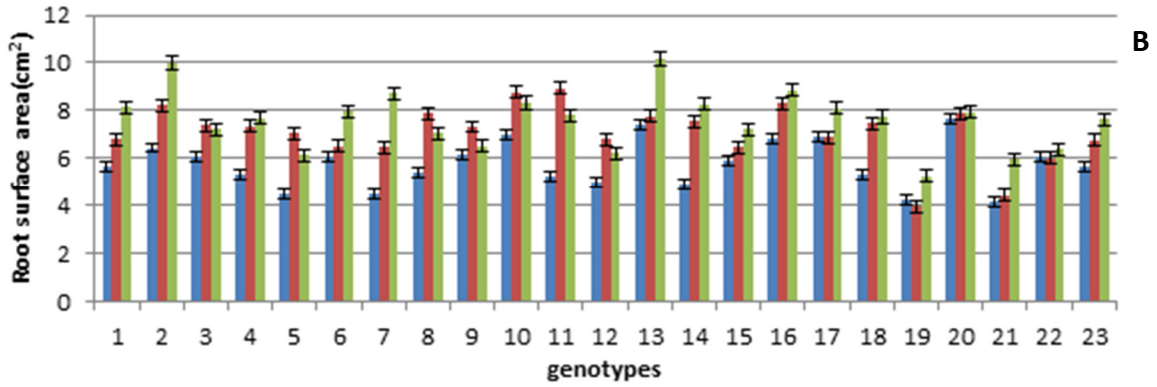
*, **, *** represent significance at P<0.05, P<0.01 and P<0.001, respectively

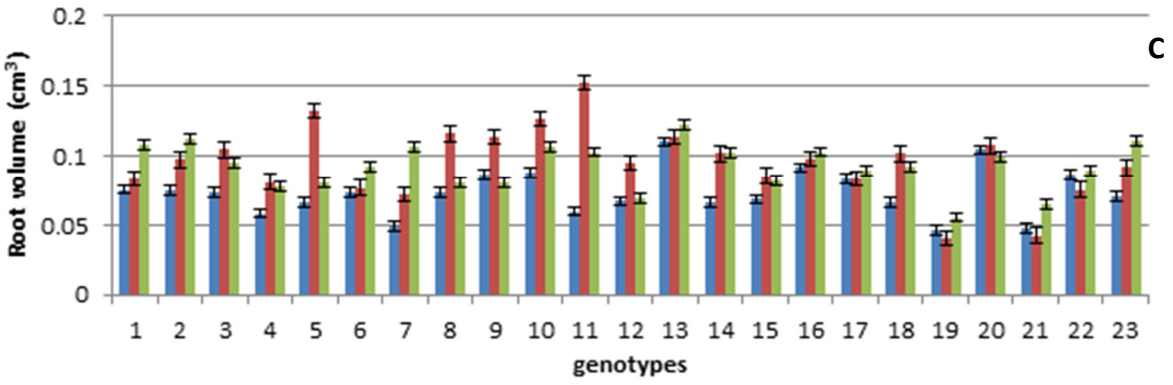
Table 3.4 Wald statistics for wheat genotype, time of sampling (DAT) and interaction effects for four root characteristics without inoculation

Fixed term	**Total Root length**	**Root surface area**	**Root volume**	**Avg diameter**
Genotype (G)	110.65***	117.06***	114.2***	166.6***
DAT	91.92***	76.72***	41.63***	22.86***
G x DAT	32.66	42.03	69.44*	90.04***

*, **, *** represent significance at P<0.05, P<0.01 and P<0.001, respectively


A
Total root length (cm)
0
20
40
60
80
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
genotypes


B
Root surface area(cm²)
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
genotypes


C
Root volume (cm³)
0
0.05
0.1
0.15
0.2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
genotypes

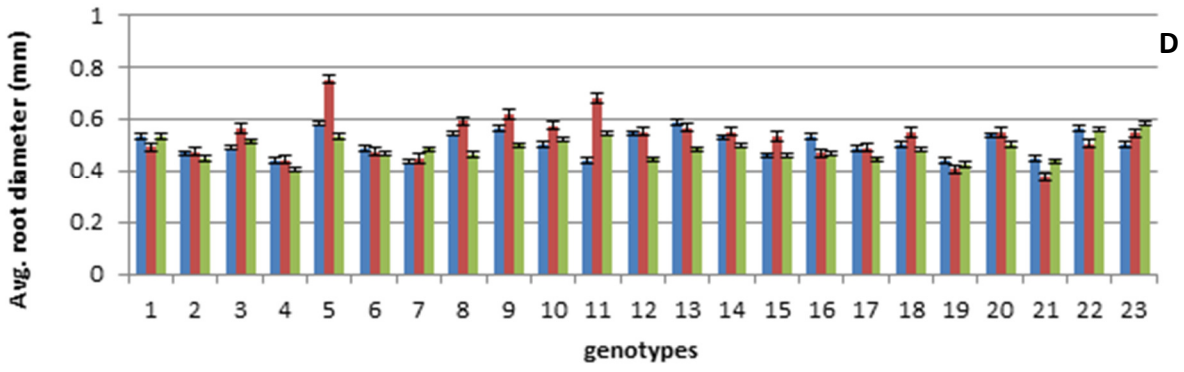

D
Avg. root diameter (mm)
0
0.2
0.4
0.6
0.8
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
genotypes

Figure 3.3. Variation in total root length (A), root surface area (B), root volume (C) and average root diameter (D) of 23 wheat genotypes at 10 (blue), 20 (red) and 30 (green) DAT to hydroponic medium.

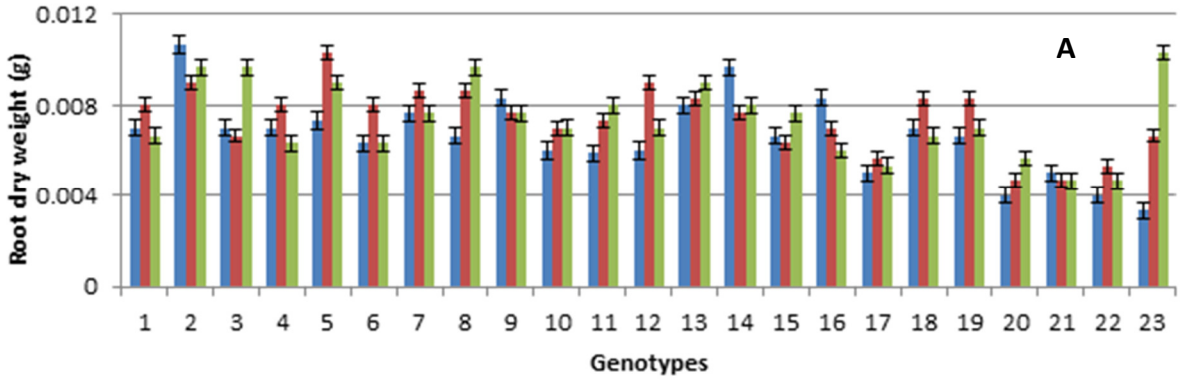
3.3.1.2. The effect of inoculation on root growth

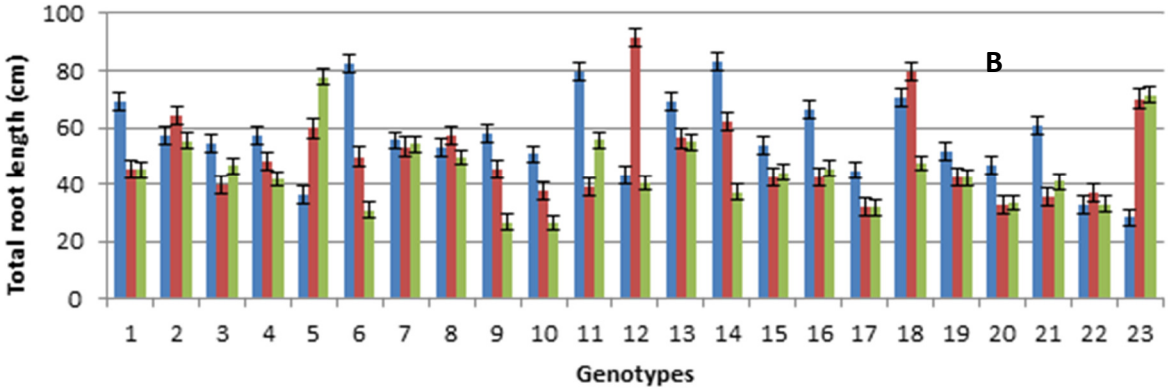
The difference in root fresh weight and dry weight was highly significant for wheat genotypes and there was a significant effect of inoculation on root dry weight. Root dry weight showed significant genotype x inoculum interaction. Roots of wheat genotypes treated with bacterial inocula were scanned and four primary root traits; total root length, surface area, volume and average diameter (Table 3.5). Genotype and genotype x inoculum effects were significant for all traits. Inoculation also had a significant effect on all root traits with the exception of volume. In some cases root traits were significantly increased in inoculated genotypes compared to the uninoculated control (Figures 3.4 A - F). For example, bacterial treatments produced significantly greater root volume in genotypes 8 and 23 than the uninoculated control; however the opposite effect was observed in genotypes 3 and 14 indicating a high level of genotype specificity to inoculation (Figure 3.4E). Inoculation had a variable effect on root traits; in some genotypes increased dry weight resulted from increased length (eg. genotype 5) and in others it was from root volume and average diameter (eg. genotype 8). Interestingly, genotype 23 maintained this responsiveness for all root traits except average root diameter. While there was no apparent trend with genetic source material, the synthetic lines 5, 6, 11, 12 and 18 generally showed greater variability in root length and potential for the longest roots.

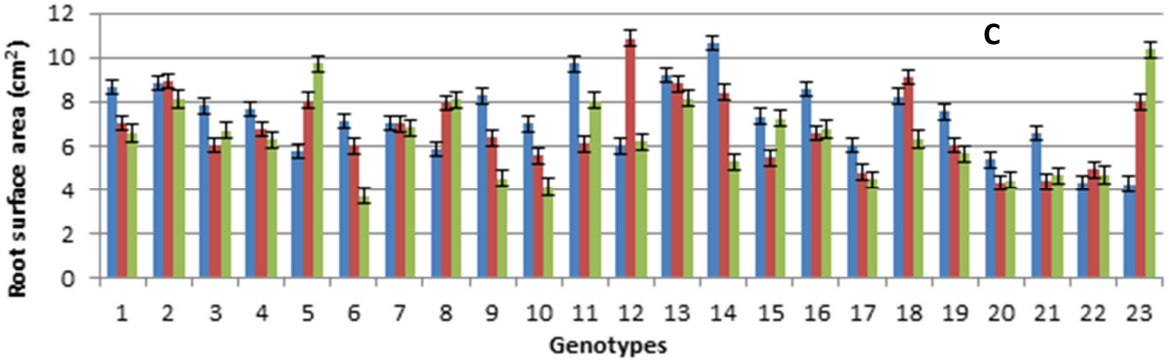
Table 3.5 Wald statistics for wheat genotype, inoculant and their interaction effects for root characteristics

Fixed term	**n.d.f**	**Root dry weight gm**	**Total root length cm**	**Root surface area cm^2**	**Root volume cm^3**	**Average Root Diameter mm**
Genotypes (G)	22	146.81***	85.31***	130.42***	162.32***	72.5***
Inoculants (I)	2	10.87**	26.99***	12.82**	0.88	20.16***
G x I	44	74.28*	143.76***	128.22***	99.65***	75.33**

*, **, *** represent significance at P<0.05, P<0.01 and P<0.001, respectively


A
Root dry weight (g)
0.012
0.008
0.004
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Genotypes


B
Total root length (cm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Genotypes


C
Root surface area (cm²)
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Genotypes

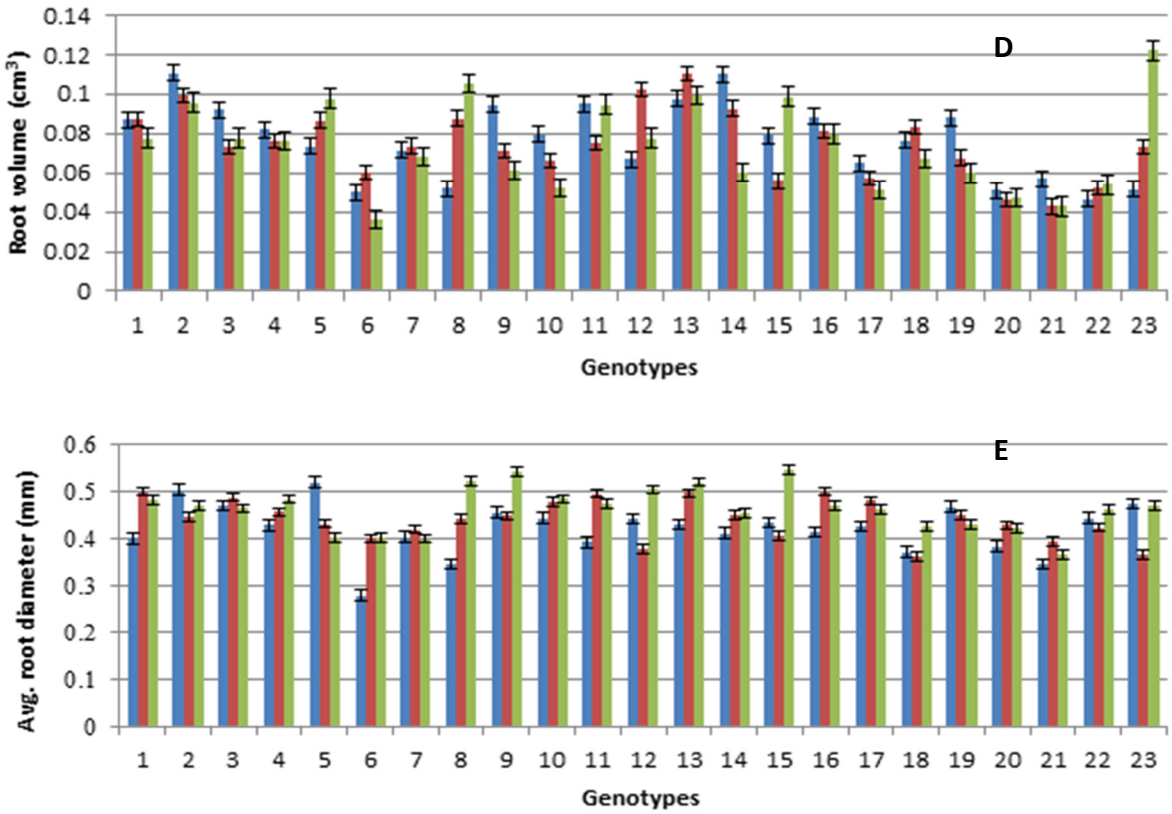


Figure 3.4 Effect of inoculation of 23 wheat genotypes with *A. brasilense* strains Sp7 and Sp7-S on root dry weight (A), total root length (B), root surface area (C), root volume (D) and root diameter (E). Treatments: Uninoculated control (blue), Sp7 (red) and Sp7-S (green).

3.3.2. Effect of inoculation on above ground growth

3.3.2.1. The effect of inoculum on plant height, shoot dry weight and relative chlorophyll

Plant height, shoot dry weight and relative chlorophyll content varied significantly with genotype (Table 3.6). However, the effect of inoculation was only significant for relative chlorophyll content. Similarly, genotype x inoculum interaction was significant for chlorophyll content. No inoculant or genotype x inoculant effects were observed for plant height and shoot dry weight indicting that bacterial inoculation had no influence on these traits. Figures 3.5C show the effects of bacterial inoculum on chlorophyll content. The significant genotype x inoculation interaction is evident in the differential responses of some genotypes. For example, genotypes 20, 22 and 23 had higher chlorophyll content when inoculated with Sp7-S, whereas genotype 4 responded better in the presence of Sp7 (Figure 3.5C).

Table 3.6 Wald statistics of the growth analysis of 23 wheat genotypes

Fixed term	**n.d.f**	**Plant height**	**Shoot dry weight**	**Relative Chlorophyll**
Genotype (G)	22	176.77***	199.89***	84.50***
Inoculant (I)	2	1.47	3.2	6.15*
G x I	44	58.57	50.9	76.31**

* is significant at P<0.05, ** P<0.01 and *** P<0.001% level respectively

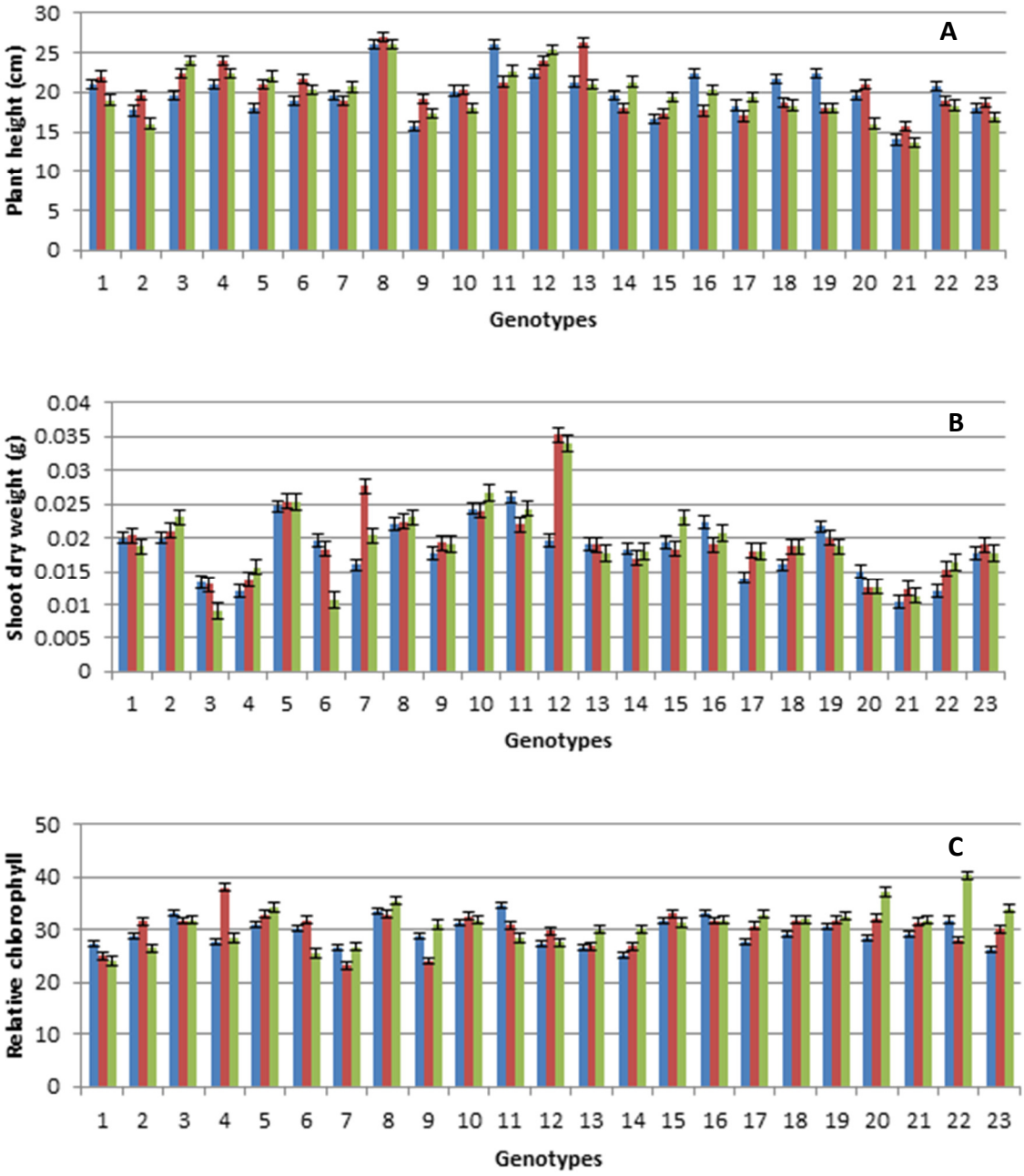


Figure 3.5 Effect of inoculation of 23 wheat genotypes with *A. brasilense* strains Sp7 and Sp7-S on plant height (A), shoot dry weight (B), relative chlorophyll (C). Treatments: Uninoculated control (blue), Sp7 (red) and Sp7-S (green).

3.3.3 Root colonisation by Sp7 and Sp7-S (bacterial enumeration)

Twenty three wheat genotypes were grown in gnotobiotic (sterile) conditions and inoculated with *Azospirillum* to determine differences in bacterial colonization of the roots and the effectiveness of the inoculation process. Bacterial number on the roots of each genotype was quantified and analysed (Table 3.7). Three roots from each treatment were sampled to count bacterial number. The data from uninoculated roots were excluded from analysis as no bacteria were detected. There was a highly significant effect of genotype and genotype x inoculant on $\log_{10}$ number of colony forming unit (cfu) of bacteria when expressed per mL of root suspension, per g of root tissue ($\log_{10}$ cfu/g) and per unit area of root surface ($\log_{10}$ cfu/cm^2) in this experiment. A significant effect was observed indicating that Sp7 and Sp7-S colonise the root surface and tissue at different levels. Though all the plants were harvested at same stage of growth (21 days after transfer to hydroponic media). These effects are evident in Figures 3.6A-C. They demonstrate that genotypes had different bacterial loads and can be broadly grouped in to low and high numbers in this experiment. There were 12 genotypes with low numbers of around $\log_{10}$ 8.0 cfu/g (genotypes 1 – 7, 11 and 13 – 16) and 11 genotypes with 100 to 10000-fold higher numbers of between $\log_{10}$ 10.0 – $\log_{10}$ 12.0 cfu/g (genotypes 8 – 10, 12 and 17 – 23). However, bacterial numbers on genotypes 6 and 13 were much higher with Sp7-S compared to Sp7. There was no trend in bacterial number according to genotype source whether they were synthetically derived, advanced lines or released varieties.

Table 3.7 Wald statistics and significance level from the analysis of the enumeration results

Fixed term	**Log cfu/g root**	**Log cfu/ml root suspension**	**Logcfu/cm^2 root surface**
Genotype (G)	993.04***	1151.31***	1115.84***
Strain±	6.19*	7*	9.03**
G x strain	73.10***	91.16***	94.73***

* represents significance at P<0.05, ** at P<0.01and *** at P<0.001%, respectively

± includes Sp-7 and Sp7-S only

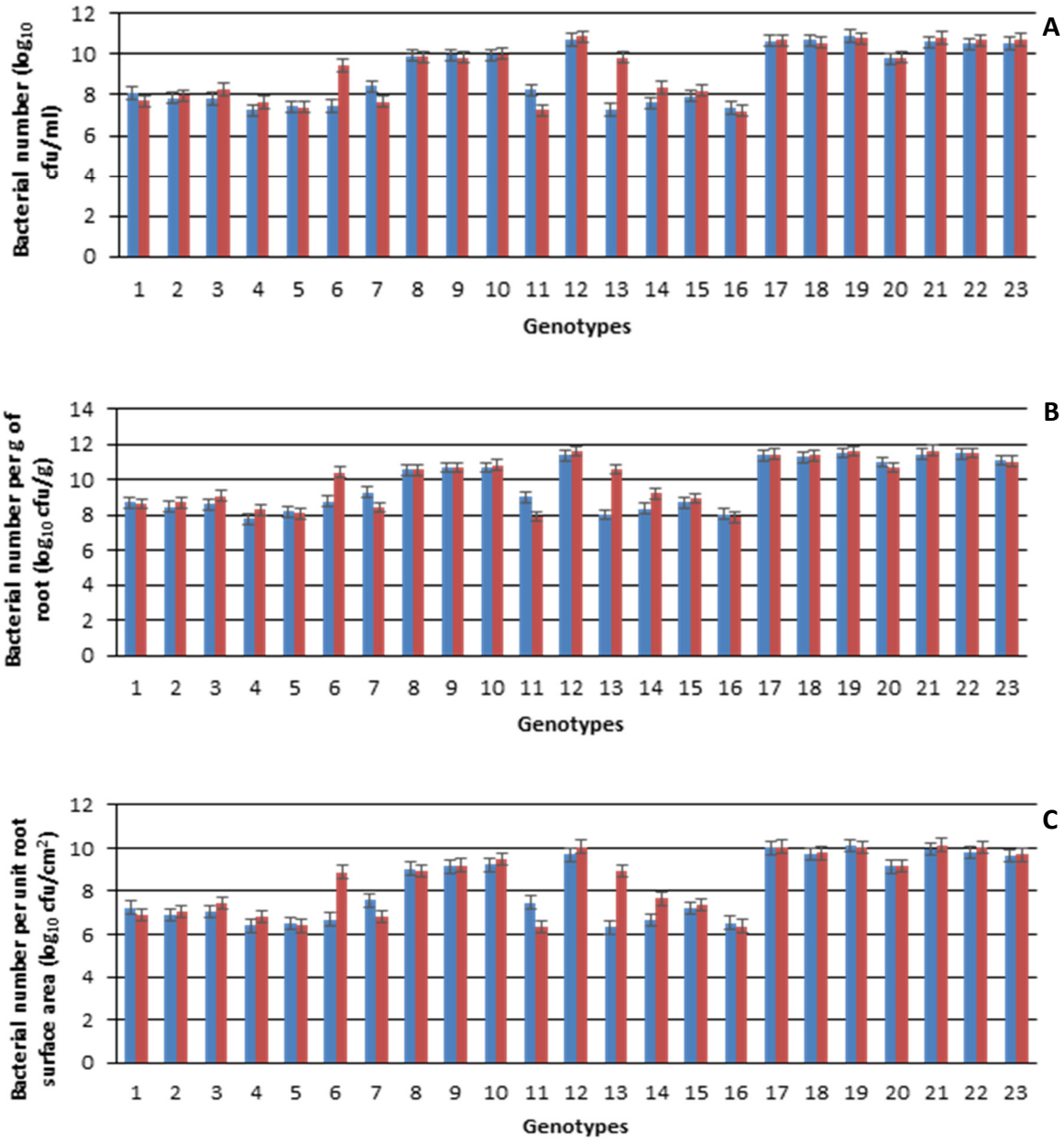


Figure 3.6 Colonisation of roots of 23 wheat genotypes by *A. brasilense* Sp7 and Sp7-S. Bacterial number per mL of root suspension. (A), bacterial numbers per gram of root (B) bacterial numbers per unit root surface area (C).

3.3.4 The effect of inoculation on plant growth parameters

3.3.4.1 Influence of inoculation on relationships between root growth parameters

The effects of inoculation on the relationship between root dry weight and other root growth parameters of plants at 20 DAT are illustrated in figure 3.7. As expected, all root traits; total root length (A, B and C) root surface area (D, E and F) and root volume (G, H and I) were positively related to root dry weight. However, stronger relationships were found between root length and root dry weight in inoculated treatments (r^2 = 0.41 and 0.42) compared to the uninoculated control (r^2 = 0.27).

3.3.4.2 Influence of inoculation on relationships between shoot dry weight and root parameters

The effects of inoculation on the relationships between root parameters and shoot dry weight are illustrated in figure 3.8. In general, increased shoot dry weight was related to increased root dry weight (A, B and C), total root length (D, E and F), root surface area (G, H and I) and root volume (J, K and L). In all cases, there was a significant positive relationship when plants were inoculated with Sp7 (B, E, H and K). Weak positive relationships were observed with uninoculated controls (A, D, G and J) and between root dry weight and root length for plants inoculated with Sp7-S (C, F). The relationship between shoot dry weight with root surface area and volume in plants inoculated with Sp7-S was slightly stronger than it was with root dry weight and length (I and L).

3.3.5 Relationship between bacterial number and different root parameters

The relationships between bacterial number per unit area of root and different root parameters are illustrated in figure 3.9. Generally, in this hydroponic system the higher the bacterial number the shorter the roots. Shorter roots may result in decreased shoot growth particularly when inoculated with high numbers of Sp7.

There was a slight non-significant negative relationship between root dry weight and bacterial number per unit root area (Fig 3.9, A and B). Both bacterial strains showed a similar relationship (r^2 = 0.16 and 0.10 for Sp7 and Sp7-S, respectively). Similarly there was a weak negative relationship between root volume and the number of *Azospirillum* per unit root area (r^2 = 0.19 and 0.16 for Sp7 and Sp7-S, respectively; Fig 3.9 G and H). The trend indicates that root growth is reduced as the number of *Azospirillum* increases.

There was very little influence of Sp7 number per unit root area on total root length and root surface area (Fig 3.9 C - F). However, weak negative relationships were observed after inoculation with Sp7-S (r^2 = 0.18 and 0.19 for total root length and surface area, respectively). In this case, root length and surface area decreased with an increase in the number of Sp7-S. There was no relationship between average root diameter and bacterial number per unit root area when different genotypes were inoculated with Sp7-S and a weak negative relationship when genotypes were inoculated with Sp7 (Fig 3.9 I and J).

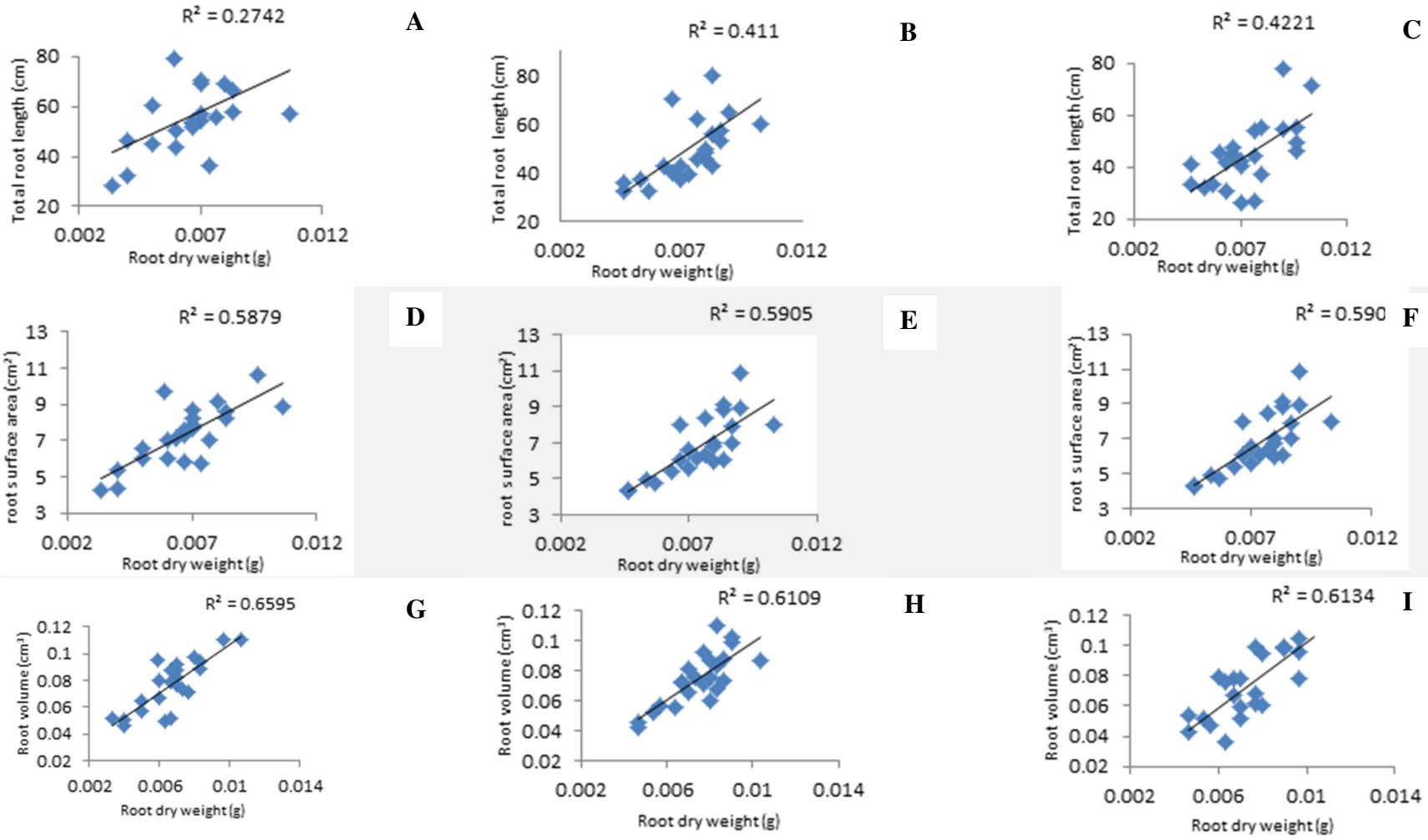
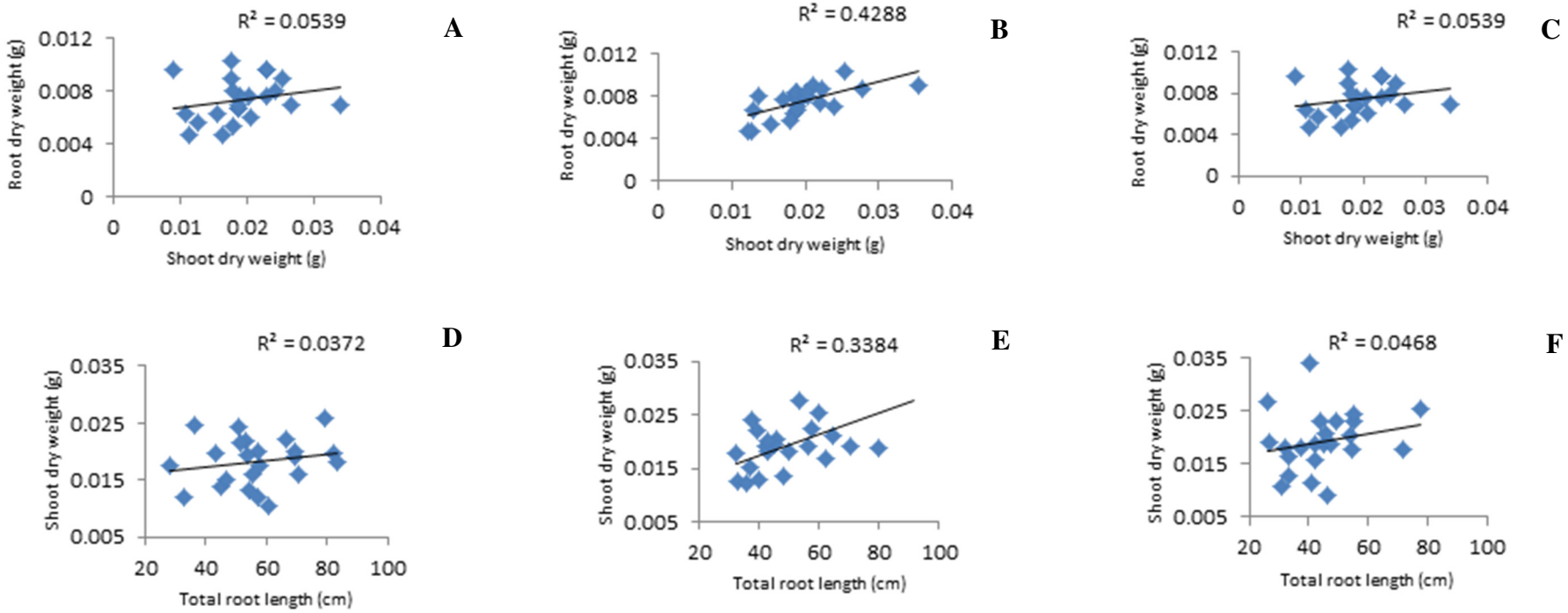

A
R² = 0.2742
Total root length (cm)
Root dry weight (g)
B
R² = 0.411
Total root length (cm)
Root dry weight (g)
C
R² = 0.4221
Total root length (cm)
Root dry weight (g)
D
R² = 0.5879
root surface area (cm²)
Root dry weight (g)
E
R² = 0.5905
root surface area (cm²)
Root dry weight (g)
F
R² = 0.590
root surface area (cm²)
Root dry weight (g)
G
R² = 0.6595
Root volume (cm³)
Root dry weight (g)
H
R² = 0.6109
Root volume (cm³)
Root dry weight (g)
I
R² = 0.6134
Root volume (cm³)
Root dry weight (g)
Control
Sp7
Sp7-S

Figure 3.7. Comparison of root dry weight with different root parameters with and without inoculation. Root parameters in rows: total root length (A, B and C); root surface area (D, E and F); root volume (G, H and I). Inoculation treatments in columns: uninoculated control (A, D and G), Sp7 (B, E and H); Sp7-S (C, F and I).



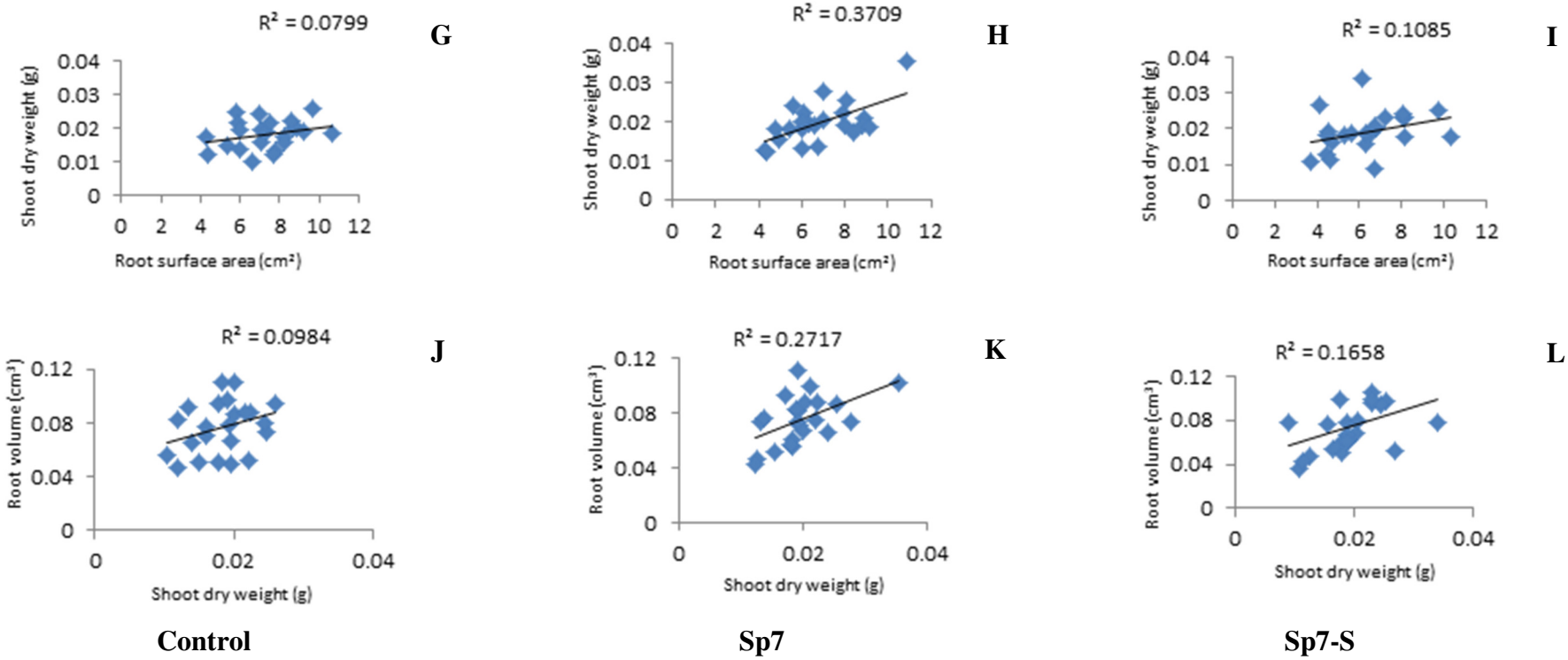
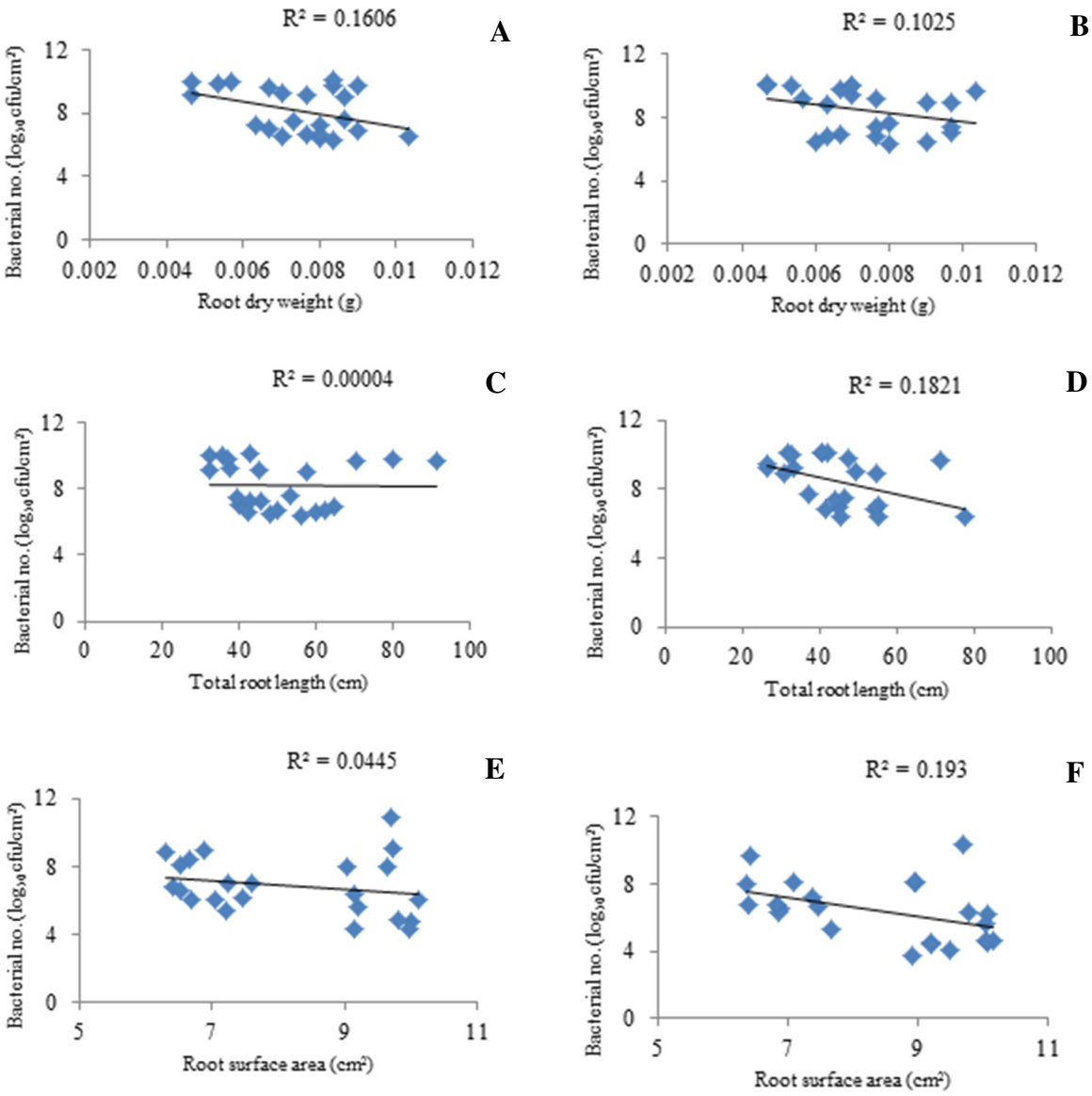


Figure 3.8. Comparison of shoot dry weight with different root parameters with and without inoculation. Root parameters in rows: root surface area (G, H and I); root volume (J, K and L). Inoculation treatments in columns: uninoculated control (A, D, G and J), Sp7 (B, E, H and K); Sp7-S (C, F, I and L).


A
R² = 0.1606
Bacterial no. (log10 cfu/cm²)
Root dry weight (g)
B
R² = 0.1025
Bacterial no. (log10 cfu/cm²)
Root dry weight (g)
C
R² = 0.00004
Bacterial no. (log10 cfu/cm²)
Total root length (cm)
D
R² = 0.1821
Bacterial no. (log10 cfu/cm²)
Total root length (cm)
E
R² = 0.0445
Bacterial no. (log10 cfu/cm²)
Root surface area (cm²)
F
R² = 0.193
Bacterial no. (log10 cfu/cm²)
Root surface area (cm²)

Sp7 **Sp7-S**

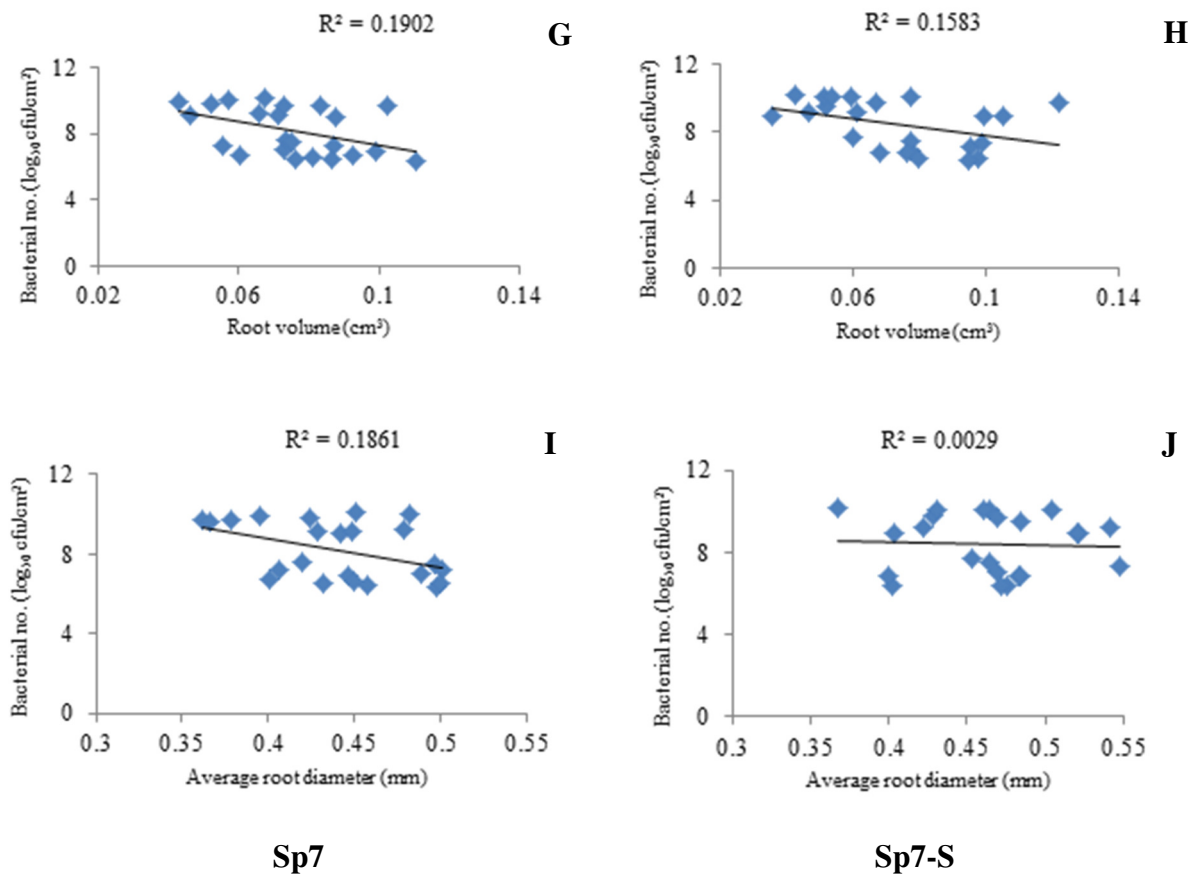


Figure 3.9. Relationship between bacterial number per unit area (log_{10} cfu/cm^2) and different root parameters. Root parameters in rows: root dry weight (A and B); total root length (C and D); root surface area (E and F); root volume (G and H); average root diameter (I and J). Inoculation treatments in columns: Sp7 (A, C, E, G and I); Sp7-S (B, D and F, H and J).

3.3.6 Observation of root colonisation by Sp7 and Sp7-S carrying pLA-*lacZ* fusions using X-gal staining

Plant roots were treated with 2% glutaraldehyde to inactivate plant β-galactosidase and then stained with X-gal solution and observed under a light microscope (Olympus DP70). Colonisation of all genotypes by Sp7 and Sp7-S showed the same pattern and representative images are presented in figure 3.10. Bacteria were not observed on uninoculated plants (Fig. 3.10A). Sp7 colonised the root surface of all 23 wheat genotypes (Figure 3.10B). In contrast, Sp7-S showed less colonization on the root surface and root hairs but strong colonization in the cracks formed by the emergence of lateral roots (Figure 3.10C).

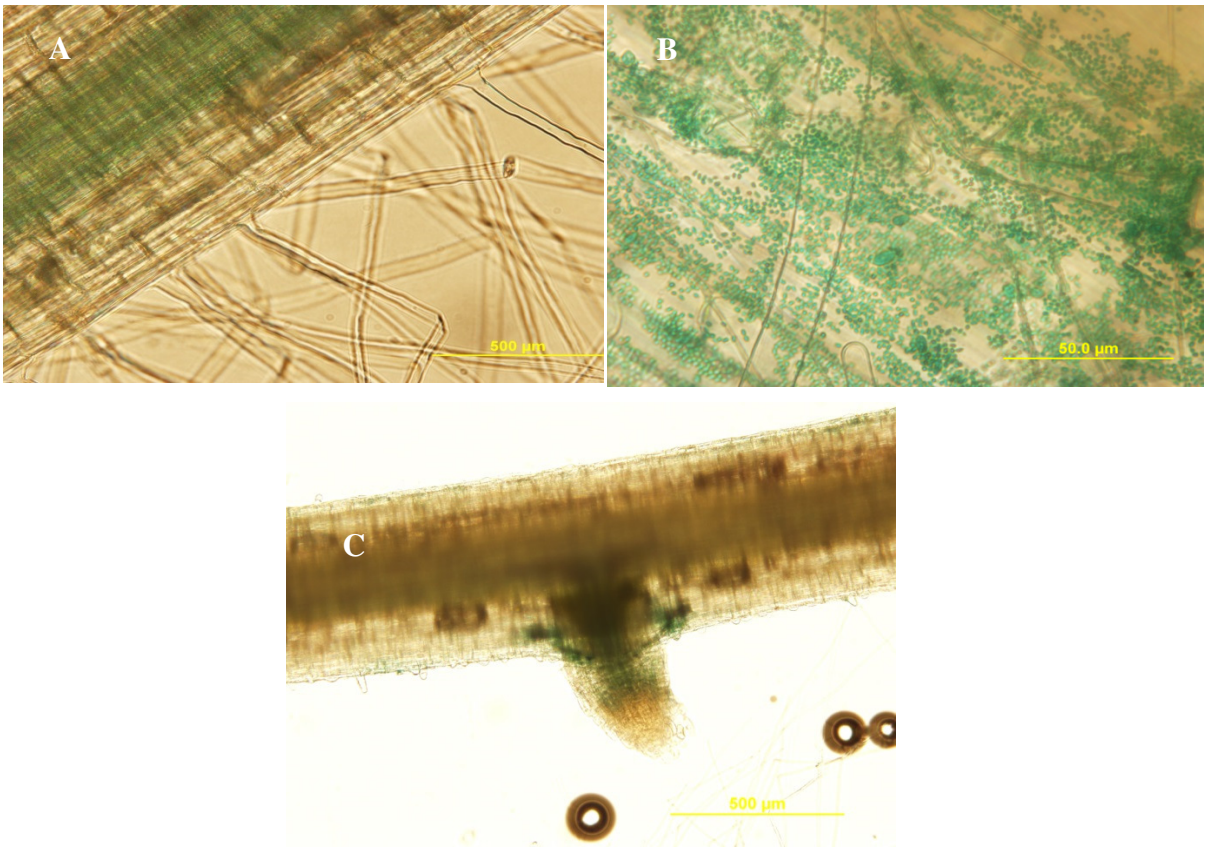


Figure 3.10 Microscopic observations of coloniastion wheat roots by *A. brasilense* Sp7 and Sp7-S after staining with X-gal. Inoculation treatment: uninoculated control (A); Sp7 pLA-*lacZ* (B); Sp7-S pLA-*lacZ*(C).

3.4 Discussion

3.4.1 Effect of inoculation on root development

The time course study conducted in the absence of inoculum demonstrated that the genetic potential for root growth varied significantly among the genotypes tested. The introduction of inoculum changed this relationship and some wheat genotypes greatly increased growth rates and root trait values while others showed no positive effects. There is clearly wheat genotype specificity for certain bacterial strains. These differential genotype responses to applied inoculum are most likely due to IAA production by *A.brasilense* (Ona et al. 2003). IAA can promote plant growth by stimulating root formation (Akbari et al. 2007). When IAA was exogenously applied in earlier work, lateral roots increased and root hairs proliferated (Jain and Patriquin 1984; Glick 1995). In the current study there was a significant but variable (both directions) effect of inoculation on total root length, root surface area, root diameter and root dry weight. There is not a general increase or decrease in these traits. The regression analyses indicate that the overall response with increasing bacterial number is a decrease in root traits. This is consistent with Dobbelaere et al. (1999). It is likely that the root exudates would vary across all genotypes and would influence the production of phytohormone by bacteria (Liljeroth et al. 1990; Bashan and Holguin 1994) and bacterial motility thus affecting colonisation. Although responses were variable, regression analyses indicated that there was a

general decrease in root traits with an increase in the number of azospirilla per unit root area. This is consistent with the results of Spaepen et al. (2008) after inoculation of wheat with *A.brasilense* Sp7 and Sp245. They observed that inoculation rate affected root length and root hair density with the longest roots observed at the lowest inoculation rate of 10^5 cfu/plant and increasing root inhibition with ten-fold increases in inoculum potential to 10^9 cfu/plant.

The genotypes selected for this experiment are genetically diverse and include synthetic derivatives, released Australian cultivars and international germplasm; this diversity has undoubtedly contributed to the strong genotypic effect for most root characteristics and the strong genotype x bacterial strain interaction. Earlier work showed that synthetic derived lines, in particular, produce greater root biomass deeper in the soil profile (Reynolds et al. 2007). It may be that this extended diversity introduced from *Triticum tauschii*, the donor of the D-genome in primary synthetic wheat (Trethowan and Kazi 2008), provides greater sensitivity to inoculation with bacteria in some genotypes (such as lines 5, 6, 11, 12 and 18 particularly for root length in this study) The *T. tauschii* used to develop the synthetic hexaploid wheat avoided the evolutionary bottleneck that gave rise to hexaploid wheat some 8,000 years ago. An evaluation of bacterial effects among *T. tauschii* accessions may provide additional useful information and genetic variation that could be introduced into modern wheat.

3.4.2 Effect of 1inoculation on above ground plant growth

While *A. brasilense* has been reported to improve grain yield following inoculation of cereals (Swędrzyńska 2000), the exact mechanisms of improved plant growth remain unclear. One possible mechanism could be stimulation of nitrogen uptake by roots (Saubidet et al. 2002) or

improved mineral balance and plant nutrient content (Bashan et al. 1990) following inoculation with *A. brasilense*. Bashan and Dubrovsky (1996) reported that *Azospirillum* spp. participates in the partitioning of dry matter (both carbon compounds and minerals) at the whole plant level by affecting root function and hence nutrient uptake.

The stimulation of root growth by IAA was discussed in the previous section. However, these effects on root growth can be reflected in better above ground biomass (Spaepen et al. 2008). While multiple mechanisms of improved plant growth in the presence of *Azospirillum* strains are possible, results vary according to plant species, plant genotype within species, *Azospirillum* strain and environmental conditions including abiotic and biotic stresses (reviewed by Bashan and De-Bashan 2010).

The very strong and significant genotype effects observed in plant growth only translated to a significant inoculum effect for root dry weight and chlorophyll content. Plant height and shoot biomass were not influenced indicating that improvements in root biomass due to inoculum did not improve shoot biomass. However, it is difficult to interpret too much from these results as this experiment was conducted under very controlled conditions in a small test tube with 15 ml of hydroponic solution for three weeks only. It is likely that plant growth was restricted and the effect of inoculum may therefore have been diminished. This system is better suited to root colonisation studies. Nevertheless, such techniques do provide an estimate of inoculum effects on large numbers of lines. More detailed field evaluations or greenhouse tests can then be conducted on the most probable materials thus identified.

3.4.3 Effect of inoculation on bacterial colonisation (visualisation and enumeration)

In this study, there were highly significant differences in bacteria number associated with the roots of different wheat genotypes two weeks after inoculation. The differences in colonisation patterns observed for the two strains, Sp7 and Sp7-S are likely linked to differences in exopolysaccharide production (Katupitya et al. 1995). Sp7-S is unable to colonise the root surface as production of fibrilliar material that allows anchoring to the root surface is reduced. The results of the current study support those of Katupitya et al. (1995) and Pereg-Gerk et al. (2000) who observed a large number of Sp7 wild type bacteria present on the root surface and relatively little surface colonisation by the mutant strain Sp7-S. Sp7-S tended to be found in root cracks and inside root tissue indicating a different mode of colonisation to the wild-type strain. Differentiation of these two strains during counting was confirmed morphologically by Congo red binding in the Nfb agar media containing Congo red dye as described by Pereg-Gerk et al. (2000). The Sp7-S strain produced pink colonies that were smooth and shiny whereas Sp-7 produced drier red colonies. Clearly, both strains persisted in the hydroponic system over the period of wheat growth, although strong wheat genotype specificity influenced colonisation density. The differences in colonisation patterns may also explain inoculum treatment effects observed for some characters. The surface colonisation of Sp7 compared to the root crevice and internal tissue patterns of Sp7-S may have influenced wheat genotype specificity and contributed to genotype x inoculum interaction. This could have been driven by minor differences in root morphology among wheat lines. For example, it would be expected that colonisation by Sp7-S on a genotype with more lateral roots and therefore more crevices would be higher than on a genotype with less lateral roots.

3.4.4 Relationships between different plant traits and the effects of bacterial inoculation.

Bacterial inoculation generally did not influence the relationships between productivity traits such shoot dry weight and root traits such as root length, surface area and volume. However, inoculum did enhance the correlation in a few cases. The increase in root dry weight with root length in the presence of inoculum indicates that longer root growth probably improved plant access to nutrient and hence overall biomass. While the significant relationship between shoot dry weight and root length and root surface area in the presence of Sp7 are encouraging, this result must be interpreted carefully as one genotype, a synthetic derived line, tended to drive the overall relationship and produced much more biomass in the presence of Sp7 than any other wheat genotype. The reduction in root traits of plants carrying higher numbers of bacteria supports earlier findings (Spaepen et al. 2008). Clearly, bacterial inoculation must be carefully managed and a balance between initial bacterial density and persistence must be found for each wheat genotype.

3.5 Conclusion

This experiment aimed to identify a subset of wheat genotypes from among the 23 evaluated in controlled conditions for more detailed field testing. However, as the field season overlapped with this evaluation a subset was identified for field evaluation based on genetic diversity determined using DArT. The five genotypes subsequently evaluated in the field at Narrabri were selected from the five diversity groups in the dendogram generated from the DNA fingerprint data. All five genotypes evaluated in field trials (see Chapter 4) showed a differential response to inoculation with the two bacterial strains under controlled conditions. However, the inclusion of the most responsive genotype (number 23) could have increased the

level of significance for some traits under field conditions. This genotype should be evaluated under field conditions in future research to confirm if responsiveness under controlled conditions translates to the field.

Chapter 4: Field responses of wheat to inoculation with different strains of bacteria

4.1 Introduction

Azospirillum is the one of the most studied plant growth promoting bacteria. A recent meta-analysis of 59 published articles evaluated the effect of *Azospirillum* on wheat growth (Veresoglou and Menexes 2010). It was reported that an increase of 8.9% in yield and 17.9% in above ground biomass is possible when wheat plants are inoculated with *Azospirillum*. It was concluded that many factors are responsible for the plant growth promoting effects of *Azospirillum* including bacterial strain, the amount of N fertilization and the genotype of the wheat cultivars evaluated. Results indicate that *Azospirillum* is an efficient growth promoting inoculant of wheat.

More than 20 years of field experiments (1974 -1994) have proven that inoculation with *Azospirillum* bacteria can improve the yield of agronomically important crops in different soils and climates. The success of inoculation depends on methods that optimize the number of viable cells and maximize subsequent root colonization. The inoculation will not replace fertiliser but should augment nutrient utilization by crop species (Okon and Labandera-Gonzalez 1994). There are several mechanisms responsible for plant growth promotion including the production of substances which improve root growth (Okon and Kapulnik 1986; Bashan and Dubrovsky 1996). Plant growth promotion is partly linked to biological N2-fixation which is a spontaneous process if soil N is limited and adequate C sources are available

(Tien et al. 1979; Okon and Kapulnik 1986; Okon and Itzigsohn 1995). *Azospirillum* improves root development by stimulating root hair length, lateral root development and root surface area colonization when optimum inoculation concentration has been maintained. *Azospirillum* produces indole 3- acetic acid and indole 3- butyric acid and these influence root morphology and physiology thus increasing water and nutrient up-take leading to faster plant growth. Under conducive conditions, these processes will increase crop yield (Okon 1985; Dodereiner and Perdosa 1987; Fallik et al. 1994; Gallo and Fendrik 1994). Plant shoot and root dry weight and root surface area increased significantly in wheat and soybean after inoculation with *Azospirillum* compared with uninoculated plants (Bashan et al. 1990). All of the *A.brasilense* strains used in their experiment, including Cd, Sp7, Sp245, Sp246, Somali 67 and OH 88028, improved plant growth and all were capable of colonizing the roots. Under stress conditions wheat plants also show increased growth linked to greater production of auxiliary photoprotective photosynthetic pigments following *A.brasilense* Cd application (Bashan et al. 2005). Inoculation of cereals (winter wheat and oats) with active strains of *A.brasilense* in the Wielkopolska region of Poland were reported to increase the vigour and yield of wheat (up to 27 %) and oats (6 %) (Swedrzynska 2000).

In a large study, Diaz-Zorita and Fernandez-Canigia (2009) evaluated 297 field experiments in the Pampas region in Argentina between 2002 and 2006 and assessed the effect of inoculation with *A.brasilense* INTA Az-39 on wheat crop productivity (*Triticum aestivum* L). Positive responses were observed in about 70% of the sites and the inoculated crop showed superior shoot and root vegetative growth (12.9% and 22% respectively) and an average improvement in grain yield of 8%. Similarly, Kapulnik et al. (1983) reported significant increases in grain

yield and foliage of two cultivars of *Triticum aestivum* and *T. turgidum* following inoculation with *Azospirillum* in field experiments in the Northern Negev and Bet Shean Valley in Israel.

In a field experiment inoculated with *A.brasilense* 1774, maize produced significantly higher above ground dry biomass compared to uninoculated plants, however similar grain yield was obtained with nitrogen fertilization treatments of 200 and 100 kg N ha^{-1} (Stancheva et al. 1992). In field experiments in Northern Negev and Bet Shean Valley in Israel, *Zea mays* (three cultivars), *Sorghum bicolor, Panicum miliaceum* and *Setaria italica* were inoculated with nitrogen-fixing bacteria of the genus *Azospirillum* and significant increases in grain yield and foliage were observed. It was concluded that inoculating summer cereal crops in Israel may save valuable nitrogen fertilizer (Kapulnik et al. 1981). Naiman et al. (2009) observed non-significant responses to plant growth promoting bacteria on wheat when inoculated with PGPB (*A.brasilense* and *Pseudomonas fluorescens*) in a field experiment in Buenos Aires, Argentina. However, the PGPB inoculation increased mean aerial biomass by 12%, root biomass by 40% and grain yield by 16% and these authors concluded that such increases may help obtain greater sustainability of the agro ecosystem. Ozturk et al. (2003) reported that yield responses of wheat and barley to inoculation with *A.brasilense* Sp 246 and *Bacillus* sp. OSU-142 were significantly higher at all levels of N fertilization in the field compared to the control but that the increases diminished with higher levels of N application. Their results suggest that there is potential for inoculation of *A.brasilense* as a biofertiliser for spring wheat and barley cultivation in organic and low N input agriculture. However, very little is known of the variation in wheat genotype response to inoculation. Salamone et al. (2012) reported that inoculation of paddy rice with PGPB *A brasilense* and *P. flueroscens* in Argentina elicited a

differential response among rice genotypes that could be associated with changes to root mass. However they found that inoculation with PGPB did not have a significant impact on the culturable microbial community and subsequent TRFLP patterns. This chapter describes two field experiments conducted to determine the effects of bacterial inoculants on the growth and grain yield of different wheat genotypes under two different levels of nitrogen in two years; 2010 and 2011.

The aim of these experiments was to determine the best combination of inoculums and genotypes under varying levels of N stress using different assessments of plant growth and grain yield. These experiments sample the variability between seasons and assess the interaction between the wheat genotype and bacterial inoculum.

4.2 Materials and Methods

4.2.1 Site description

Experiments were conducted during the wheat growing season of May-November in 2010 and 2011 at the IA Watson Grains Research Centre located near Narrabri in northwestern NSW (30° 19′ 0″ latitude and 149° 46′ 0″ E longitude). The soil was a black vertosol with 2.7% organic matter, 3.7 N w/v (weight per volume) and pH 7.5 in the top 15 cm. Irrigation was applied as required to alleviate drought stress.

4.2.2 Crop rotation

The experiments sown in both years were conducted on different sites within the Narrabri

research station but following the same 4-year crop rotation: barley; field pea; fallow; wheat. The site was managed using zero-tillage and weeds were chemically controlled as needed during the rotational period and during the winter season when experiments were sown.

4.2.3 Climate description

Figures 4.1 and 4.2 show the annual fluctuations in temperature and rainfall at Narrabri in 2010 and 2011, respectively (data collected from bureau of meteorology, Australia). Both years were relatively wet and temperatures were subsequently lower than the long-term average (Figure 4.1 and 4.2). Experiments were sown on May 20th in both years and harvested on November 20th 2010 and November 17th 2011. Irrigation was not applied in either year as rainfall was sufficient throughout the growth period in both years. Narrabri is a summer dominant rainfall environment and experiments were sown on a full moisture profile.

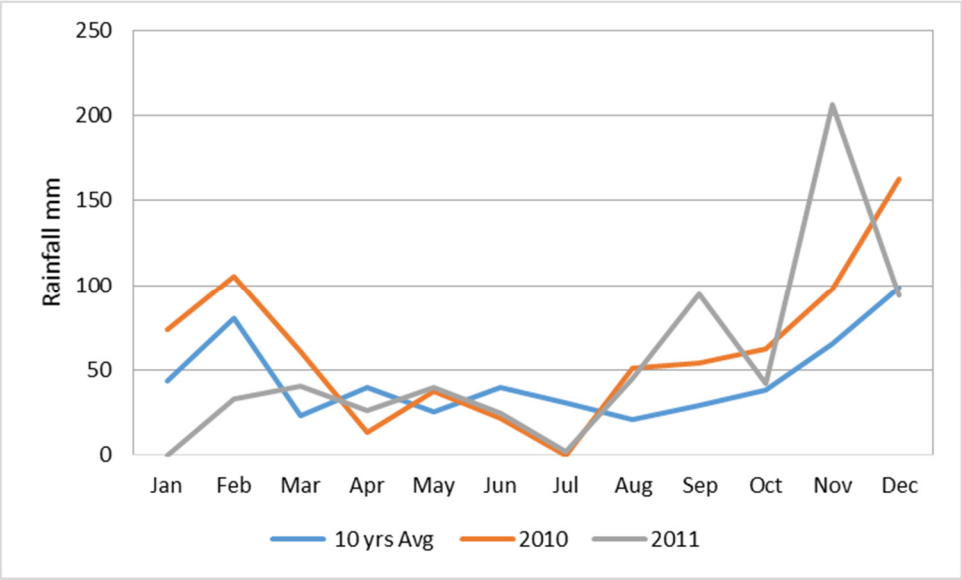


Figure 4.1 Rainfall at Narrabri during 2010, 2011 and 10 year average rainfall (data source http://www.bom.gov.au/climate/data/)

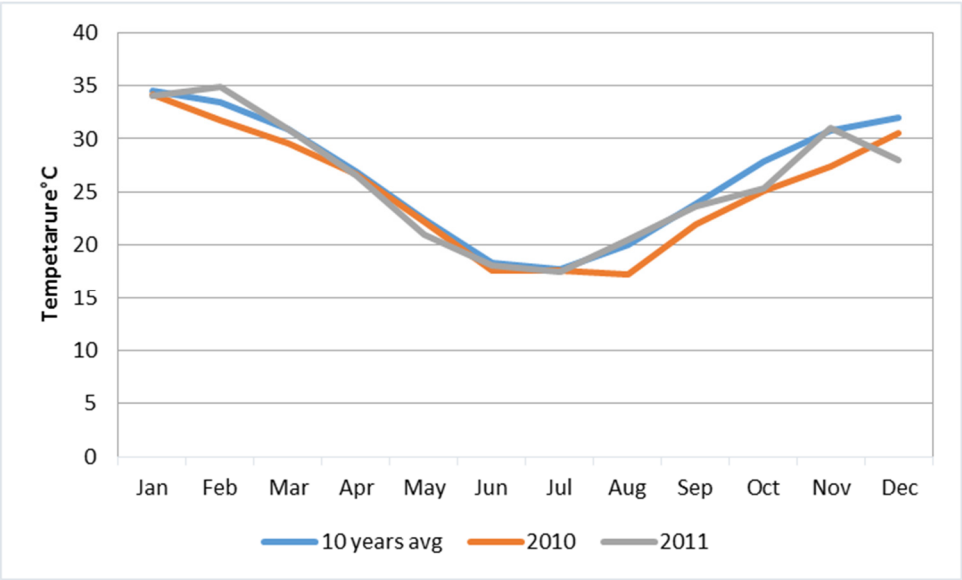


Figure 4.2 Temperature at Narrabri during 2010, 2011 and 10 year average temperature (data source http://www.bom.gov.au/climate/data/)

4.2.4 Genotypes

Five wheat genotypes were selected for the experiments in 2010 and 2011 (Table 4.1).

Table 4.1 List of the genotypes tested in 2010 and 2011.

Pedigree	Agency & year	Description
1. EGA GREGORY	DEEDI (Department of Employment, Economic Development and Innovation)	Released Australian cultivar 2004
2. CBRD/KAUZ//KASO2	International Center for Maize and wheat Improvement	Advanced line
3. CROC_1/AE.SQUARROSA(205)// KAUZ/3/2*METSO	International Center for Maize and wheat Improvement	Synthetic derivative
4. SOKOLL	International Center for Maize and wheat Improvement	Synthetic derivative
5 KRICHAUFF	University of Adelaide	Released Australian cultivar 1997

These five genotypes were selected from a range of 23 diverse wheat genotypes which were

grown in a controlled environment with bacterial treatments (see Chapter 3). These were reduced using DNA fingerprints (based on Diversity Arrays technology or DArT profiles) to ensure maximum genetic diversity and likelihood of responsiveness among the five selected genotypes (Figure 4.3). The variation in response of these genotypes to bacterial inoculation was subsequently confirmed based on the results from Chapter 3. The five genotypes differed for various morphological characteristics including plant height and seed size. EGA GREGORY is currently the most widely grown cultivar in the Narrabri region, SOKOLL and CROC_1/AE.SQUARROSA(205)//KAUZ/3/2*METSO are synthetic derivatives developed by the International Maize and Wheat Improvement Centre (CIMMYT) and therefore represent new D-genome diversity, Krichauff is a cultivar released in southern Australia and CDRD/KAUZ//KASO2 is an advanced line with high yield potential from CIMMYT.

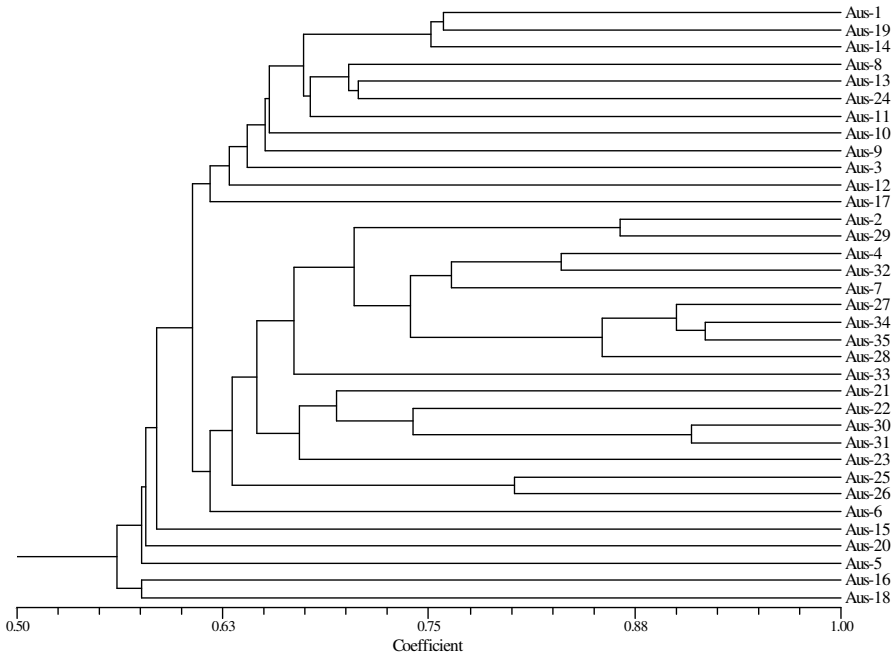


Figure 4.3 Dendrogram of candidate genotypes for field testing based on DArT genotyping; Aus-1 (EGA Gregory); Aus-17 (CBRD/KAUZ//KASO2); Aus-18 (CROC_1/AE.SQUARROSA (205)//KAUZ/3/2*METSO); Aus-20 (SOKOLL); Aus-22 (KRICHAUFF).

4.2.5 Experimental design and plot management

Wheat genotypes were established in two split plot designs with bacterial treatments assigned to whole plots and wheat genotypes to subplots within whole plots and four replicates during both 2010 and 2011. The plots were 12m in length and 2m wide and a $20m^2$ area was harvested for yield following removal of the first and last 1m of plot. The experiments were conducted in different locations on the same soil type within the research station in both years following a standard crop rotation as described earlier. Each plot was separated by a buffer plot of the

cultivar Sunlin to avoid contamination among the bacterial treatments. In 2010, no N treatment was established and the same rate of N (75 kg of urea at sowing) was applied to both experiments resulting in a mild N stress. However, in 2011 an N treatment was established through the application of 75 kg urea to the high N treatment and 33 kg urea to the low N treatment after sowing. Both treatments received 50 kg of N at sowing and the differential treatment of 75 and 33 kg was applied at the jointing stage.

4.2.6 Bacterial treatments

In 2010, two bacterial treatments *A.brasilense* Sp7 and its mutant strain *A.brasilense* Sp7-S were selected for inoculation in both experiments. This resulted in three treatments in 2010: Sp7, Sp7-S and an uninoculated control. In 2011 *A.brasilense* Sp245 was included as an additional treatment resulting in four treatments: Sp7, Sp7-S, Sp245 and an uninoculated control. Peat was used as the inoculum carrier. Bacteria were initially grown in nutrient broth for 48 hours with shaking at 350 rpm following which 100 ml of bacterial culture equivalent to 10^9cfu/ml was injected aseptically in 150 g of dry sterile peat and allowed to grow for 10 days in a 30 °C incubator until the culture reached 10^9 cfu/g.

4.2.7 Sowing and establishment of inoculation treatments

Experiments were sown on the 20th May in both 2010 and 2011 using a cone seeder at the rate of 150 plants/ m^2 in 24m^2 plots. On the day of sowing six bags of peat (each bag 250 g) per treatment containing 10^9 cfu/g (colony forming unit/gram) bacteria were mixed with 60 L water then applied at 200 mL per m thus distributing 3 g peat per meter of plot (or 33.3 mL per linear meter from each tube of five). The liquid inoculum was delivered directly into the furrow with

the seed via a tube attached to each set of discs. Uninoculated plots received water containing sterile peat and these were sown first. Then tanks and tubes were flushed with ethanol (or methylated spirits) between each bacterial treatment. Samples from the inoculation tube were collected to verify the bacteria number per ml of liquid inoculum and to ensure that each plot received equal amounts of bacteria. The inoculation tube was sampled three times (at the beginning of sowing, mid-sowing and at the end of sowing) and counts of culturable microorganisms and contaminants were performed (Table 4.2). Bacterial counts recorded were 10^{6-7} cfu/ml on Nfb agar medium with congo red and no contaminants were detected in the inoculants before applying in the field. The recommended bacterial concentration for inoculation of whole plot treatments was produced by dilution with water.

Table: 4.2 Inoculation rate measured at different stages of sowing in 2010 and 2011.

	2010		2011		
Sample collection	Sp7 cfu/ml	Sp7-S cfu/ml	Sp7 cfu/ml	Sp7-S cfu/ml	Sp245 cfu/ml
Beginning	1.1×10^7	6×10^6	1.1×10^7	1×10^7	1.2×10^7
Middle	6×10^6	7×10^6	5×10^7	3×10^7	1.5×10^7
End	1.5×10^6	2.7×10^6	1.1×10^7	1×10^7	1.7×10^7

4.2.8 Plant and Soil Sampling

Plants emerged one week after sowing and plant matter and rhizosphere soil samples were collected 43 days after sowing (DAS). This procedure was repeated (at 103DAS, 133DAS, and

harvest ripeness) throughout the crop cycle to estimate plant growth. Plant matter was sampled to estimate growth and rhizosphere samples collected to estimate the bacterial number.

4.2.8.1 Tillering Stage (43 DAS)

Twenty plants were randomly selected from each plot resulting in 2400 plants collected from 120 plots in 2010 and 3200 plants from 160 plots in 2011. Plants were uprooted with a shovel by digging to 20-30 cm depth to sample as much of the root system as possible while minimizing damage. One person collected all plants in the same treatment to avoid cross contamination of inoculum by the shovel. The roots of 10 plants were separated from the shoot and washed with running tap water and kept in 50% ethanol for subsequent root analysis, roots of a further 5 plants were stored in a refrigerator for microbial analysis and the roots of the remaining five plants were stored at -80°C in liquid nitrogen immediately after sampling for subsequent molecular analysis. All the shoots were dried in a dehydrator oven at 75°C for four days to measure biomass accumulation.

4.2.8.2 Anthesis time (103DAS)

Ten plants were randomly selected from each plot, uprooted and separated from the shoot. Shoots were dried in the dehydrator for one week and the roots of 5 plants kept at 4°C in a refrigerator for microbial analysis. The roots of the remaining five plants were frozen in liquid nitrogen immediately after sampling for subsequent molecular analysis

4.2.8.3 Post anthesis (133DAS)

Ten plants were randomly selected from each plot, uprooted and separated from the shoot.

Shoots were dried in the dehydrator at 75°C for one week and the roots of five plants kept at 4°C in the refrigerator for microbial analysis. The roots of the remaining five plants including soil were frozen at -80°C in liquid Nitrogen immediately after sampling for molecular analysis.

4.2.8.4 Maturity stage (harvest)

Plants were harvested on 9th November in 2011 and 20th November in 2010. At maturity, twenty plants were selected randomly from each plot and only the above ground biomass was dried in the dehydrator oven at 75°C for one week. After drying, total biomass, grain weight and harvest index were calculated.

4.3 Measurements

Percent emergence was completed 10 days after sowing and recorded. Heading dates were recorded and plant height, relative chlorophyll content, and above ground dry weight were measured at each sampling time. Plant height was estimated using a meter ruler and relative chlorophyll was measured using a chlorophyll meter SPAD -502 plus from Konica Minolta Sensing Inc. in the field. Roots were scanned and analysed with Winrhizo software (Regents Instruments, Canada) at 43 DAS in both years. Harvest index, grain yield, above ground biomass and 1000 grain weight were assessed after maturity.

4.4 Data analysis

Data were analysed using Genstat version 14. The split plot design analyses were conducted using the REML linear mixed model algorithm. Bacterial treatments were assigned to whole

plots and wheat genotypes were randomly allocated as sub-plots within whole plots within each replication. N treatments and/or environments were discreet experiments and were analyzed independently and in combination to examine genotype x year x bacterial treatment x N level interactions. Subsequent correlations among variables were calculated using the correlation function in Genstat.

4.5 Results

4.5.1 Root analysis

Scanned root images were analysed with WinRHIZO software (Regents Instruments,Canada) and total root length, root surface area, root volume and average root diameter were measured. The Wald statistics of the 3-way interactions for these root characters are presented in Tables 4.3 and 4.4.

4.5.1.1 Season 1 (2010)

In 2010 the bacterial treatments significantly increased total root length, root surface area and root volume but not root diameter (Table 4.3). However, as no N treatment was established in 2010, both experiments were considered to be different environments and the effect of environment was significant only for root volume and average root diameter. No significant genotype effects were observed although the genotype x inoculant interaction effect was significant for root volume. All other interactions were non-significant. The significant inoculation effects were largely driven by positive genotype responses to inoculation with Sp7

compared to the control.

Table 4.3 Wald statistics and their significance from analysis of root traits in 2010

Fixed term	Total root length	Root surface area	Root volume	Average Root Diameter
Genotype (G)	1.94	2.38	2.16	0.95
Inoculant (I)	16.13*	25.86**	26.84**	0.25
Environment (E)	0.14	2.56	7.81**	8.88**
G x I	3.71	9.31	17.43*	7.13
G x E	0.58	0.75	2.19	4.00
I x E	0.16	1.97	5.66	4.97
G x I x E	6.17	6.78	7.20	3.29

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively.

4.5.1.2 Season 2 (2011)

In 2011, a significant genotype effect was observed for root volume and average root diameter which both increased with inoculation (Table 4.4). The bacterial treatment effects were significantly positive for the same root characters as 2010; total root length, surface area and volume. However, in 2011 there were pronounced N effects for root surface area and volume. A significant genotype x inoculant interaction was observed for both total root length and surface area. The same traits also showed a significant inoculant x N interaction. The significant inoculation effect in this year was largely driven by positive plant responses to Sp245.

Table 4.4 Wald statistics and their significance from analysis of root traits in 2011

Fixed term	Total root length	Root surface area	Root volume	Average Root Diameter
Genotypes (G)	3.15	9.48	15.37**	17.01*
Inoculants (I)	48.59***	21.10**	58.48***	4.50
Nitrogen (N)	0.11	1469.42***	1516.71***	0.64
G x I	31.80**	24.63*	7.20	8.30
G x N	3.37	5.92	2.57	1.16
I x N	15.58**	11.50**	5.25	3.77
G x I x N	7.47	11.80	6.50	13.89

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively.

4.5.2 Plant growth analysis

4.5.2.1 Plant height

A very strong genotype effect was observed for plant height at every stage of growth in both seasons (Table 4.5). However, inoculation produced a significant negative effect at flowering in 2010 and no inoculation effect was detected in 2011. There was a significant environment effect at the tillering and grain filling stages in 2010; however no significant difference was observed between the N treatments in 2011. Nevertheless, a positive effect on plant height at flowering (103 DAS) was observed due to inoculation with Sp7 and Sp7-S on a few genotypes (3, 4, and 5). No significant interactions were observed in either season at any growth stage. A trend to shorter plants in the presence of inoculum was observed in both years although there were some exceptions (genotypes 2 and 5 at103 DAS and genotype 1 at 133 DAS in 2010;

genotype 2 at 43 DAS genotypes 3, 4, 5 at 103 DAS and genotype 4 with Sp245 inoculation in 2011).

Table 4.5. Wald statistics and their significance from analysis of plant heights in 2010 and 2011

	Season 1 (2010)			Season 2 (2011)		
Fixed term	43 DAS	103 DAS	133 DAS	43 DAS	103DAS	133 DAS
Genotypes (G)	22.97**	274.56***	184.34***	45.61***	3.45	10.02*
Inoculants (I)	5.75	12.77*	0.58	0.93	4.82	4.84
E (2010); N (2011)	4.34*	0.21	12.66***	3.16	0.01	2.45
G x I	8.77	7.76	8.05	15.38	21.87	4.79
G x N (E)	1.37	1.77	2.78	0.44	1.01	5.82
I x N (E)	3.74	0.34	5.10	2.07	2.13	1.17
G x I x N (E)	6.02	6.40	7.50	12.44	10.26	11.91

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

4.5.2.2 Plant Dry weight

Plant genotype main effects were significantly different over the growing season in both years (Table 4.6) and a significant environment effect was noted in early development in 2010 and in both early and late development in 2011; likely attributable to N differences between trials in 2011with higher N producing greater dry weights. However, there was no significant inoculant effect, inoculant x genotype or genotype x environment x inoculant interaction effect detected for plant dry weight at any growth stage in 2010 and only at 133 DAS in 2011. At some growth stages, particularly flowering, Sp7 produced positive increases in above ground dry matter accumulation. However, the responses were inconsistent and non-significant. Although non-significant, some genotypes responded positively to inoculation including genotype 4 (133DAS in 2011) and genotypes 1 and 3 in 2010, while others showed a reduction in dry weight with inoculation (genotypes 4 and 5).

Table 4.6 Wald statistics and their significance from the analysis of plant dry weights in 2010 and 2011.

	Season 1 (2010)			**Season 2 (2011)**		
Fixed term	43 DAS	103 DAS	133DAS	43 DAS	103 DAS	133DAS
Genotype (G)	31.14***	28.87***	23.97***	48.71***	1.00	24.65***
Inoculant (I)	0.90	6.50	0.69	0.67	3.61	0.23
E (2010); N (2011)	6.61**	2.79	0.01	17.64***	0.15	18.86***
G x I	15.65	5.82	13.17	4.82	16.88	19.04
G x N (E)	1.85	3.67	6.50	8.81	2.46	1.98
I x N (E)	1.79	2.73	2.21	0.44	0.21	9.06*
G x I x N (E)	5.00	5.13	9.56	3.38	9.51	4.74

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively1 all these values are mean square

4.5.2.3 Relative chlorophyll

A portable chlorophyll meter (SPAD 502) was used to estimate chlorophyll concentration and nitrogen status of the leaves. The SPAD 502 has been used by farmers for early detection of fertilizer needs (Argenta et al. 2004). A highly significant positive effect of plant genotype on relative chlorophyll was observed at each time of sampling in both seasons with the exception of 43 DAS in 2011 (Table 5.7). No inoculant effect was observed at any growth stage in any year although a significant inoculant x genotype interaction was detected at flowering in 2011 and an inoculant x genotype x environment effect at flowering in 2010. Although there was a non-significant positive response to inoculation observed in early growth in 2010, this diminished with time. In 2011 during early growth a positive response to inoculation was observed in genotypes 2 and 4 and this was maintained until 133 DAS in genotype 4. However, other genotypes produced non-significant but negative responses to inoculum.

Table 4.7 Wald statistics and their significance from analysis of relative chlorophyll in 2010 and 2011.

	Season 1(2010)			Season 2(2011)		
Fixed term	43 DAS	103 DAS	133DAS	43 DAS	103 DAS	133DAS
Genotype (G)	13.97*	306.17***	10.34*	7.17	37.60***	28.10***
Inoculant (I)	2.50	8.01	0.17	0.73	1.03	1.97
E (2010); N (2011)	0.53	0.19	0.94	2.47	1.46	0.12
G x I	4.90	6.19	4.55	9.94	24.58*	17.72
G x N (E)	0.39	0.75	7.76	0.53	3.41	2.52
I x N (E)	0.62	4.72	2.21	1.80	1.67	0.97
G x I x N (E)	12.02	20.16*	7.89	9.62	8.53	11.72

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

4.5.2.4 NDVI Index

Normalised difference vegetation index (NDVI), was measured at flowering in both years using a Green Seeker, Variation Rate Application System, 010-3-106. A highly significant difference among genotypes was found in 2011 but not 2010 (Table 4.8). No significant interaction effects were observed in any season.

Table 4.8 Wald statistics and their significance from analysis of NDVI index taken at 103 DAS in 2010 and 2011

Fixed term	2010	2011
Genotype (G)	3.94	88.81***
Inoculant (I)	6.83	3.19
E (2010); N (2011)	0.57	11.78
G x I	1.90	7.70
G x N (E)	2.07	0.69
I x N (E)	1.20	6.93
G x I x N (E)	4.38	8.69

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respecti

4.5.2.5 Tiller number/plant

Tiller number per plant was measured before harvest and a significant positive genotype effect was observed in 2010 but not 2011(Table 4.9). A significant environment effect was observed in the 2010 season only and no significant interaction effects were found in either season. Although non-significant, genotypes in both years tended to produce positive responses to

inoculation.

Table 4.9 Wald statistics and their significance from analysis of tiller number/plant in 2010 and 2011.

Fixed term	2010	2011
Genotype (G)	28.16***	7.03
Inoculant (I)	2.39	3.18
E (2010); N (2011)	6.49**	1.01
G x I	4.14	14.15
G x N (E)	1.75	2.03
I x N (E)	1.61	7.12
G x I x N (E)	5.15	5.10

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

4.5.2.6 Grain yield and thousand kernel weight, 2010

Genotype effects for grain yield were highly significant in 2010. While no inoculant or environment effect nor any interaction effects were observed (Table 4.10), most genotypes showed a positive or neutral response to inoculation. The most promising being the synthetic genotype 3. Only one line, genotype 2, showed a negative response to inoculation.

Thousand kernel weight (TKW) effects were significant for genotype, inoculant and genotype x inoculant interaction (Table 4.10). However, no significant environment effect was observed

for TKW. Genotypes 3 and 4 produced bigger seed than the uninoculated control, while other genotypes showed very little or no response. However, no genotype showed a negative response to inoculation.

Table 4.10 Wald statistics and their significance from analysis of grain yield and thousand kernel weight (TKW) in 2010.

Fixed term	Grain Yield kg/ha	TKW
Genotype (G)	22.43***	252.96***
Inoculant (I)	2.67	10.81*
Environments (E)	0.28	0.08
G x I	12.31	44.60***
G x E	2.69	4.82
I x E	0.65	0.15
G x I x E	4.82	3.22

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

4.5.2.7 Grain yield and thousand kernel weight, 2011

Significant genotype, N and genotype x N effects were observed for grain yield. Although inoculant effects were non-significant in 2011, only genotype 3 showed a negative response to inoculation (Table 4.11).

For TKW, only genotype main effects and inoculant x N interaction were significant and unlike

2010, no significant genotype x inoculant interaction was observed. The genotype response to inoculation is also inconsistent, as genotypes 1 and 2 produced smaller seed and genotype 3, 4, and 5 larger seed in response to inoculation. Only the three-way interaction; genotype x inoculant x N was significant for shoot biomass and the genotype main effect for harvest index. In biomass production at maturity the trend of the responses was positive, however harvest index tended to reduce with inoculation. No significant genotype x inoculant interaction was observed in 2011 for any character.

Table 4.11 Wald statistics and their significance from analysis of yield attributes in 2011

Fixed term	Grain yield	TKW[±]	Biomass	HI[#]
Genotypes (G)	393.30***	30.12***	5.95	52.06***
Inoculants(I)	2.83	4.38	4.65	3.68
N rate[±](N)	20.05***	0.22	1.74	0.20
G x I	8.74	9.36	8.60	5.75
G x N	16.80**	7.16	3.35	4.09
I x.N	6.07	7.97*	0.48	0.77
G x I.x N	18.48	11.80	24.79*	15.25

[#]Harvest Index

[±]Thousand kernel weight

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

Table 4.12 Mean grain yield (t/ha) of five wheat genotypes with different bacterial treatments in 2010 and 2011

	2010			2011			
Genotypes	Control	Sp7	Sp7-S	Control	Sp245	Sp7	Sp7-S
1	5.014aA	4.900bcA	5.083bA	4.071bA	4.066 bA	3.912 aB	4.038bA
2	5.184aA	5.055bA	5.085bA	3.743c A	3.852c A	3.757 aB	3.844 cA
3	5.111aB	5.332aB	5.799aA	4.591aA	4.558aA	4.548 aA	4.592 aA
4	5.013aB	4.967bA	5.142bA	4.175 bA	4.160 bA	4.072 aB	4.023bA
5	4.911baA	5.034bA	4.919bcA	3.528dA	3.688 dA	3.594 dA	3.719 cA

*Means in the same column with the same lowercase letter are not significantly different at P<0.05

Means in the same row and year followed by the same uppercase letter are not significantly different at P<0.05

Highly significant (P <.001) genotype responses were detected in both years; however only genotype 3 responded significantly to inoculation in 2010 and was observed to be 4.3% and 13.5% higher yielding than the control in the presence of *A.brasilense* Sp7 and Sp7-S respectively (Table 4.12). In the second year none of the genotype inoculation combinations produced a significantly higher yield than the controls. However, two of five genotypes responded to the bacterial inoculum treatment. Genotype two produced a 2.9%, 4% and 2.7% yield increase compared to the control when inoculated with *A.brasilense* sp245, Sp7 and Sp7-S, respectively. Similarly, genotype five was 4.5%, 1.9% and 5.4% higher yielding than the control when inoculated with *A.brasilense* Sp245.Sp7 and Sp7-S, respectively.

4.5.3 Relationships among the variables measured under different inoculation treatments.

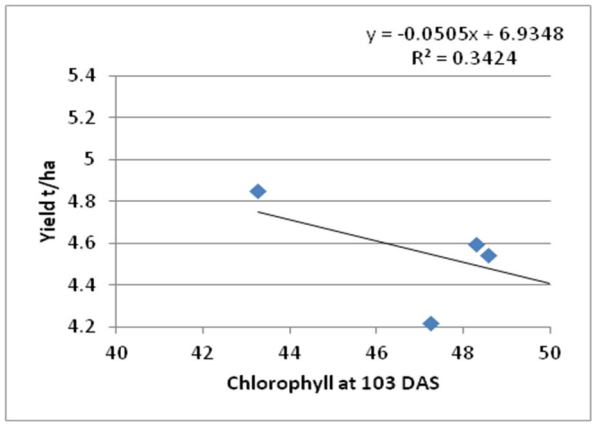
Based on significant correlations among variables (see Appendix Table 8) a series of relationships are presented in this section that relate physiological and/or morphological measures to productivity traits such as grain yield and dry matter accumulation.

4.5.3.1 Relationship of grain yield to relative chlorophyll at 103 DAS

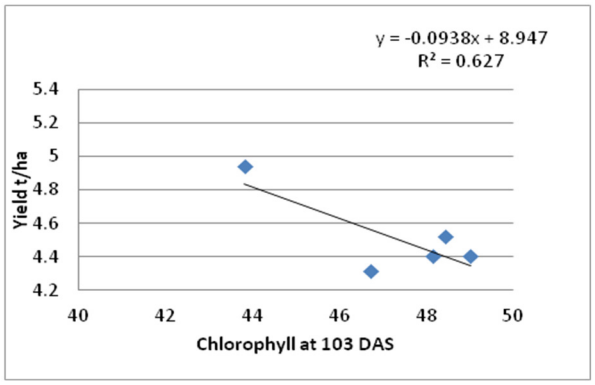
Figures were based on the combined analysis of years 2010 and 2011. Figure 4.4 shows a negative relationship between grain yield and chlorophyll content for the control and each bacterial inoculant. The results could be artifactual due to the small number of genotypes tested; however the negative trend is consistent across all three treatments and skewed by the performance of the synthetic derivative Sokoll, which produced high yield with relatively low

leaf chlorophyll content.

4.4.a: Control



4.4.b: Sp7



4.4.c:Sp7-S

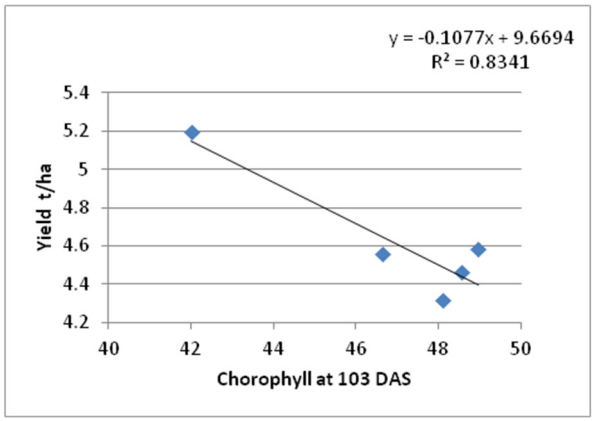
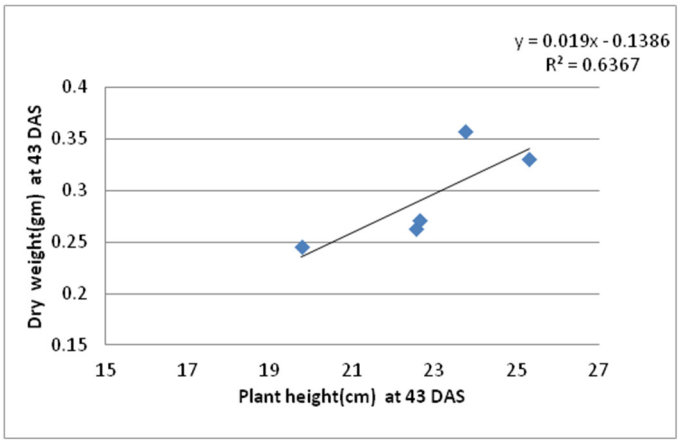


Figure 4.4 Comparisons of chlorophyll content and grain yield under different bacterial treatments: 4.4a, 4.4b and 4.4c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

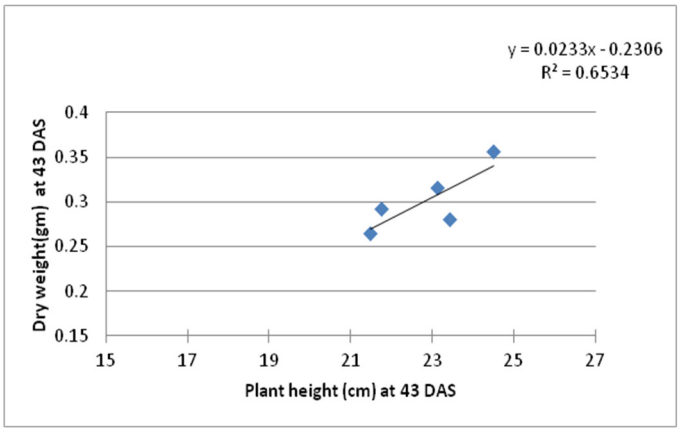
4.5.3.2 Relationship of plant height and dry weight at 43 DAS in the combined analysis across 2010 and 2011.

Figure 4.5 demonstrates a relatively strong and positive relationship between plant height and early season dry matter accumulation. Clearly, the taller plants tend to produce more early-season dry matter than shorter materials. The result could be influenced by inherent yield or growth rate differences between the shorter and taller materials in the small group under study. However, the trend is significant across bacterial treatments.

4.5.a: Control



4.5.b: Sp7



4.5.c:Sp7-S

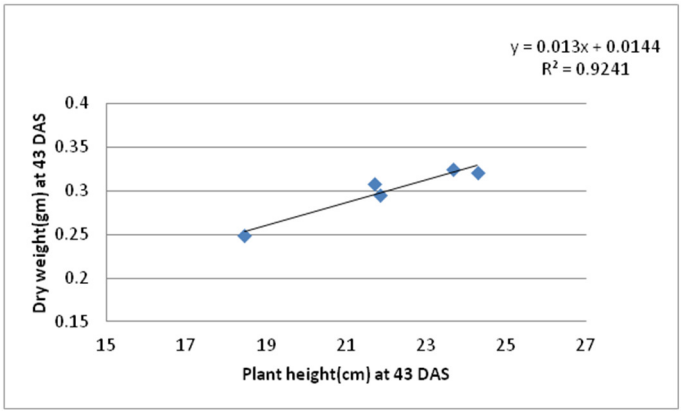
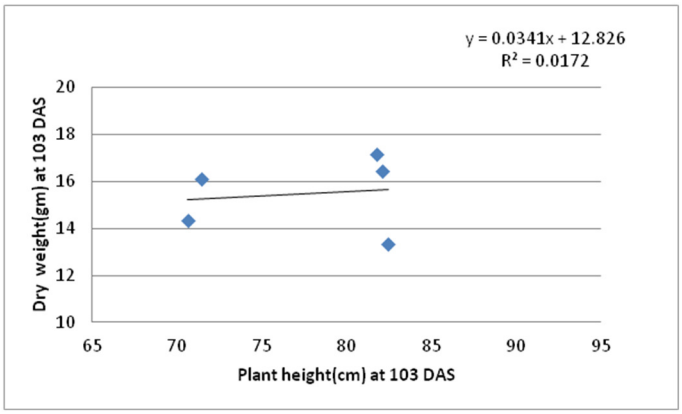


Figure 4.5 Comparisons of plant height and dry weight at 43 DAS under different bacterial treatments: 4.5.a, 4.5.b and 4.5.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

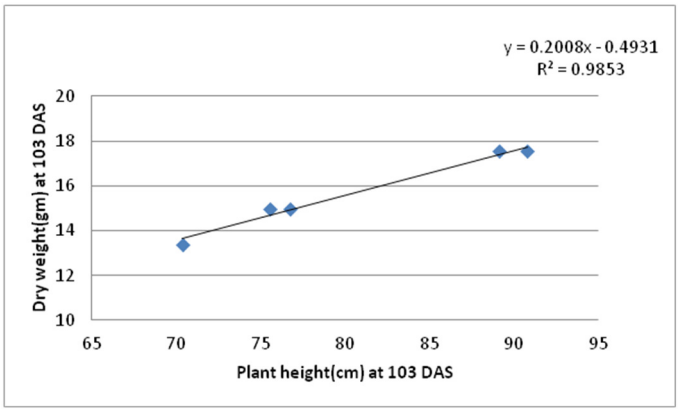
4.5.3.3 Relationship of plant height and dry weight at 103 DAS in combined analysis over years (2010 and 2011)

A similar trend to that observed between plant height and dry matter accumulation early in the season was observed at 103 DAS (Figure 4.6). However, no correlation was observed in the control treatment. Both bacterial treatments demonstrated higher dry weight in taller plants at or close to anthesis.

4.6.a: Control



4.6.b: Sp7



4.6.c:Sp7-S

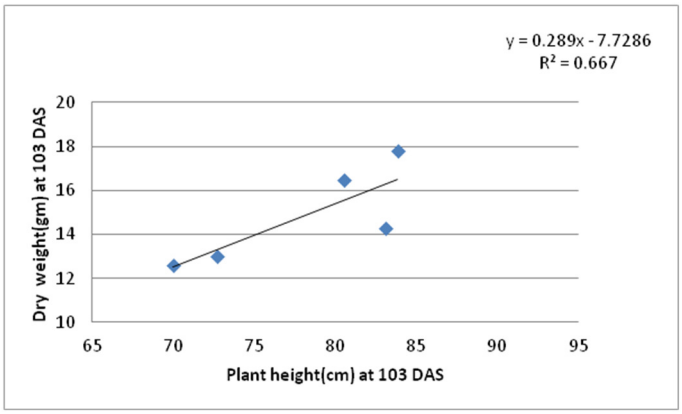
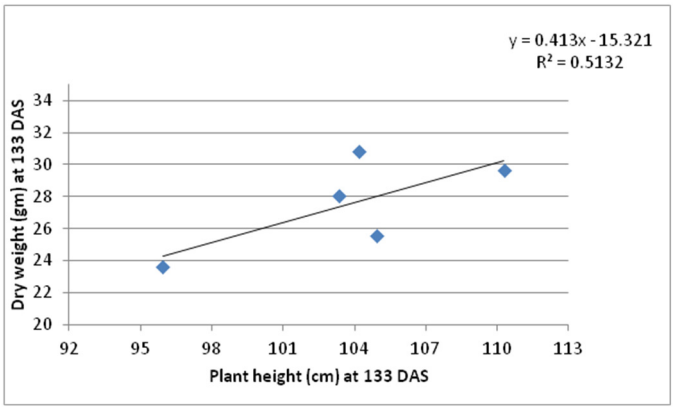


Figure 4.6: Comparisons of plant height and dry weight at 103 DAS under different bacterial treatments: 4.6.a, 4.6.b and 4.6.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

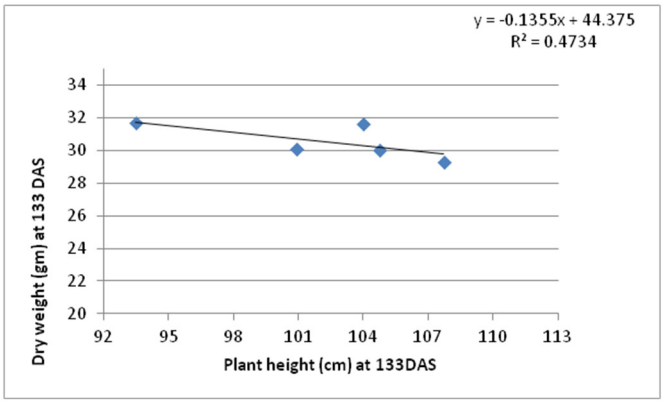
4.5.3.4 Relationship of plant height and dry weight at 133 DAS in combined analysis over years (2010 and 2011)

The relationship between plant height and late season dry matter is illustrated in Figure 4.7. The relationship was considerably weaker than that observed earlier in the cropping season. Clearly any effect of inoculation observed early in the season has dissipated by 133 DAS.

4.7.a: Control



4.7.b: Sp7



4.7:Sp7-S

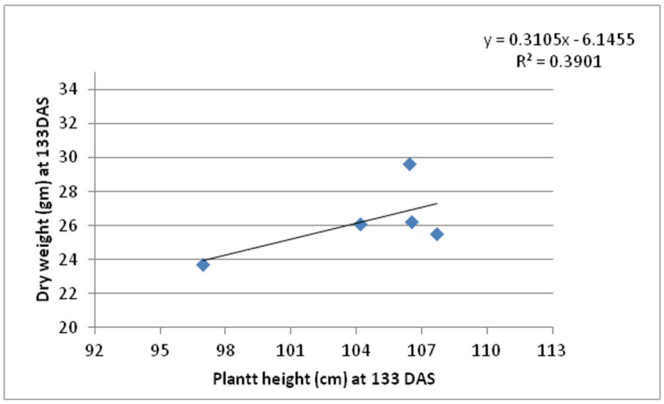


Figure 4.7: Comparisons of plant height and dry weight at 133 DAS under different bacterial treatments: 4.7.a, 4.7.b and 4.7.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

4.5.3.5 Relationship of NDVI with yield in combined analysis (2010 and 2011)

There was no discernible relationship between NDVI assessed at anthesis and grain yield in all bacterial treatments. This was consistent across both years including 2011 where a positive significant genotype effect was observed (see Table 4.9).

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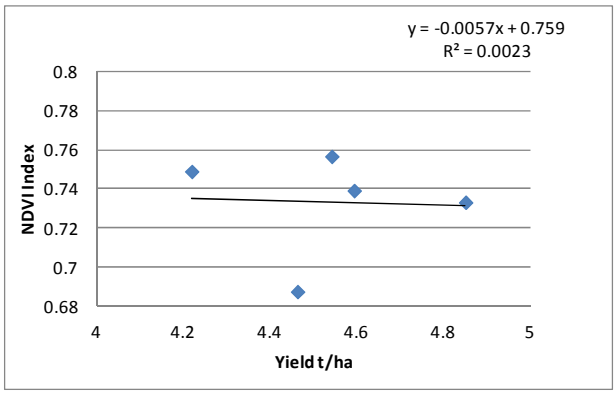
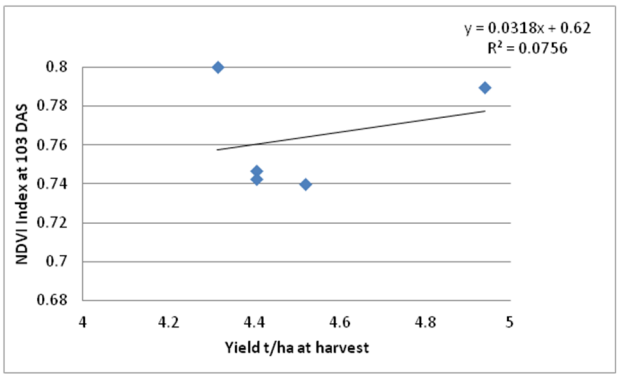
4.8.a: Control



4.8.b: Sp7



4.8.c:Sp7-S

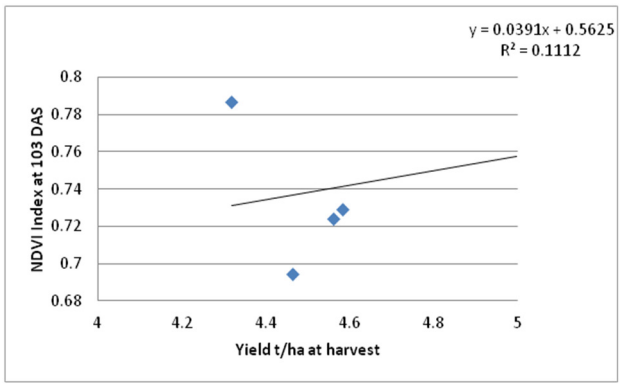
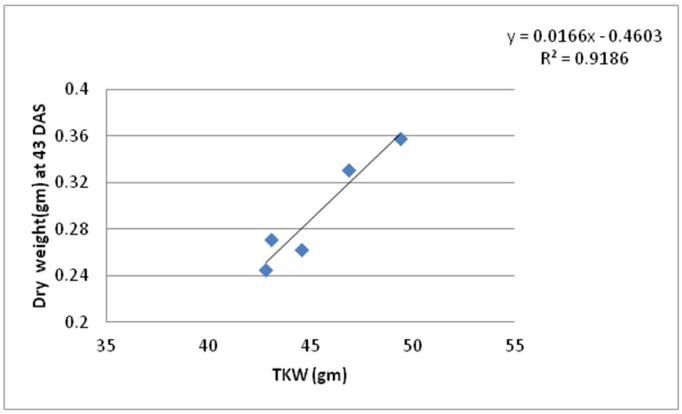


Figure 4.8 Comparison of NDVI Index at 103 DAS and yield under different bacterial treatments: 4.8.a, 4.8.b and 4.8.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

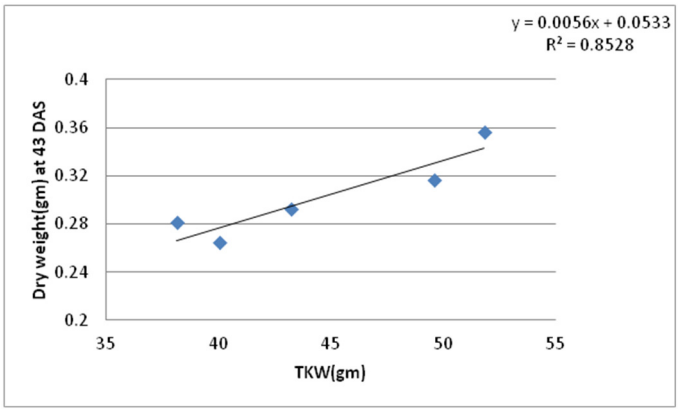
4.5.3.6 Relationship of thousand kernel weight (TKW) with plant dry weight at 43 DAS combined across years.

Results demonstrate a strong and positive relationship between early dry matter accumulation and TKW in the control and both bacterial treatments (Figure 4.9). This observation was consistent across bacterial treatments.

4.9.a: Control



4.9.b: Sp7



4.9.c:Sp7-S

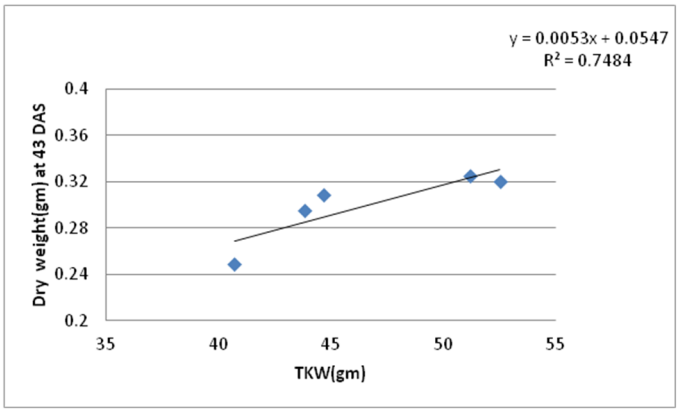
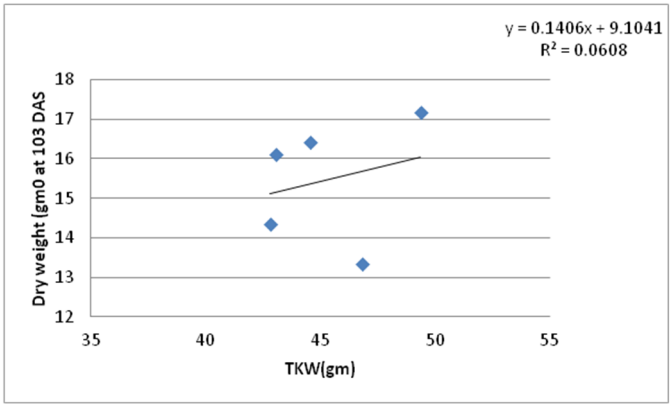


Figure 4.9 Comparisons of TKW and plant dry weight at 43 DAS under different bacterial treatments: 4.9.a, 4.9.b and 4.9.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

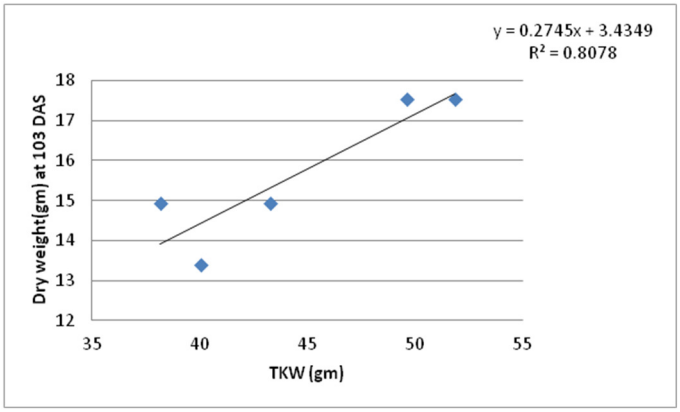
4.5.3.7 Relationship of TKW with plant dry weight at 103 DAS in combined analysis

There was a weaker relationship between TKW and dry matter accumulation close to or at anthesis than observed at 43 DAS (Figure 4.10). The control treatment showed no association and while Sp7 was significant, the relationship in the presence of Sp7-S was weaker and non-significant.

3.10.a: Control



3.10.b: Sp7



3.10.c:Sp7-S

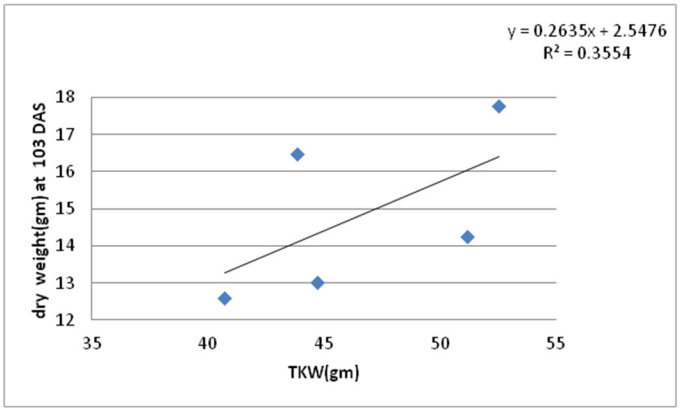
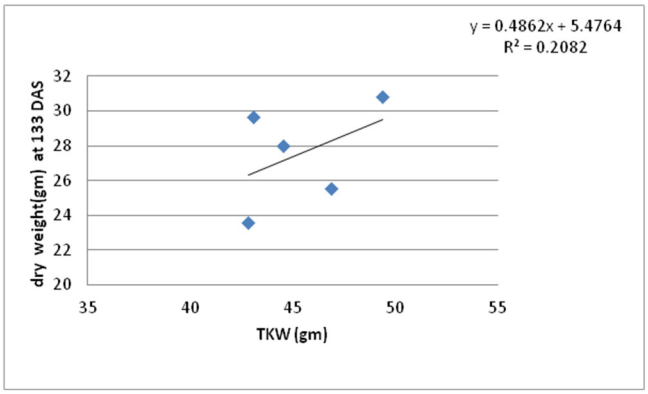


Figure 4.10: Comparisons of TKW and plant dry weight at 103 DAS under different bacterial treatments: 4.10.a, 4.10.b and 4.10.c are control, Sp7 and Sp7-S treatments respectively. P values for each comparison are listed in the Appendix.

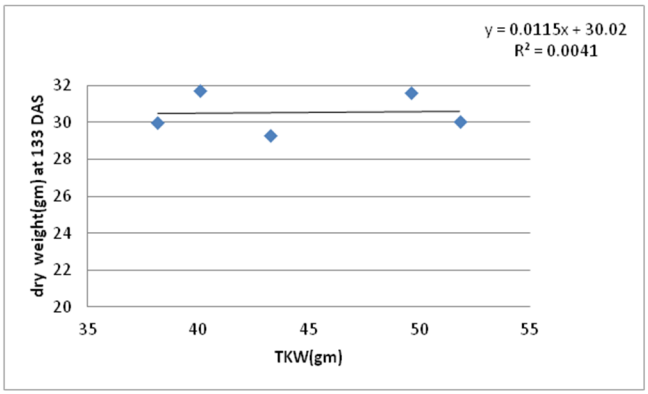
4.5.3.8 Relationship of TKW with dry weight at 133 DAS combined over years.

Figure 4.11 demonstrates a significant relationship between TKW and plant dry weight in Sp7-S treated plants compared to the control and Sp7 treatments.

4.11.a: Control



4.11.b: Sp7



4.11.c:Sp7-S

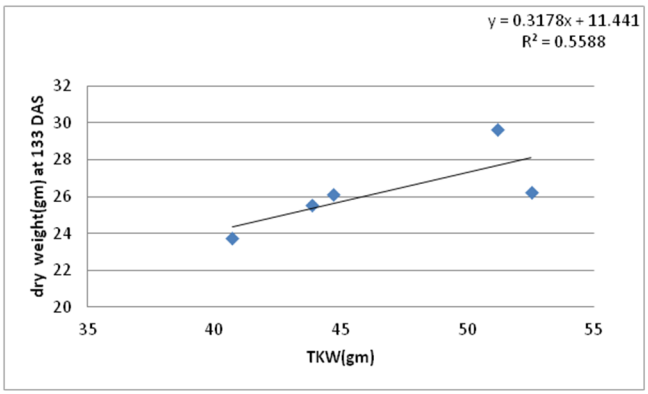


Figure 4.11: Comparisons of TKW and plant dry weight at 133 DAS under different bacterial treatments: 4.11.a, 4.11.b and 4.11.c are control, Sp7 and Sp7-S treatments, respectively. P values for each comparison are listed in the Appendix.

4.6 Discussion

4.6.1 Effect of bacterial inoculation on root development

Different in season and rainfall patterns in 2010 and 2011 led to differences in genotype responses to inoculation. The lack of significance among genotypes for root characters assessed in 2010 is likely a result of the well-watered conditions experienced throughout the cropping cycle (Figure 4.1), whereas, in 2011 differences among genotypes were observed for root volume and average root diameter. This season was relatively dry pre-flowering, after which no stress was observed due to high and frequent rainfall (Figure 4.1). The early stress likely helped differentiate genotypes as those with more vigorous roots accessed water deeper in the profile, thereby increasing root volume and average diameter. However, the significant effect of inoculant observed in both years for total root length, surface area and volume compared to the uninoculated control indicates that the bacterial strains used have altered root growth. This finding is similar to that observed by Dobbelaere et al. (1999) and Kapulnik et al. (1985) using a different strain of *A. brasilense*. *Azospirillum* species produce plant growth promoting substances in culture, mainly auxins (indole-3-acetic acid, IAA) (Lambrecht et al. 2000), but also (in smaller amounts) cytokinins and gibberellins (GA_3) (Bottini et al. 1989). These hormones play a major role in plant root development (Dobbelaere et al. 1999) and are most probably the cause of differentiation among genotypes observed in the current study.

Quite clearly, inoculation does influence root growth although the genotype x inoculation effects were somewhat inconclusive. Only root volume was affected in 2010 and two different traits, total root length and root surface area, in 2011. Nevertheless, there does appear to be an influence of genotype on the effectiveness of inoculation. We believe this is the first evidence reported of such an interaction with root growth.

However, the significant inoculation effects on total root length, surface area and volume may have been influenced by the sampling procedure. Samples were taken 43 DAS and to avoid contamination among bacterial inoculants, a different person sampled each bacterial whole-plot treatment. This may have influenced some of the observed variation among bacterial treatments.

4.6.2 Effect of bacterial inoculation on above ground plant growth and development.

4.6.2.1 Plant Height

While genotypes differed for plant height there was very little effect of environment and inoculation on plant height expression. The lack of significant genotype x inoculation interaction in either year indicates that plant height is not responsive to these bacterial strains. These findings contrast with those of Dobbelaere et al. (2001) who reported an 18% increase in plant height following inoculation with *A.irakense*. However, the genotypes evaluated in our study were semi-dwarf in stature and carried the GA-insensitive dwarfing genes Rht1 or Rht2; these materials may be less responsive than the largely non-semi dwarf materials evaluated in

the Dobbelaere study.

4.6.2.2 Dry Weight

While wheat genotypes differed for dry weight, the lack of an inoculation effect and no significant genotype x inoculation interaction indicates that the *Azospirillum* strains used do not influence biomass accumulation at any stage of plant development. These findings contrast with those of Naiman et al. (2009) who observed a 12% increase in the aerial biomass of the Argentinian cultivar Buck Sureño following inoculation with *A.brasilense* and *Pseudomonas fluorescens* Pf. However, the genetic materials assessed in the Naiman et al. (2009) study were different and the inoculation method also differed; they used a single inoculation of seeds with a high number of bacteria (10^9 cfu/ml).

4.6.2.3 Relative chlorophyll

The wheat genotypes differed significantly for chlorophyll content; however the bacterial treatments did not influence the expression of chlorophyll nor was a consistent genotype x inoculation interaction observed. The lack of a main effect response to inoculation in the current study contrasts with the findings of Bashan et al. (2005) who observed an increased quantity of photosynthetic pigment in wheat seedlings following inoculation with *A.brasilense* Cd as liquid or alginate microbeads.

4.6.2.4 NDVI Index

One of the goals in agriculture is to maximize yield production at minimum cost. Early

detection of crop yield and those factors that limit yield can help increase food production while minimizing environmental impacts. Lobell et al. (2005) used early detection of yield limiting factors to adjust crop management to significantly improve wheat production in the Yaqui valley in Mexico. The Normalized Difference Vegetation Index (NDVI) is one of the tools commonly used for assessing the health, vigour and N requirements of plants. Lofton et al. (2012) successfully used NDVI to assess the adaptability of sugarcane under stress in USA.

There was a strong genotypic effect observed in 2011 indicating that the genotypes in the current study differed genetically for NDVI response. However, as with dry matter and chlorophyll content, the lack of an inoculation effect and interaction with genotype indicates no measurable effect of inoculation. When NDVI index and grain yield were regressed no relationship was observed; this contrasts with the observations of Marti et al. (2007). However, while there was no significant effect of inoculum, the NVDI values were always higher in inoculated plots than the uninoculated control. NDVI was assessed at anthesis in both years and this may not have been the most discriminatory stage of development. NDVI assessed in early growth, when the inoculum effects are potentially at their strongest, may have produced significant results.

4.6.2.5 Grain yield and yield attributes

Improving grain yield through the application of bacterial inoculants is the primary economic benefit targeted by this research. Yield is strongly influenced by the environment and most multi-environment studies report significant year, environment and genotype x environment effects. Ozturk et al. (2003) described the importance of limiting N in detecting inoculation

effects in a study of wheat and barley inoculated with *Azospirillum* and *Bacillus*. The experimental site at Narrabri was relatively high in N and although an N response was noted for some characters (in 2011), the lower limit may not have been sufficient to detect the advantage of inoculation.

Garcia de Salomone and Dobereiner (1996) reported a strong and consistent plant genotype x Azospirillum spp strain interaction in northern Argentina in maize. Similarly, Remans et al. (2008) reported a genotype × inoculum interaction for yield in beans. They conducted a genetic analysis of this response and reported the presence of Quantitative Trait Loci linked to *Azospirillum* responsiveness in beans. However, little genotype x inoculation interaction was observed for most traits in the current study and no effect noted for grain yield.

In the current experiment results differed by year. While the genotypes differed in yield potential; demonstrated by significant genotype effects; the effect of inoculants and genotype x inoculation interaction varied. As discussed earlier, the seasons varied in the distribution of rainfall. However, there was no water stress in either year during the anthesis and grain-filling periods. The longer than usual grain-filling period, attributable to the non-water limiting conditions and milder temperatures, most likely diluted any effect of increased nutrient availability linked to inoculation. In contrast, Bashan et al. (1989) analyzed 56 different field experiments and reported positive responses to bacterial inoculation in many crops including tomato, eggplant, pepper and cotton. They reported yield increases as high as 30%, although some responses were negligible. In an extensive study in wheat, Diaz-Zorita and Fernandez-

Canigia (2009) analyzed 297 experiments inoculated with liquid *A.brasilense* and reported an increase in grain number and yield of 6.1 and 8%, respectively. In the current study, yield increases ranged from 0.4 to 13.5% in inoculated plants. Although a significant N effect was observed in 2011; the year when an N treatment was imposed, the lower N treatment did not promote an inoculum effect. We know that the *Azospirillum* bacteria were present in these treatments throughout the cropping cycle (see Chapter 5) and can only conclude that N was not sufficiently limiting in the low N treatment to elicit a response to inoculum. However, the highly significant genotype x inoculant effect for TKW observed in 2011 indicates that components of yield are influenced by inoculum and that genotypes do respond differently depending on the environmental conditions. This result is consistent with the conclusions of a Meta- analysis conducted by Veresoglou and Menexes (2010) spanning 1981 - 2008. They reported that the key determinants for a plant growth promoting effect are the amount of the N fertilization, wheat cultivar and the *Azospirillum* isolates used.

4.6.3 The relationship among the traits assessed and the influence of inoculation

4.6.3.1 The relationship between physiological and morphological traits and productivity.

The strong positive relationship observed between TKW and early dry matter accumulation is likely a function of larger embryos and therefore more vigorous early growth in larger seeded genotypes. Richards et al. (2002) observed that bigger embryos tended to produce better early vigour and more rapid rates of plant growth thus supporting these observations. The fact that the relationship between TKW and dry matter reduced as the season progressed suggests that the advantage of early vigour is lost with time. The lack of relationship between NDVI index

and grain yield in the current study indicates that NDVI cannot be used as an indirect selection criterion for grain yield, although this conclusion must be viewed in the context of the genotypes, seasons and treatments used. Others reported that NDVI can be used to predict yield (González et al. 2002; Panda et al. 2010). Okada et al. (2008) reported that NDVI assessed in wheat at jointing correlated well with eventual grain yield in the Kanto region of Japan.

The positive relationship between plant height at different stages of development and dry matter, particularly in inoculated plots, indicates that *Azospirillum* inoculation is influencing early growth. However, as the season progressed, the rainfall in both years increased and the growing season lengthen thus reducing this effect.

4.7 Conclusion

While inoculation effects were observed for some traits, particularly those assessed early in the growth cycle, these diminished with time and no effect on final biomass and grain yield was observed. Those traits most influenced by inoculation were the root traits; total root length, surface area and volume, with some effect observed on early season plant height and TKW. However, as noted in the materials and methods, the significant root traits may have included sampling error as a different person sampled roots for assessment from each bacterial treatment to avoid cross-contamination. However, the genotypes selected for study varied significantly for most of the traits assessed and the strongest genotype x inoculation interactions were noted for root characteristics; total root length, root surface area and root volume. Chlorophyll and TKW were also influenced to some degree. However, these positive influences on yield related traits did not translate into higher yield nor a genotype specific response to inoculation. The cool growing season with non-limiting moisture throughout the anthesis and grain-filling

periods in both years extended the plant growth cycle, thus diminishing the early season effects of inoculation. The bacteria did persist throughout the growth period (see Chapter 5) so we can only conclude that growing conditions were too favorable to elicit and economic response to inoculation.

Chapter 5: Colonisation and persistence of inoculant bacteria in the rhizosphere pre and post-anthesis.

5.1 Introduction

Since the1970's *Azospirillum* been isolated and used to inoculate plants. It is best characterized as a free living nitrogen fixing genus among plant growth promoting rhizobacteria (Steenhoudt and Vanderleyden 2000; Dobereiner and Day 1976). Over the past 40 years, results from field inoculation with *Azospirillum* indicate that these bacteria are capable of promoting the growth and yield of agronomically important crops in different soil and climatic conditions around the world (Okon 1985; Okon and Labandera-Gonzalez 1994). Inoculation does not replace fertiliser but instead augments the availability of nutrient to crops (Okon and Labandera-Gonzalez 1994). The success of inoculation depends on the optimization of cell number and maintenance of cell viability in the root zone, thus ensuring root colonization. It has been acknowledged that root colonization by plant-growth promoting bacteria is the first step towards positive plant responses (Kloepper and Beauchamp 1992). It is therefore important to assess bacterial numbers throughout the plant growth cycle when determining the effectiveness of inoculants.

The significant enhancement of plant growth has been demonstrated by application of PGPR in both laboratory and green house trials (Almaghrabi et al. 2013; Ferreira et al. 2013).

However, the results from field applications are not always consistent (Bowen and Rovira 1999; Diaz-Zorita and Fernandez-Canigia 2009). This inconsistency could be due to the failure of inoculant microorganisms to survive in competition with indigenous microorganisms in the soil and biotic and abiotic stresses may enhance this effect (Nelson 2004). These factors likely explain the lack of plant growth effects in the field following PGPR inoculation in many studies. However, the development of better microbial inoculant formulations and improved crop management could enhance the survival and activity of inoculants in the rhizosphere (Bashan 1998; Bowen and Rovira 1999; Yardin et al. 2000; Gardener and Fravel 2002). Understanding the colonisation ability of the PGPR, their mode of action and optimized formulation and application could increase the reliability of PGPR thus improving the sustainability of agricultural systems (Nelson 2004).

Monitoring plant growth promoting bacterial populations in the rhizosphere and their colonization and persistence after application is difficult because of the complexity of the indigenous bacterial community. Enumeration methods need to be not only specific to a particular species or strain but also determine viability. Culture dependent methods are ideal for determining viability but no single medium is suitable for isolating individual microbial strains from a complex community (Deaker et al. 2008). In addition, the method should be rapid, inexpensive, easily applied and provide consistent results under diverse conditions (Sutton 2010). An improved technique for isolation and enumeration of *Azospirilla brasilense* was developed based on nitrogen free semisolid media using a counting method termed the most probable number (MPN) (Bashan and Levanony 1985).

There are many different techniques or methods that can be used for enumeration and all have limitations and advantages. In the current study, a multiple tube fermentation method was used to estimate the MPN of viable azospirilla in the rhizosphere to gain some insight into colonisation and persistence from sowing to grain filling. This method is simple, rapid, inexpensive and relatively easy to use (Colwell 1979; Sutton 2010). In the current study, *Azospirillum* spp. were selected for field application because of their demonstrated ability to promote plant growth in previous studies. They are microaerophillic diazotrophs and thus able to grow in N-free semi-solid agar to the exclusion of non-nitrogen fixing bacteria. This multipletube fermentation technique is based on the presence of the viable cell in the diluted sample. Accuracy increases with the number of tubes inoculated at each dilution, or by reducing the base of the dilution ratio (Gonzalez 1996).

The multiple tube fermentation method is widely used in microbial ecology and sanitary bacteriology to estimate the bacterial numbers in milk, water and food (Townsend et al. 1998; Goodridge et al. 2003; Seo KH 2006: Wohlsen et al. 2006). This technique has been used to measure the MPN of hydrocarbon degraders (Johnson and Henriksen 2009), nitrifiers (Feray et al. 1999), iron-reducers and iron-oxidisers (Nielsen et al. 2002; Weiss et al. 2003), arsenic reducing bacteria (Kuai et al. 2001) and methanotrophs (Escoffier et al. 1997). The multiple tube method is frequently used to determine the MPN of nitrogen fixing rhizobia (Brockwell 1963) using legume root infection as an indicator of rhizobial identity and viability. Growth of *Azospirilllum* in N free semi-solid agar as a rising pellicle is a useful characteristic to apply in a multiple tube fermentation technique. Using this approach, *Azospirillum* and other diazotrophs were enumerated from the roots and rhizosphere of oats and taro (Soares et al.

2006; Jolly et al. 2010). However, this technique does not differentiate between inoculant strains of azospirilla and other microaerophillic diazotrophs.

The soil microbial community is complex and dynamic and shows differences in composition which is a real challenge in soil ecology and the plant-soil-biota interaction is not well understood (Trabelsi and Mhamdi 2013). Another approach to determine whether the inoculant strain has colonised and persists in the rhizosphere is to measure its effect on the microbial community. In this study, T-RFLP (Terminal-Restriction Fragment Length Polymorphism) was used to identify and observe the effect of the introduced bacteria on the rhizospheric community.

T-RFLP is a PCR based DNA fingerprinting technique which can be used for rapid analysis of the diversity of complex bacterial communities in different environments (Liu et al. 1997; Osborne et al. 2000) and has been widely adopted for soil microbial studies. In this technique one of the primer pairs (one forward and one reverse primer) is labeled with fluorescent dye at the 5′end and is used to amplify a selected region of the bacterial gene of interest from the total DNA. The PCR product is then digested with a restriction enzyme (endoneuclease) and the fluorescently labeled Terminal Restriction Fragments (T-RFs) are precisely measured or separated using an automated DNA sequencer (commonly Beckman CEQ™, ABI PRISM®, or MegaBACE™). The number and peak heights of the T-RFs patterns can be analyzed to evaluate the microbial diversity in a community or environment. The PCR based T-RFLP technique is suitable for monitoring highly diverse soil microbial communities over time

(Lukow et al. 2000). Many researchers agree that community analysis by T-RFLP is highly reproducible, robust and sensitive enough to investigate microbial communities and that the choice of restriction enzymes should be considered carefully for effective community analysis (Sessitsch et al. 2001; Osborne et al. 2006; Zhang et al. 2008). More recently, the T-RFLP method has been used to analyze communities of bacteria, archaea, fungi, other phylogenetic groups or subgroups, as well as functional genes in different environments as the method is rapid and highly reproducible (Smalla et al. 2007; Sun and Liu 2013).

The aims of this study were to (i) monitor the colonization and persistence of *Azospirillum* in the wheat rhizosphere in the field using the multiple tube fermentation method, and (ii) assess the usefulness of T-RFLP as an alternative method for estimating the colonization and persistence of inoculant bacteria throughout the crop growing season.

5.1 Materials and Methods

Rhizosphere samples were collected periodically from field experiments designed to test the effect of *Azospirillum* inoculation on wheat growth and yield (previously described chapter 4). The most probable number (MPN) of microorganisms able to grow in semi-solid nitrogen-free medium (Nfb) in the rhizosphere over the crop growth cycle was estimated using a multiple tube fermentation method and the effect of inoculation on nitrogen fixing communities in the rhizosphere was determined at selected time points using *nif*H T-RFLP.

5.2.1 Estimating the MPN of Nfb positive microorganisms in the rhizosphere

A multiple tube fermentation technique was used to estimate the MPN of Nfb positive microorganisms in rhizosphere soil during the crop growth cycle. The technique estimates viable cell numbers assuming a poisson distribution and the probability that one living cell will develop a positive response in a tube.

5.2.1.1 Media preparation and procedure for estimation of MPN

Semi-solid NFb (Nitrogen -free broth) media was used to determine the presence of viable azospirilla (Baldani and Dobereiner 1980). Here malic acid was used as the sole carbon source and yeast extract was replaced with vitamin solution (media recipe in appendix A). Semi-solid NFb media (3.5mL) was transferred to acid washed culture tubes by pipette. The tubes were then fitted with lids and autoclaved in racks of up to 72 culture tubes to sterilize the medium. Filter sterilized vitamin solution (1.0ml/L) was subsequently added.

5.2.1.2 Sample preparation and MPN dilution series

Rhizosphere samples were weighed and approximately 5 g of rhizosphere soil and roots were transferred into 50 ml sterile falcon tubes with ten sterile glass beads (10 mm) following which sterile water was added to make up 50 ml. The falcon tubes were then vortex for 30 seconds to extract the bacteria from the roots and soil and rested for 2-3 minutes to allow the bacterial and soil particle fractions to separate. The soil suspension was serially diluted ten-fold until a dilution of 10^{-8} was reached. Aliquots (0.1 ml) of dilution 10^{-3} to 10^{-8} were transferred to triplicate tubes containing semi-solid Nfb medium. The tubes were then incubated at 30°C for 48 hours. Positive tubes produced a subsurface white pellicle and an increase in pH from the

production of ammonium as a result of nitrogen fixation (Hartmann and Baldani 2006). The number of the positive tubes at each dilution level was recorded. The MPN was determined using MPNes software produced by NifTAL (Woomer et al. 1990).

5.2.1.3 MPN data analysis

The MPN obtained for each experimental plot was analysed by REML for each sampling time using Genstat software version 14. The analyses of the split plot designs were conducted using the REML linear mixed model algorithm. Bacterial treatments were assigned to whole plots and wheat genotypes were randomly allocated as sub-plots within whole plots within each replication. Environments were discreet experiments and were analyzed independently and in combination to examine genotype x year x bacterial treatment x environment interactions. As described in chapter 4, the environments were two different experiments in 2010 and two levels of N in 2011. Subsequent correlations among variables were calculated using the correlation function in Genstat 14th edition.

5.2.2 Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

5.2.2.1 DNA extraction from soil

A MoBio Powersoil® DNA Isolation Kit (MO BIO Laboratories, Inc) was used to extract the DNA from 0.25 g of each soil sample according to the manufacturer's protocols. Purified soil DNA was stored at −20°C and the amounts of extracted DNA were qualitatively evaluated on a 1.0% agarose gel run at 220 V for 20 min and stained with ethidium bromide.

5.2.2.2 Reagents and Mastermix preparation for PCR

PCR was prepared for a 25 µl reaction and mastermix and reagents were prepared as follows. Before making the mastermix the reagents were thawed and mixed well. All the reagents excluding the DNA sample were combined in 1.5 ml sterile tubes according to the number of reactions required. Sufficient mastermix was prepared (including some extra to account for pipetting errors) and positive and negative controls included. The mastermix was prepared with Mango Taq^{TM} DNA polymerase from Bioline, as (5U/µl) , $Mgcl_2$(50mM), BSA (10mg/ml) and 5X PCR buffer used in 25µl reaction tubes along with primers to amplify the *nifH* gene.

5.2.2.3 Primer Selections

A nested PCR approach was used to perform and obtain the PCR product because insufficient product was produced from the first PCR (Table 5.1). In the second PCR reaction, 1 µl of PCR product was used to amplify the *nifH* gene. Two different *nifH* primers, PolF and PolR for first PCR and PolF1 and AQER for the second, were used to analyze the nitrogen fixing community in the rhizosphere (Poly et al. 2001).

Table 5.1 Primers used for T-RFLP

Primer Name	Sequence	Target	Start/end position in gene	Application	Reference
PolF	TGCGAYCCSA ARGCBGACTC	universal' *nifH*	118-137	*nifH* nested PCR (1st)	Poly et al. 2001
PolR	ATSGCCATCAT YTCRCCGGA		476-457	*nifH* nested PCR (1st)	Poly et al. 2001
PolFI	TGCGAICCSAAI GCIGACTC	universal' *nifH*	118-137	*nifH* nested PCR (2nd)	Poly et al. 2001
AQER	GACGATGTAG ATYTCCTG		453-436	*nifH* nested PCR (2nd)	Poly et al. 2001

For T-RFLP analysis of the nitrogen fixing community in the rhizosphere, the second PCR primer, AQER, was labelled at the 5′ end with 5- carboxyfluorescein (FAM). We ran a T-RFLP of the *nifH* genes amplified from pure culture DNA.

5.2.2.4 PCR cycling

Initial denaturation was conducted at 95°C for 3 minutes; denaturation at 95°C for 30 seconds with an annealing temperature of 55°C for 30 seconds; 35 cycle and extension and final extension at 72 °C for 45 seconds and 5:00 minutes. The PCR reaction was conducted in a S1000 Touch™ Thermal Cycler (Bio-Rad). PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide (Sigma), using ChemiDoc™ MP (Bio-Rad). Any extractions that yielded insufficient DNA were re-extracted.

5.2.2.5 Digestion of the PCR product

PCR products (40-55 µL) were purified by ethanol precipitation. A further 15 µL of Milli-Q water was then added to dissolve the precipitate. The resultant purified DNA concentration was 100−200 ng μl^{-1}. The purified PCR product was then digested with 5 U of restriction enzyme *Hha I*, 0.25µl of BSA, 2.5 µL of 10×NEB buffer 4, and 7 µL of Milli-Q water. The bacterial DNA samples were incubated at 37 °C overnight prior to T-RFLP analysis.

5.2.2.6 Analysis of T-RFLP data

T-RFLP data was analysed using the analysis expedite (T-REX): a web based software for the processing and analysis of T-RFLP data (Culman et al. 2009). Noise filtering (determining true peaks from background noise) was performed using the approach developed by Abdo et al. 2006. Briefly, true peaks were identified by an iterative approach and were determined as those peaks whose height area exceeded the standard deviation computed over all peaks (with a standard deviation multiplier of 1.1). T-RFs were aligned using the T-align algorithm (Smith et al. 2005) with a clustering threshold of 0.5 thus allowing more than one peak to be classified as a single T-RF. Data matrices were constructed in T-REX using peak presence/absence and relativised peak height and peak area. Double-centred principle components analysis (AMMI) was performed within T-REX, and non-metric MDS (using the Bray-Curtis measure of dissimilarity) was performed using the PAST software package (Hammer et al. 2001).

5.3 Results

5.3.1 MPN of Nfb positive microorganisms in wheat rhizospheres in 2010

The MPN of Nfb positive microorganisms in the rhizosphere of wheat was estimated using the multiple tube fermentation at method at three different plant growth stages. In 2010, significant differences among genotypes and inoculation treatments were observed in the early growth stages at tillering (43 DAS) and flowering (103 DAS). However, this effect reduced at grain filling (133 DAS). Mean $\log_{10}$ MPN indicated that numbers were always higher in the inoculated plots than the control plots in the early stages of growth and that these effects reduced later in the season (Tables 5.2; 5.3; 5.4). The effect of environment on MPN was highly significant at grain filling (133 DAS) but not earlier in the season. Significant interactions were observed for inoculant x environment at flowering (103 DAS) and genotype x inoculant x environment early in the season at tillering (43 DAS). Genotypes 3 and 5 in particular maintained significantly higher numbers compared to the other genotypes tested in this experiment. At tillering (43 DAS) in environment 1, undetectable or negligible numbers were observed in the uninoculated plots. However, later in the season at the flowering and grain filling stages (103 and 133 DAS) numbers had increased (Figure 5.1). When the means were plotted it was obvious that numbers had increased significantly as the plants grew (Figure 5.1).

Table 5.2 Wald statistics and their significance from analysis of $\log_{10}$ MPN in 2010 at different sampling times

Fixed term	n.d.f.	43 DAS	103 DAS	133 DAS
Genotype (G)	4	81.23***	24.73***	3.62
Inoculant (I)	2	24.65**	49.51**	1.62
Environment (E)	1	0.00	3.05	15.58***
G x I	8	12.03	11.93	9.94
G x E	4	9.54	14.15	8.73
I x E	2	4.82	1.40**	1.35
G x I x E	8	21.89 **	14.56	1.15

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

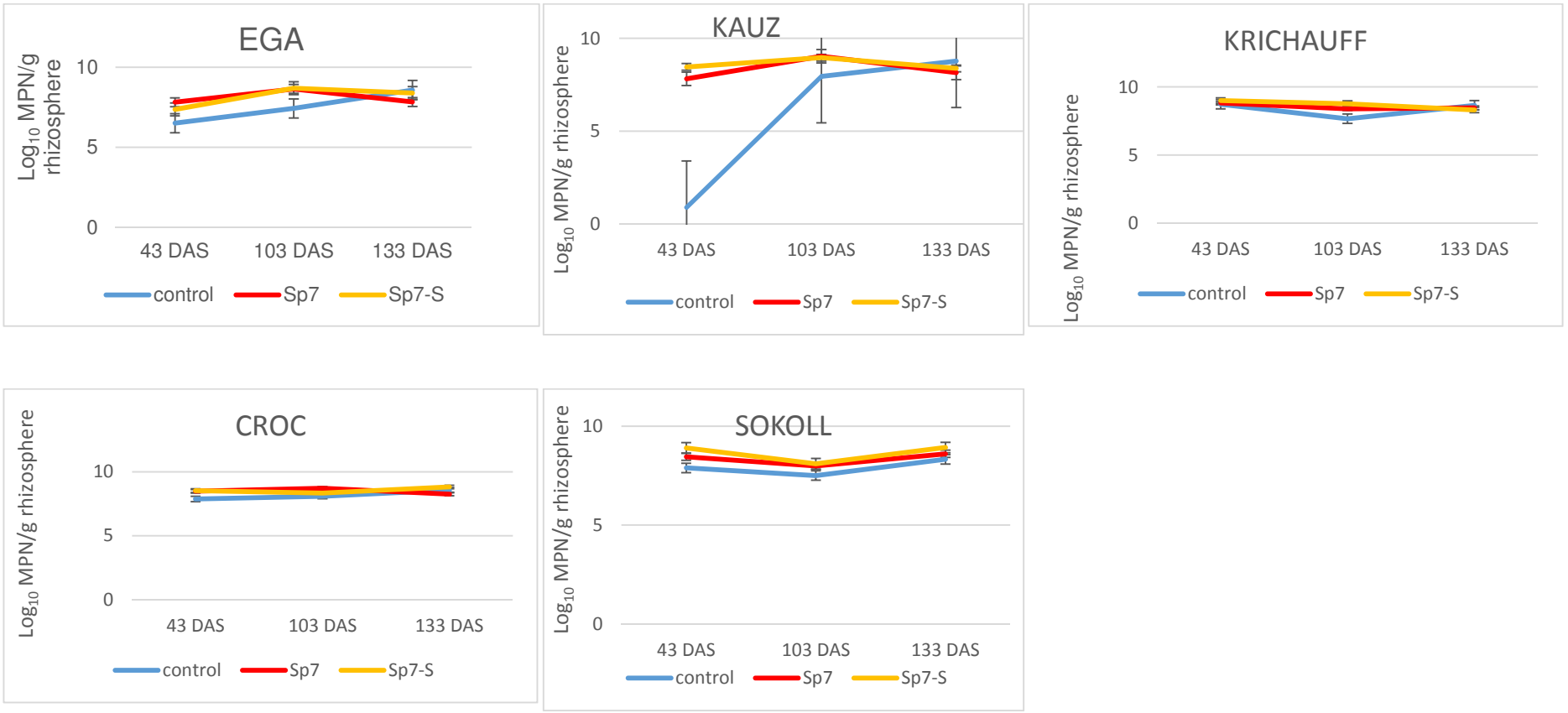


Figure 5.1 Number of Nfb positive bacteria (Log_{10} MPN) in 2010 at different sampling times for different genotypes (limit of detection 3.56 MPN/ml of original solution)

Table 5.3 Predicted mean log_{10} MPN of Nfb positive bacteria in 2010 at 43 DAS

Genotypes	Control	Sp7	Sp7-S
1	6.504 cC	7.814 bA	7.367 cB
2	0.905 cC	7.829 bB	8.466 bA
3	7.876 bB	8.477 aA	8.52 bA
4	7.892 bB	8.456 aA	8.893 aA
5	8.733 aA	8.822 aA	9.002 aA

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

Table 5.4 Predicted mean log_{10} MPN of Nfb positive bacteria in 2010 at 103 DAS

Genotypes	Control	Sp7	Sp7-S
1	7.428bB	8.643bA	8.693aA
2	7.95aB	9.043aA	8.964aA
3	8.089aB	8.715aA	8.327bA
4	7.504bB	7.991cA	8.093bA
5	7.669bC	8.399bB	8.774aA

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

Table 5.5 Predicted mean log_{10} MPN of Nfb positive bacteria in 2010 at 133 DAS

Genotypes	Control	Sp7	Sp7-S
1	8.573aA	7.826bA	8.395bA
2	8.782aA	8.151bA	8.385bA
3	8.570aA	8.255aA	8.812aA
4	8.322aA	8.604aA	8.919aA
5	8.652aA	8.469aA	8.312bA

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

5.3.2 MPN of Nfb positive microorganisms in wheat rhizospheres in 2011

In the 2011 field experiment, rhizosphere soil samples were also taken at three different plant growth stages to estimate the MPN of Nfb positive microorganisms. In the early stages of growth (43 DAS), MPN was significantly higher than the control (Table 5.6). However, similar to 2010, this effect disappeared later in the season as numbers of indigenous Nfb positive microorganisms increased. Nevertheless, there were significant interactions including inoculant x N and genotype x inoculant x N at the flowering and grain filling periods (103 and 133 DAS). No significant genotype effect was observed at any growth stage and inoculant effects were significant at tillering (43 DAS) only. The mean MPN data showed that numbers of Nfb positive microorganisms were lower in uninoculated plots early in the season (Tables 5.7; 5.8; 5.9). For all genotypes, bacterial numbers increased as the plant developed (Figure 5.2).

Table 5.6 Wald statistics and their significance from analysis of $\log_{10}$ MPN in 2011 at different sampling times: 43,103,133 DAS

Fixed term	n.d.f.	43 DAS	103DAS	133DAS
Genotype (G)	4	2.04	2.14	2.48
Inoculant (I)	3	126.56***	2.97	4.04
N	1	1.20	1.63	3.61
G x I	12	15.99	13.31	10.98
G x N	4	4.54	7.43	4.88
I x N	3	1.20	9.29*	8.46*
G x I x N	12	20.92	27.50*	11.91

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respective

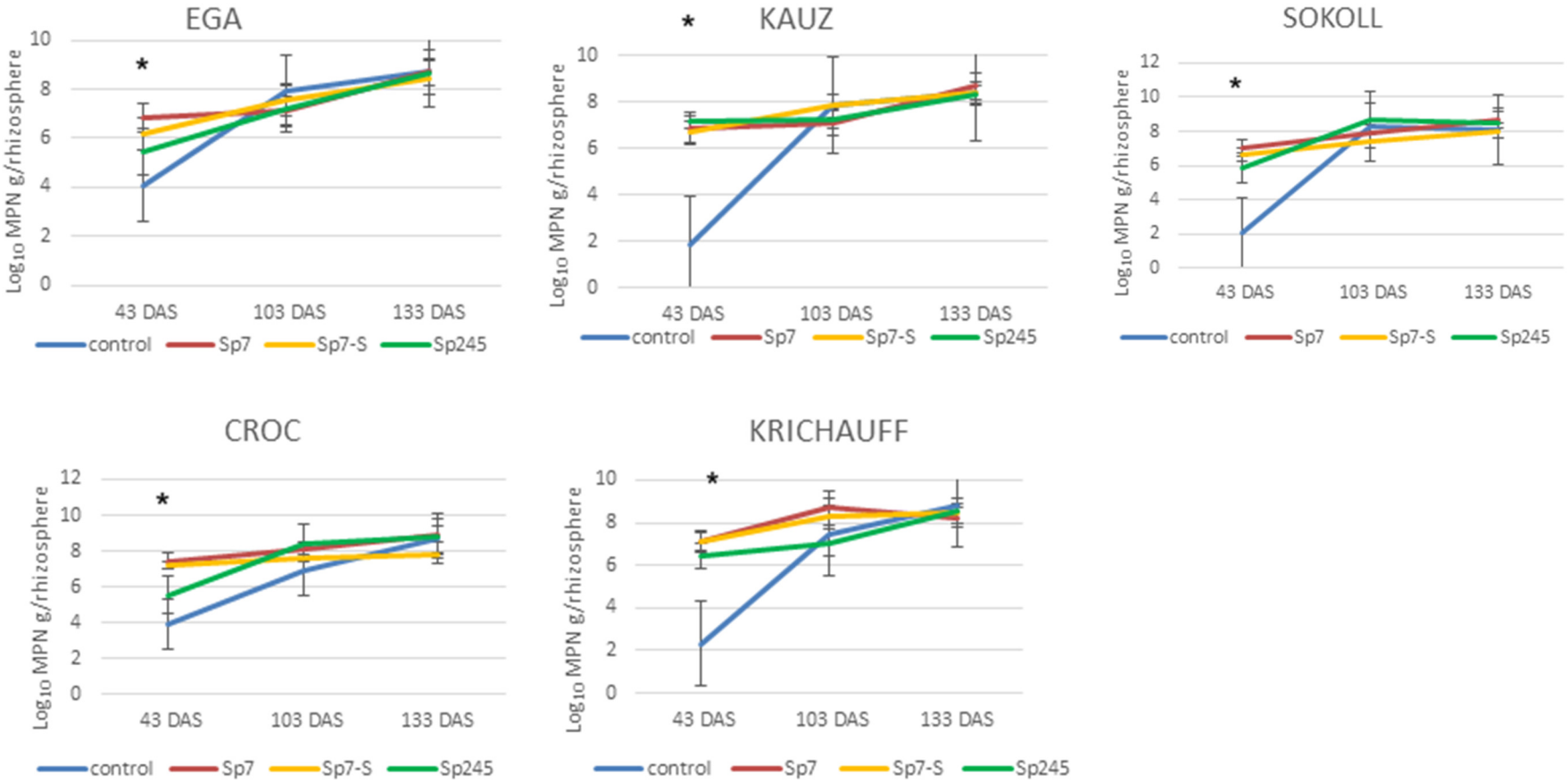


Figure 5.2 Number of Nfb positive bacteria (log_{10} MPN) in 2011at different sampling times in different genotypes (limit of detection 3.56 MPN/ml of original solution). * indicates significance at P<0.05

Table 5.7 Predicted mean log_{10} MPN of Nfb positive bacteria in 2011 at 43 DAS

Genotypes	Control	Sp245	Sp7	Sp7-S
1	4.055aC	5.463bB	6.828aA	6.192bA
2	1.859bB	7.201aA	7.351aA	6.693aA
3	3.935aC	5.540bB	7.432aA	7.187aA
4	2.069bC	5.860bB	7.006aA	6.632aA
5	2.323bB	6.449aA	7.152aA	7.080aA

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

Table 5.8 Predicted mean log_{10} MPN of Nfb positive bacteria in 2011 at 103 DAS

Genotypes	Control	Sp245	Sp7	Sp7-S
1	7.927aA	7.216bA	7.109bA	7.569aA
2	7.871aB	7.233bB	8.838aA	7.837aB
3	6.915bB	8.422aA	8.057aA	7.631aA
4	8.310aA	8.717aA	7.914aA	7.462aB
5	7.495aB	7.051bB	8.722aA	8.337aA

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

Table 5.9 Predicted mean log_{10} MPN of Nfb positive bacteria in 2011 at 133 DAS

Genotypes	Control	Sp245	Sp7	Sp7-S
1	8.710aA	8.686abA	8.698bA	8.475aAB
2	8.435bB	8.346cB	8.663bA	8.376aB
3	8.704aAB	8.808aA	8.942aA	7.768cB
4	8.136cB	8.490bcAB	8.704abA	8.039bB
5	8.806aA	8.546abAB	8.226cC	8.433aAB

Different lower case letters in columns indicates significance at P<0.05; Different upper case letters in rows indicates significance at P<0.05

5.3.3 Inferred presence of inoculant strain by T-RFLP

Based on the MPN results, samples with differential responses to inoculation at 43 DAS were chosen for T-RFLP analysis. The persistence of *Azospirillum* inoculants in soil was inferred by examining T-RFLP profiles for peaks of an expected size. We ran a T-RFLP of the nif H genes amplified from the DNA of the pure culture of *A.brasilense* Sp7, Sp7-S and Sp 245, the T-RF size of different *Azospirillum* strains was then determined empirically (Table 5.10). These (bp and peak height) values were used to interrogate T-RFLP profiles from soil DNA of inoculated and uninoculated plots. Table 5.11 shows the sizes and heights of peaks closest in size to those expected from the inoculants. The base pair sizes for all 52 samples in both 2010 and 2011 are listed in Appendix table 11.

In most cases the environmental sample taken from inoculated plots shows the presence of the same base pair sizes as the pure strain. In control plots where no inoculation was applied, the observed base pair size generally differed from the pure strain, although there were exceptions indicating that *Azospirillum* was also indigenous. The results therefore confirmed that *Azospirillum* was present in the rhizospheric soil samples taken from the inoculated plots; however this result could be influenced by indigenous *Azospirillum.* It must be noted that inferences of identity from T-RFLP profiles must be interpreted cautiously as multiple species can have T-RFs of identical length.

Table 5.10 Size and peak height of *nifH* gene from pure cultures of different *Azospirillum* strains

Bacterial strain(pure)	Size (bp)	Peak height
A.brasilense Sp7	128	20675
A. brasilense Sp7-S	128	21957
A. brasilense Sp245	115	21918

Table 5.11 Size and peak height of potential *Azospirillum* inoculant T-RFs from environmental samples

	2010		2011	
Plots	Size (Base pair)	Peak height (fluorescence units)	Size (Base pair)	Peak height (fluorescence units)
Control	127	18157	129	7554
Inoculated with Sp7	128	30877	128	11879
Inoculated with Sp7-S	128	5009	128	11445
Inoculated with Sp245	Na	na	115	18449

na – not applicable in 2010

5.3.4 T-RFLP analyses, 2010 and 2011

AMMI analysis of the T-RFLP data is presented in Table 4.12. Bacterial T-RFLP analysis yielded a total of 110 and 124 distinct T-RFs in 2010 and 2011, respectively. The average T-RF richness per sample of 33.59% was slightly higher in 2011 than the 31.62% recorded in 2010. Beta diversity, a measure of species richness, was observed to be 2.47 and 2.69 in 2010 and 2011, respectively (Table 5.12). Similar overall diversity was obtained in both years as values between 2 and 3 are fairly typical.

Table 5.12. Characteristics of the T-RFLP datasets in 2010 and 2011.

Experiment Year	Total T-RFs	Richness	beta diversity/sample heterogenocity
2010	110	31.62	2.47
2011	124	33.59	2.69

Table 5.13 shows the distribution of variation within the T-RFLP datasets from ANOVA. In 2011 and 2010, the nitrogen fixing bacterial community main effects were 93.35% and 88.46%, respectively. The main source of variation was attributable to T-RF diversity between samples. This is a typical characteristic of T-RFLP datasets and has the potential to mask trends resulting from differences between experimental treatments. A double-centered principle components analysis (AMMI) measures variation caused by the interaction between T-RFs and treatments and can tease out trends in the data which are otherwise masked by T-RF variability. However, it can be seen from Table 5.13 that low or very low signal values (a measure of the degree of

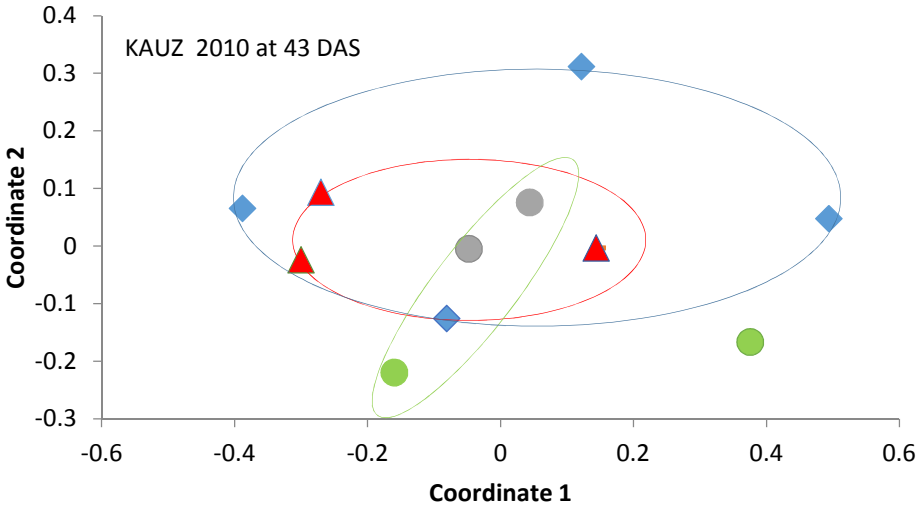
interaction between different treatments) were obtained for 2010 (0.87%) and 2011 (3.81%) samples. As a result, nMDS analysis was used to generate ordination plots for different sample sets as this technique allows the similarity between replicate samples to be visualized, while AMMI analysis does not.

Table 5.13 Percent variation in peak height of T-RFLP datasets from analysis of variance

Experiment year	T-RFs	Environments	Signal	Noise
2010	93.35%	0	0.87%	5.77%
2011	88.46%	0	3.81%	7.73%.

The community structures of nitrogen fixing bacteria, based on T-RFLP analysis, are displayed using nMDS (Fig 5.3 and 5.4). While some clustering of the samples from different treatments (inoculants and controls) was observed, no shift in the nitrogen fixing bacterial community was observed with the addition of inoculum in either 2010 or 2011. The conclusion from this is that inoculation had little effect on the N-fixing soil bacterial community. However, there may have been differences between the treatments and the method used (T-RFLP) was simply not sensitive enough to detect them.

5.3.a



5.3.b

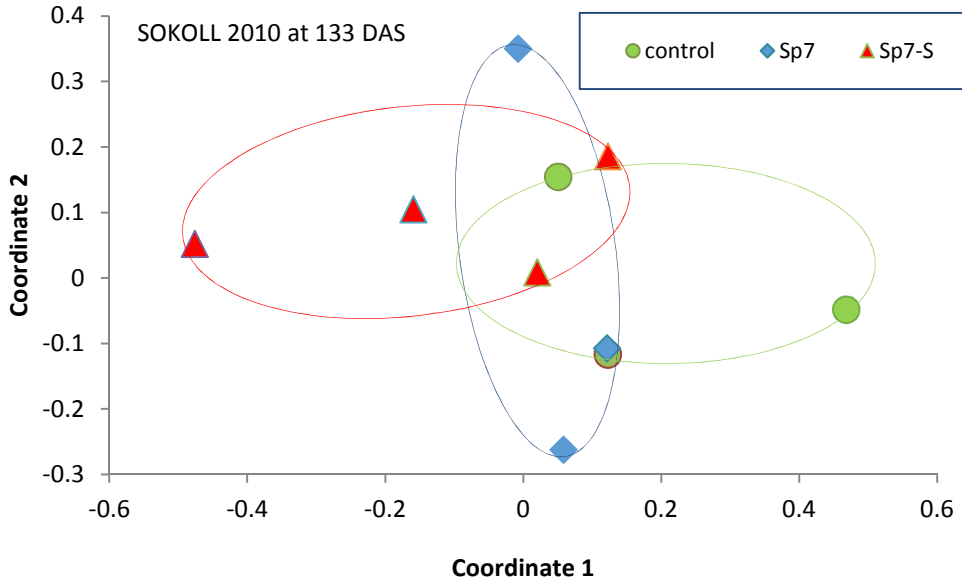
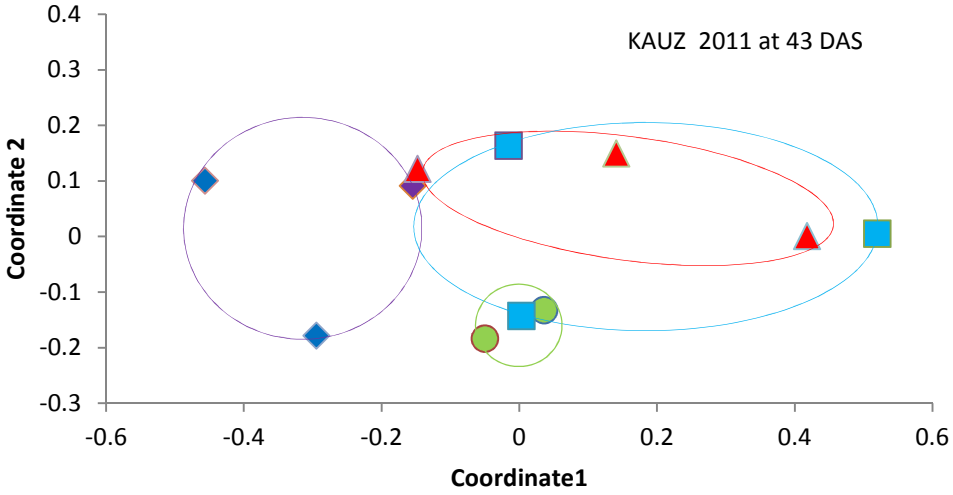


Figure 5.3 Ordinations of bacterial T-RFLP data with nMSDS analysis 2010. 5.3.a represents KAUZ at 43 DAS in 2010 and 5.3.b SOKOLL at 133 DAS in 2010

5.4.a



5.4.b

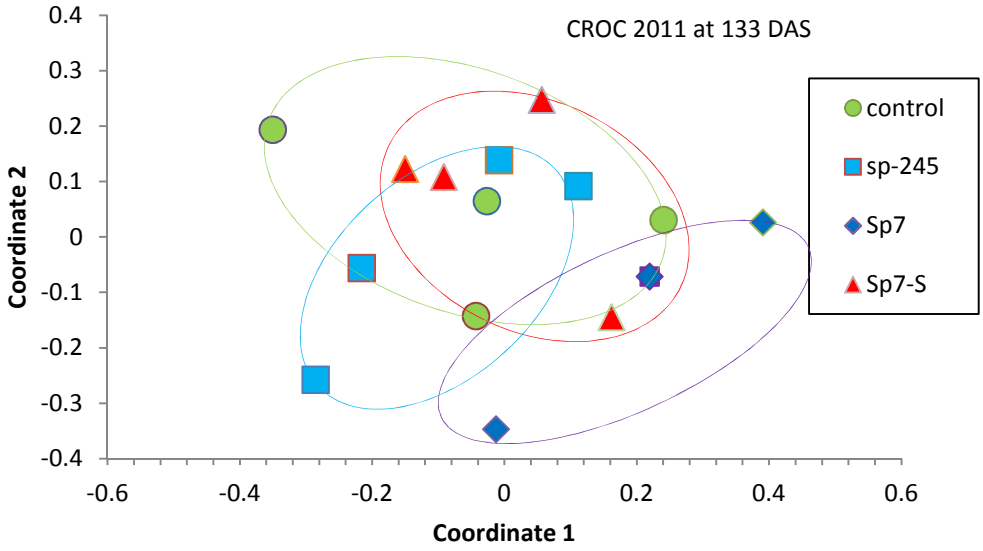


Figure 5.4 Ordinations of bacterial T-RFLP data with nMSDS analysis in 2011. 5.4.a represents KAUZ at 43 DAS and 5.4.b CROC at 133 DAS.

5.4 Discussion

5.4.1 Number of *Azospirillum* associated with the root rhizosphere

In both 2010 and 2011 the MPN of *Azospirillum* in the rhizosphere was estimated using the multiple tube fermentation method in semi-solid Nfb medium. This medium is selective for a range of nitrogen fixing microorganisms including *Azospirillum* and further purification steps are required when isolating *Azospirillum* from soil (Baldani and Dobereiner 1980). This multiple tube fermentation method using Nfb was applied to determine if inoculation with *Azospirillum* would result in measurable differences in MPN in the rhizosphere. It was observed that the MPN was lower in the early stages of plant growth than later in the season. Numbers were lower in the control treatments than inoculated plots at the early sampling times suggesting that the introduced *Azospirillum* contributed to the increased MPN and that inoculation was successful at delivering high numbers of viable azospirilla to the rhizosphere. However, this difference tended to decrease as the season progressed as indigenous nitrogen fixing bacteria increased in number. Nevertheless, it was not possible to differentiate between inoculant *Azospirillum* and diazotrophic *Azospirillum* species using the multiple tube fermentation method and while total numbers did not vary significantly at later plant growth stages, the proportion of inoculated *Azospirillum* may have been higher in the inoculated treatments. These observations support the work of Kundu and Gaur (1980) who observed that bacterial number remained high for a long period of plant growth following inoculation with *Azotobacter chroococcum* and phosphobacteria (*P.striata* and *B. polymyxa*). Others also reported that environmental factors, plant cultivar, inoculation technique and the indigenous micro flora influence the establishment of the *A.brasilense* strains on wheat roots. The indigenous strains most likely compete with inoculated strains for space although interactions,

including suppression, are also possible. *A.brasilense* Sp245 was reported to have the highest and most persistent colonizing potential compared with other strains of *A.brasilense* Sp7 (Schloter and Hertmann 1998). In a review of beneficial bacteria with agricultural importance, Babalola (2010) discussed the role of quorum sensing, chemotaxis and root exudates in determining root colonization in the rhizosphere. In the field there is a complex interaction between the plant species and genotype, microbial strain and the environment (soil fertility, N application, soil moisture, day length, light intensity, length of growing season and temperature) that effects the rhizosphere and plant growth promotion (Babalola 2010). N application and inoculation with *A. brasilense* can have a positive impact on the culturable microbial community and *Azospirillum* number in the rhizosphere of wheat as reported by Naiman et al. (2009); an observation supported by the current study. Beschoren et al. (2013) found that fertilization affects PGP traits of diazotrophic communities and this should be considered when selecting the PGP for inoculation in the field. However, while N application did generally influence bacterial colonization in the current study, the trends may have been reduced by generally high levels of residual N at the location where the experiments were conducted in both years.

5.4.2 Nitrogen fixing bacterial community analysis with T-RFLP

There are many studies on bacterial community structure using the 16S gene that indicate there is little effect from soil inoculation or seed inoculation to the structure of microbial communities (review Trabelsi and Mhamdi 2013). Reports indicate that application of rhizobial inoculants can temporarily influence the phylotype richness of the microbial communities in potato and *Phaseolus vulgaris* rhizosperes using 16S rRNA gene TRFLP profiling (Trabelsi et

al. 2011; Trabelsi et al. 2012). No prominent effect on the rhizosphere community was observed after inoculation with *A.brasilense* on Maize (Lerner et al. 2006).

Here we targeted the functional gene *nifH,* present in the inoculant bacteria, to narrow the gene pool and bacterial community under investigation. It was assumed that *Azospirillum* inoculants would be more likely to affect the structure of the nitrogen fixing community than the entire bacterial community. However, no influence of inoculation on nitrogen fixing bacterial communities as measured by *nifH* gene diversity in wheat roots was observed. Field inoculation with *A.brasilense* similarly showed no significant effects on bacterial communities of maize in different soils and in different growth systems (Lerner et al. 2006). Inoculation with *A.brasilense* and the biocontrol agent *Bacillus subtilis* did not significantly impact on soil microbial communities and *nifH* T-RFLP-patterns of diazotrophic bacteria (16S rRNA) and fungal (18S rRNA) communities in the rhizosphere (Felici et al. 2008; Salamone et al. 2012); a finding supported by the current study. The overall microbial community diversity of specific functional groups is most likely influenced by the change in available nitrogen and carbon source in the rhizosphere rather than the direct effect of inoculation (Tribalsi and Mhamdi 2013). Different soil type and management conditions may influence the plant response to inoculation and the microbial community in the rhizosphere. These effects were noted in previous studies when comparing rich soil with poor soil or acid soil (Crits-Christoph et al. 2013; Bardhan et al. 2012; Salamone et al. 2012). Bremer et al. (2007) observed crop species specific effects on *nifK*-type denitrifiers (a group of functional soil microorganisms) and additional significant effects were due to environmental conditions such as sampling time. Knauth et al. (2005) reported varietal differences in root associated *nifH*-gene expressing

communities in rice roots under field conditions while analysing T-RFLP and RT-PCR products and Tan et al. (2003) reported that N-fertilizer has a strong influence on the diversity of diazotrophic populations. Similarly, Lovell et al. (2000) observed positive effects of N fertilizer applications on rice roots and associated diazotrophs; both increased with N level. However, N level did not significantly influence microbial communities in the current study and as stated earlier, the relatively high levels of residual N at the experimental site (this site was considered to have a rich soil) may have reduced treatment differences and therefore the sensitivity of the analysis. Although the effect of wheat genotype was relatively weak, some differences were evident and a genotype x sampling time x year interaction is evident in Figures 5.3 and 5.4.

The specific mechanisms affecting changes in the rhizospheric community with inoculation are not clear and require further study. While there are clearly demonstrated effects from modifying the soil environment by varying plant genotype and applying fertilisers, more subtle effects may occur through the application of inoculants and more sensitive methods may be required to detect these.

5.5 Conclusion

The microbial and molecular analysis showed a complex interaction between plant genotypes, microorganisms, environments and soil properties (such as nutrient availability). The microbial analysis indicated an inoculant effect in the early stages of growth which later disappeared as the season progressed. The long, wet grain filling periods in both 2010 and 2011 (see Chapter

4) will have influenced the colonization patterns in both years and most likely reduced the impact of inoculation. However, the molecular analysis indicated a clear effect of genotype and environment on bacterial colonization although no significant inoculation effect was found. The genotype effect on the functional groups of the microbial community requires more research across a wider range of environments before firm conclusions can be drawn. Further research should also target root exudates of individual plant genotypes as these could hold the answer to understanding the complex plant-microbe relationship.

Chapter 6: General Discussion and Conclusion

Plant growth promoting (PGP) bacteria are defined as root colonising microorganisms that have beneficial effects on plant growth and development (Kloepper, 1994). Plant growth promoting rhizobacteria (PGPR) long ago gained worldwide attention and acceptance for their agricultural benefits and role in sustainability (Das et al. 2013). However, the influence of plant genotype and bacterial strain on growth promotion is not clear. This project examined the influence of wheat genotype and up to three strains of *Azospirillum* on plant growth under both controlled and field conditions.

6.1 PGP in controlled conditions

The observed plant genotype responses to inoculation were both positive and negative depending on the trait assessed and the genotype/inoculum combination. Clearly, the genotype/inoculum combination is vital in conferring a positive plant response and in some cases inoculation can even reduce the expression of economically important traits. However, the positive responses to inoculum observed for chlorophyll and some root characteristics indicate that nitrogen was used more efficiently in many genotype/inoculum combinations. These results are supported by the earlier findings of Kim et al. (2012) who inoculated switch grass with *Burkholderia phytofirmans* strain PsJN and found a highly significant and positive effect on plant growth promotion in both the growth chamber and green house. They also reported that growth promotion using PsJN was genotype-dependent after testing seven other

switch grass cultivars for their growth responses. This observation is similar to the current study where a wheat genotype interaction with *A. brasilense* Sp7 and Sp7-S was found. The wheat cultivar Sunco showed a negative response in most growth parameters whereas the cultivar Tasman was generally positive for the same traits. As both cultivars are parents of a genetic mapping population, developed initially to identify QTL for rust resistance, there is an opportunity to identify QTL linked to plant growth promotion if the progeny of the population are assessed. The positive and negative responses to inoculum in the current study support earlier observations under greenhouse conditions in paddy rice (Keyeo et al. 2011). Keyeo et al. (2011) reported negative plant responses to inoculation with Sp7 and *H. seropedicae* Z78. The large amount of IAA produced in this study actually supressed plant root growth and overall plant development.

Previous studies also examined optimal inoculation rates for eliciting a plant response. Pereyra et al. (2007) reported that the optimum concentration of *A. brasilense* Sp245 was 10^5cells/seedling. Their study showed that inoculation at higher rates (10^6 -10^8 cell/seedling) had no growth promoting effects. Similarly, Kapulnik et al. (1985) reported that inoculation of wheat seeds (*Triticum aestivum* L.) with *A. brasilense* Sp7, Cd and Cd-1 at 10^5 to 10^6 colony-forming units caused the largest root elongation and total root surface area of seedlings, whereas 10^8 to 10^9 colony-forming units inhibited root development. Inoculation with a high concentration of *A. brasilense* Sp245 and Sp7 strains was also shown to suppress wheat root length and density (Dobbelaere et al. 1999). In the current study, 13ml of hydroponic media in a small test tube was used to grow plants for three weeks only; a very short time to elicit growth promotion effects. In hindsight, sterile sand or soil would have been a better medium for

observing plant growth promotion effects as suggested by Soubidet et al. (2002). These authors concluded that bigger pots give better plant responses to *Azospirillum*.

Future studies should therefore use larger pots with sterile sand or soil with a reduced concentration of bacteria and a longer sampling time adopted to elicit an improved response to *Azospirillum* in wheat.

6.3 PGPs in the field

Five genotypes were selected for detailed field experiments based on their genetic diversity and plant growth promoting responses in controlled environments over two years (detail in Chapter 4). The sowing season at Narrabri made it impossible to wait until the controlled environment assessments were complete before finalizing the five genotypes, although some preliminary information was available. Nevertheless, the five genotypes selected on the basis of genetic diversity, were subsequently shown to have a differential response to inoculation, even though the most responsive genotype, Tasman, was not included. The field responses to inoculation were inconsistent and weaker than those observed under controlled conditions with the exception of root traits. There was also inconsistency between years as exemplified by TKW where a significant inoculation effect and genotype interaction was observed in one year only. Grain yield, the ultimate measure of the effects of plant growth promotion, was higher than average in both 2010 and 2011 as the growing season rainfall was above average (see figures 5.1). The high rainfall extended the grain filling period in both years and likely reduced the effects of inoculation on grain yield. Although many genotype/inoculation effects were diminished and non-significant in both years there were some interesting trends. Genotype 3

(CROC), a synthetic derivate, showed a significant positive effect on grain yield in 2010 and was 4.3% and 13.5% higher yielding than the uninoculated control in the presence of Sp7 and Sp7-S, respectively. In 2011, genotype 2 (KAUZ) and 5 (KRICHAUFF) showed positive (although non-significant) increases in yield due to inoculation; genotype 2 (KAUZ) showed 2.9%, 4% and 2.7% grain yield increases over the control when inoculated with Sp245, Sp7 and Sp7-S, respectively. Genotype 5 (KRICHAUFF) was 4.5%, 1.9% and 5.4% higher yielding than the control when inoculated with Sp245, Sp7 and Sp7-S, respectively. Clearly, the trends indicate a positive effect of inoculation in the field and a drier year with a shorter grain filling period and better inoculum persistence may produce statistically significant results. While reports of genotype/inoculum effects under controlled conditions abound for a range of crops, there is very little published on the effect of genotype on plant growth promotion in the field. In a review of 56 articles of field experiments Bashan et al. (1989) concluded that positive responses to inoculation occur in about 71-75% of studies. However, the responses varied depending on plant species and inoculum strain. Probably the most extensive study of plant growth promotion in wheat is that published by Diaz-Zorita and Fernandez-Canigia (2009). They analysed 297 field experiments in Argentina inoculated with *A.brasilense* INTA Az39 and found positive responses on grain yield in 70% of trials. The average yield increase in the presence of inoculum was 8%. They reported a significant effect of season and concluded that plant response is dependent on season, genotype and bacterial strain. These conclusions are supported by the meta-analysis of Veresolglou and Menexes (2010) based on articles published in the ISI web of science (1981-2008). They reported that the key determinants of plant growth promotion are N fertilization, wheat cultivar and the *Azospirillum* isolate used. Similarly, Dobbelaere et al. (2001) reviewed trials in Belgium, Uruguay, Mexico and Israel on different

crop species inoculated with *Azospirillum* in the field and green house in the period 1994-2001. They reported positive responses but conclude that response was dependent on environmental conditions including fertiliser and soil type. All studies assumed that improved plant growth was mainly the effect of plant growth promoting substances that improved root development and subsequent water and mineral uptake. Nevertheless, the wheat genotype effects reported are largely circumstantial and based on unbalanced and relatively small numbers of lines.

Based on these earlier findings and the improved growth promotion observed in Chapter 3, a positive response to inoculation in the field was expected but not consistently observed (Chapter 5) in the current study. As mentioned earlier, the unusually productive growing conditions of 2010 and 2011 probably minimised the effect of inoculation on production traits such as grain yield, total above ground biomass and TKW. Both years recorded at least 150 mm more rainfall than the 10 year average which resulted in an excessively long grain-filling period and much higher grain yield, biomass and seed weight than normally expected. While some effect of inoculation was observed in the early stages of growth, this had disappeared later in the season. Cultivation practise could have also masked the inoculation effect. The experimental site was characterized by a best practice wheat/pulse/fallow rotation with minimum tillage including stubble surface retention. This practise may have resulted in higher levels of naturally occurring PGP bacteria thereby diminishing the effect of inoculation.

Nevertheless, a significant inoculation effect on root development was observed early in the season in both years and supports the observations made under controlled conditions and the findings of previous studies. However, the size of the genotype/inoculation effect for root traits

may have been exaggerated due to the sampling method. To avoid cross contamination (also important for subsequent microbial and molecular analyses) a different person sampled each treatment in the field by digging out soil and root matter to a specified depth. Therefore, the observed significant treatment effects may also include a sampler effect. This could have been avoided if one person has sampled all treatments (changing or cleaning the equipment between treatments). However, the scale of the experiment and time and weather constraints made this impractical.

6.4. The relationship between traits assessed under controlled conditions and the field

As expected there was little relationship between plant response to inoculation assessed under controlled conditions and that observed in the field. The lack of relationship to some extent reflects the lack of significance in the field trials for the same traits assessed. A more extensive examination of field response using more genotypes and inoculum strains and a wider range of environments may enhance the relationship.

Root traits including root length, area, volume and diameter and plant height, above ground dry weight and relative chlorophyll were assessed in both the growth chamber and the field. Table 6.1 presents the correlations between the two evaluation methods for the 5 selected genotypes for each bacterial inoculation treatment. Very little relationship was observed between the growth chamber and field. Only plant height in Sp-7 was significantly positively correlated ($r = 0.87$), although the relationship was also close to significant in the control ($r =$

0.71). Interestingly, in Sp-7 a number of root traits, including area, volume and diameter and total dry weight, although non-significant are trending negative. This trend becomes significantly negative for root length (r = -0.86) and area (r = -0.96) in Sp7-S.

Clearly, there is little relationship, apart from plant height, among the variables tested in the growth chamber and field and the trend to negative correlations among some variables indicates that testing under controlled conditions is actually counter-productive. The influence of soil (opposed to media) appears to completely change the plant response. There are bacterial populations in soil that interact with the inoculated strain altering behaviour and colonisation patterns, whereas in the hydroponic media only the inoculated strain was present. This lack of relationship underscores the need to conduct initial plant genotype screening using a controlled system that is more representative of the field environment. However, as discussed earlier, the controlled environment responses could also be improved by assessing materials in sterilised sand or soil in much larger pots. Another option could be to evaluate the use of non-sterile soil in the growth chamber with careful monitoring of the persistence of the inoculated strain.

Table 6.1. The relationship between traits assessed in the growth chamber on sterile medium and the field.

a. Control

		Controlled condition						
		Root length	Root area	Root volume	Root diameter	Plant height	Dry weight	SPAD
Field	Root length	0.20						
	Root area		-0.65					
	Root volume			0.64				
	Root diameter				0.24			
	Plant height					0.7		
	Dry weight						0.00	
	SPAD							0.13

b. Sp7

		Controlled condition						
		Root length	Root area	Root volume	Root diameter	Plant height	Dry weight	SPAD
Field	Root length	0.61						
	Root area		-0.71					
	Root volume			-0.52				
	Root diameter				-0.64			
	Plant height					0.87*		
	Dry weight						-0.68	
	SPAD							0.47

c. Sp7-S

		Controlled condition						
		Root length	Root area	Root volume	Root diameter	Plant height	Dry weight	SPAD
	Root length	-0.86*						
	Root area		-0.96**					
Field	Root volume			0.58				
	Root diameter				0.31			
	Plant height					-0.67		
	Dry weight						-0.80	
	SPAD							-0.51

* indicates significance at P<0.05, ** at P<0.01 and *** at P<0.001% level respectively

6.5 Persistence of the inoculum in the field

The success of inoculation and subsequent plant growth promotion depends on the persistence of the bacterial strain throughout the crop growing season which is influenced by root colonisation ability and competition with indigenous species found in the soil. It is therefore, very important to monitor the inoculated bacteria throughout the season to assess competition with native rhizosphere bacteria. Inoculation timing appears to be crucial in determining successful root colonization and subsequent plant growth promotion. Bashan (1986) reported that early inoculation on the seed and successive inoculations on the growing wheat plant gives better responses and that 10^5 - 10^6 cfu/ml is the optimum rate for inoculation. In the current study, the seed and soil were inoculated at the suggested 10^5 - 10^6 cfu/plant at sowing. The numbers of *Azospirillum* were estimated three times; at tillering, heading/flowering and grain

filling using the MPN technique. The inoculation was successful as high numbers of *Azospirillum* were observed in the rhizosphere compared with the control at the early stages of wheat growth. However, this effect did not persist as numbers of *Azospirillum* were equivalent to the uninoculated control by grain filling and in some cases by anthesis. This reduction in persistence is partially explained by the findings of Swędrzyńska and Sawicka (2001) who reported that persistence of *A.brasilense* following inoculation is dependent upon plant species, stage of development and the availability of N (also observed by Kolb and Martin 1988). The current study is similar in that the number of *Azospirillum* increased in the soil with plant growth and development, reaching a maximum at flowering and then decreasing with maturation; a phenomenon also reported by Liljeroth et al. (1990). However, the early differences in *Azospirillum* based on MPN could not be confirmed in the current study using T-RFLP. The T-RFLP procedure may not be sensitive enough to quantify differences in *Azospirillum* and other molecular techniques, such as pyrosequencing, may be better alternatives for confirming the presence of inoculum in the field at different stages of growth.

Recommendations for future field based testing for PGP effects follow:

1. The Tasman and Sunco genotypes should be included in field experiments and should their response differ (as observed under controlled conditions), then the available mapping population could be assessed to identify QTL linked to plant response in the field.
2. A broader range of *Azospirillum* strains could be tested

3. Root sampling in the field could be conducted by one person at an earlier stage of crop development (thus allowing more complete extraction of the root systems and removing any sampler bias)

4. The inoculum was applied in peat diluted with water that was incorporated with the seed at sowing. Different inoculum application methods could be examined and even a second application made at anthesis.

5. Field experiments need to be repeated across a greater range of seasons to observe the effects of inoculum and wheat genotype under sub-optimal moisture and nutrient conditions.

7. Conclusion

The current study found a positive effect of wheat genotype on various aspects of plant development and that more genetically diverse materials, such as the CROC synthetic derived line, show greater response to inoculum. The better response of the synthetic line may indicate that vital genetic variation for plant response to *Azospirillum* has been lost through intensive selection for yield and market traits in modern times. A more extensive examination of ancestral wheat materials, including the traditional landrace cultivars found in many countries, may provide greater genetic diversity for PGP responses for plant breeders. Nevertheless, the combination of genotype and bacterial strain is vital as many negative effects of inoculation were observed for some traits. It is unlikely that these negative effects are due to higher than optimal concentrations of bacteria as the previously published optimal concentration of 10^5 - 10^6 cfu/ml was used for all assessments. The lack of relationship between the controlled environment testing and the field is a combination of factors including environmental

constraints and limited plant genotype and strain numbers. However, lack of persistence (as compare to control treatments) beyond anthesis in the field may also reduce plant growth promotion.

References

Abdo, Z., U. M. E. Schuette, S. J. Bent, C. J. Williams, L. J. Forney, and P. J. (2006). Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environmental Microbiology*, *8*, 929–938.

Abeles, F. B., Morgan, P. W., & M. E. Saltveit Jr. (1992). *Ethylene in Plant Biology,* (2nd editio.). New York, NY, USA,: Academic Press,.

Afzal, A., & Bano, A. (2008). Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum* L.). *International Journal of Agriculture and Biology*, *10,*, 85–88.

Ahemad, M., & Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. *Journal of King Saud University - Science*, *26*(1), 1–20. doi:10.1016/j.jksus.2013.05.001

Ahemad, M. , Khan, M. S. (2010). Phosphate-Solubilizing and Plant-Growth-Promoting *Pseudomonas aeruginosa* PS1 Improves Greengram Performance in Quizalafop-p-ethyl and Clodinafop Amended Soil. *Archives of Environmental Contamination and Toxicology*, *58,*(2), 361–372.

Ahemad, M. , Khan, M. S. (2011). *Pseudomonas aeruginosa* strain PS1 enhances growth parameters of greengram [*Vigna radiata* (L.) Wilczek] in insecticide-stressed soils. *Journal of Pest Science*, *84*(1), 123–131.

Ahemad, M., Khan, M. S. (2012). Alleviation of fungicide-induced phytotoxicity in greengram [*Vigna radiata* (L.) Wilczek] using fungicide-tolerant and plant growth promoting *Pseudomonas* strain. *Saudi Journal of Biological Sciences*, *19*(4), 451–459.

Ahmad, F., Ahmad, I., & Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research*, *163,*(2), 173–181.

Akbari, G. A., Arab, S. M., Alikhani, H. A., Allahdadi, I., & Arzanesh, M. H. (2007). Isolation and Selection of Indigenous *Azospirillum* spp . and the IAA of Superior Strains Effects on Wheat Roots. *World Journal of Agricultural Sciences*, *3*(4), 523–529.

Almaghrabi, O. A., Massoud, S. I., & Abdelmoneim, T. S. (2013). Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. *Saudi Journal of Biological Sciences*, *20*(1), 57–61. doi:10.1016/j.sjbs.2012.10.004

Anjum, M. A., Sajjad, M. R., Akhtar, N., Qureshi, M. A., Igbal, A., Jami, R. A., & Hasan, M. U. (2007). Response of cotton to plant growth promoting rhizobacteria (PGPR) inoculation under different levels of nitrogen. *J. Agric. Res.*, *Volume 45,*(2), Pages 135–143.

Argenta, G., Silva Paulo Regis Ferreira, & Sangoi, L. (2004). Leaf relative chlorophyll content as an indicator parameter to predict nitrogen fertilization in maize. *Cliencia Rural, Santa Maria*, *34*(5), 1379–1387.

Arsene, F., Katupitiya, S., Kennedy, I., & Elmerich, C. (1994). Use of LacZ fusions to study the expression of nif Genes of *Azospirillum brasilense* in association of plants. *Molecular Plant-Microbe Interactions*, *7*(6), 748–757.

Ashrafuzzaman, M., Hossen, F. A., Ismail, M. R., Hoque, M. A., Islam, M. Z., Shahidullah, S. M. & Meon, S. (2009). Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. *African Journal of Biotechnology*, *8,*, 1247–1252.

Babalola, O. O. (2010). "Beneficial bacteria of agricultural importance. *"Biotechnology Letters*, *32.*(11), 1559–1570..

Bakker, P. A. H. M., Pieterse, C. M. J., & Loon, L. C. van. (2007). “Induced systemic resistance by fluorescent Pseudomonas spp,.” *Phytopathlogy,*, *vol. 97,*(2), 239–243.

Baldani, V. L. D., Baldani, J. I., & Döbereiner, J. (1987). Inoculation of field-grown wheat (*Triticum aestivum)* with *Azospirillum* spp. in Brazil. *Biology and Fertility of Soils*, *4,*(1-2), 37–40.

Baldani, V. L. D., & Döbereiner, J. (1980). Host-plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biology and Biochemistry,*, *12*(4), 433–439.

Banerjee, M. R., Yesmin, L., & Vessey, J. K. (2006). Plant-growth-promoting rhizobacteria as biofertilizers and biopesticides. In M. K. R. (ed.) (Ed.), *Handbook of Microbial Biofertilizers.* (pp. p. 137–183.). New York.: Food Products Press,.

Bardhana, S., Josea, S., Jenkinsb, M. A., Websterc, C. R., Udawattaa, R. P., & Stehnc, S. E. (2012). Microbial community diversity and composition across a gradient of soil acidity in spruce–fir forests of the southern Appalachian Mountains. *Applied Soil Ecology*, *61*, 60–68.

Bashan, Y. (1986). Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biology and Biochemistry*, *18:*, 297–301.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnol. Adv.*, *16*, 729–770.

Bashan, Y., Bustillos, J. J., Leyva, L. a., Hernandez, J.-P., & Bacilio, M. (2005). Increase in auxiliary photoprotective photosynthetic pigments in wheat seedlings induced by *Azospirillum brasilense*. *Biology and Fertility of Soils*, *42*(4), 279–285. doi:10.1007/s00374-005-0025-x

Bashan, Y., & de-Bashan, L. E. (2010). How the Plant Growth-Promoting Bacterium *Azospirillum* Promotes Plant Growth — A Critical Assessment. *Advances in Agronomy* (1st ed., Vol. 108, pp. 77–136). Elsevier Inc. doi:10.1016/S0065-2113(10)08002-8

Bashan, Y. and Dubrovsky, J. G. (1996). Azospirillum spp. participation in dry matter partitioning in grasses at the whole plant level. *Biology and Fertility of Soils*, *23*, 435–440.

Bashan, Y., & Gonzalez, L. E. (1999). Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. *Applied Microbiology and Biotechnology*, *51*(2), 262–266. doi:10.1007/s002530051391

Bashan, Y., Harrison, S. K., & Whitmoyer, R. E. (1990). Enhanced Growth of Wheat and Soybean Plants Inoculated with *Azospirillum brasilense* Is Not Necessarily Due to General Enhancement of Mineral Uptake †. *Applied and Environmental Microbiology*, *56*(3), 769–775.

Bashan, Y., & Holguin, G. (1994). Root-to-Root Travel of the Beneficial Bacterium *Azospirillum brasilense*. *Applied and Environmental Microbiology*, *60*(6), 2120–31. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=201610&tool=pmcentrez&rendertype=abstract

Bashan, Y., & Holguin, G. (1997). *Azospirillum* -plant relationships: environmental and physiological advances (1990-1996). *Canadian Journal of Microbiology*, *43*, 103–121.

Bashan, Y., Holguin, G., & De-Bashan, L. E. (2004). *Azospirillum*-plant relationships: physiological, molecular, agricultural, and enviromental advances (1997 - 2003). *Canadian Journal of Microbiology*, *50:*, 521 – 577.

Bashan, Y., & Levanony, H. (1985). An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*. *Canadian Journal of Microbiology,*, *31*(10), 947–952.

Bashan, Y., & Levanony, H. (1990). Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. *Canadian Journal of Microbiology*, *36:*, 591–608.

Bashan, Y., Ream, Y., Levanony, H., & Sade, A. (1989). Nonspecific responses in plant growth, yield, and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. *Canadian Journal of Botany,*, *67*(5), 1317–1324.

Bashan, Y, Singh, M., Levanony, H. (1989). Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation. *Canadian Journal of Botany*, *67,*, 2429–2434.

Bashan, Y., & Holguin, G. (1995). Inter-root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. *Microbial Ecology*, *29:*, 269–281.

Beijerinck, M. W. (1925). Zentralblatt fur Bakteriologie, Parasitenkunde, Infektion-skrankheiten und Hygiene. Abteilung, *11*(63), 353–359.

Beschoren, da C. P., & Anelise Beneduzi, Rocheli de Souza, Rodrigo Schoenfeld, Luciano Kayser Vargas, and L. M. P. (2013). “The effects of different fertilization conditions on

bacterial plant growth promoting traits: guidelines for directed bacterial prospection and testing." *Plant and Soil*, *1-*, 14.

Biswas, J., Ladha, J., & Dazzo, F. (2000). Rhizobial inoculation influences seedling vigor and yield of rice. *Agron J*, *92*, 880–886.

Bláha, L., Babica, P., & Maršálek, B. (2009). Toxins produced in cyanobacterial water blooms - toxicity and risks. *Interdisciplinary Toxicology*, *2*(2), 36–41. doi:10.2478/v10102-009-0006-2

Bláhová, L., & Babica P, Maršálková E, Smutná M, Maršálek B, B. L. (2007). Concentrations and seasonal trends of extracellular microcystins in freshwaters of the Czech Republic – results of the national monitoring program. *CLEAN -Soil, Air, Water.*, *35:*, 348–354.

Bloemberg, G., & Lugtenberg, B. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr Opin Plant Biol.*, *4*((4)), 343–50.

Boddey, R. M., Baldani, V. L. D., Baldani, J. I., & Döbereiner, J. (1986). Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field-grown wheat. *Plant and Soil*, *Volume 95,*(Issue 1,), pp 109–121.

Boivin, C., Camut, S., Malpica, C. A., Truchet, G., & Rosenberg, C. (1990). Rhizobium meliloti Genes Encoding Catabolism of Trigonelline Are Induced under Symbiotic Conditions. *The Plant Cell*, *2*(12), 1157–1170.

Bottini, R., Fulchieri, M., Pearce, D., & Pharis, and R. P. (1989). Identification of Gibberellins A1, A3, and Iso-A3 in Cultures of *Azospirillum lipoferum*. *Plant Physiol.*, *90*((1):), 45–47.

Bowen, G. D., and Rovira, A. D. (1999). The rhizosphere and its management to improve plant growth. *Advances in Agronomy,*, *66*, 1–102.

Bremer, C., Braker, G., Matthies, D., Reuter, A., Engels, C., & Conrad, R. (2007). Impact of plant functional group, plant species, and sampling time on the composition of nirK-type denitrifier communities in soil. *Applied and Environmental Microbiology*, *73*(21), 6876–84. doi:10.1128/AEM.01536-07

Brockwell, J. (1963). Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*. *Appl Microbiol*, *11:*, 377–383.

Broek, A. Vande, Gysegom, P., Ona, O., Hendrickx, N., Prinsen, E., Impe, J. Van, & Vanderleyden, J. (2005). Transcriptional Analysis of the *Azospirillum brasilense* Indole-3-Pyruvate Decarboxylase Gene and Identification of a cis-Acting Sequence Involved in Auxin Responsive Expression. *Molecular Plant-Microbe Interactions*, *18,*(4), Pages 311–323.

Camargo, J. A., & Alonso, Á. (2006). Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. *Environment International*, *32*, 831–849.

Chen C, Bauske EM, Musson G, Rodriguez-Kaban˜a R, K. J. (1994). Biological control of Fusarium on cotton by use of endophytic bacteria. *Biol Control*, *5:*, 83–91.

Chin-A-Woeng, T. F. C., Bloemberg, G. V. and Lugtenberg, B. J. J. (2003). Phenazines and their role in biocontrol by Pseudomonas bacteria. *New Phytologist*, *157,*, 503–523.

Chorus, I. (2001). *Cyanotoxins - Occurrence, Causes, Consequences*. (Ingrid Chorus, Ed.) (Springer; ., p. 357).

Codd, G. A., Morrison, L. F., & Metcalf, J. S. (2005). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology*, *203*(3), 264–72. doi:10.1016/j.taap.2004.02.016

Colwell, R. R. (1979). Enumeration of specific populations by the most probable number (MPN) method. In Native aquatic bacteria: Enumeration, activity, and ecology. Edited by J.W. Costerton and R.R. Colwell. ASTM STP 695. pp. *American Society for Testing and Materials, Philadelphia.*, 5661.

Compant, S., Duffy, B., Nowak, J., Clement, C., & Barka, E. A. (2005). Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases : Principles , Mechanisms of Action , and Future Prospects MINIREVIEW Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases : Principles , Mechanisms of Action , and. *Appl Environ Microbiol.*, *71*(9), 4951–4959. doi:10.1128/AEM.71.9.4951

Creus, C., Sueldo, R., & Barassi, C. (1997). Shoot growth and water status in *Azospirillum*-inoculated wheat seedlings grown under osmotic and salt stresses. *Plant Physiol Biochem.*, *35*(9), 39–944.

Crits-Christoph, A., Robinson, C., Barnum, T., Fricke, W., Davila, A., Jedynak, B., … DiRuggiero, J. (2013). Colonization patterns of soil microbial communities in the Atacama Desert. *Microbiome*, *1*(1), 28. Retrieved from http://www.microbiomejournal.com/content/1/1/28

Culman, S. W., Bukowski, R., Gauch, H. G., Cadillo-Quiroz, H., & Buckley, D. H. (2009). T-REX: software for the processing and analysis of T-RFLP data. *BMC Bioinformatics*, *10*, 171. doi:10.1186/1471-2105-10-171

Dalla Santa, O. R., Hernández, R. F., Alvarez, G. L. M., Junior, P. R., & Soccol, C. R. (2004). *Azospirillum* sp . Inoculation in Wheat , Barley and Oats Seeds Greenhouse Experiments. *Brazilian Archives Ofbiology and Technilogy*, *47*(11), 843–850.

Dary, M. Chamber-Pérez, M.A., Palomares, A.J., P. (2010). “In situ” phytostabilisation of heavy metal polluted soils using Lupinus luteus inoculated with metal resistant plant-growth promoting rhizobacteria. *Journal of Hazardous Materials*, *177*(1-3), 323–330.

Das, A. J., Kumar, M., & Kumar, R. (2013). Plant Growth Promoting Rhizobacteria (PGPR): An Alternative of Chemical Fertilizer for Sustainable , Environment Friendly Agriculture. *Research Journal of Agriculture and Forestry Sciences*, *1*(4), 21–23.

Datta C, Basu. P. (2000). Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, Cajanus cajan. *Microbiol Res.*, *155*(2), 123–7.

Davies, P. J. (2004). *Plant Hormones: Biosynthesis, Signal Transduction,Action!, , 2004.* (p. 750 pages). Kluwer Academic, Dordrecht, the Netherlands: Springer,.

De Freitas, J. R., & Germida, J. J. (1992). Growth promotion of winter wheat by fluorescent pseudomonads under growth chamber conditions. *Soil Biology and Biochemistry*, *24*(11), 1127–1135. doi:10.1016/0038-0717(92)90063-4

Deaker, R., & Casteriano, G. M. and A. (2008). Estimating the most probable number of bacteria in multistrain biofertiliser inoculants using a multiple-tube fermentation test. In *ACIAR Proceedings :efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers* (pp. 108–116).

Deaker, R. J., & and Kennedy, I. R. (2001). Improved Potential for Nitrogen Fixation in Azospirillum brasilense Sp7-S Associated with Wheat nifH Expression as a Function of Oxygen Pressure. *Acta Biotechnologica*, *21.*(1), 3–17.

De-Bashan, L.E.Hernandez, P., J., & Bashan, Y. (2012). The potential contribution of plant growth-promoting bacteria to reduce environmental degradation – A comprehensive evaluation. *Applied Soil Ecology*, *61:*, 171–189.

Dekhil, B. S., Cahill, M., Stackebrandt, E., & Sly, L. I. (1997). Transfer of *Conglomeromonas largomobilis* subsp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largimobile* comb. nov., and elevation of *Conglomeromonas largomobilis* subsp. parooensis to the

new type species of *Conglomeromonas*, *Conglomeromonas* paro. *Appl Microbiol*, *20,*, 72–77.

Diaz, R. J., & Rosenberg, R. (2008). Review Spreading Dead Zones and Consequences for Marine Ecosystems,. *Science*, *321*.(5891), 926 – 929.

Diaz-Zorita, M., & Fernandez-Canigia, M.V. (2009). Field performance of a liquid formulation of *Azospirillum brasilense* on dryland wheat productivity. *European Journal of Soil Biology*, *45*(1), 3–11. doi:10.1016/j.ejsobi.2008.07.001

Dobbelaere, S., Croonenborghs, A., & Amber Thys, David Ptacek, Jos Vanderleyden, Pablo Dutto, Carlos Labandera-Gonzalez, Jesus Caballero-Mellado, Juan Francisco Aguirre, Yoram Kapulnik, Shimon Brener, Saul Burdman, Daniel Kadouri, S. S. and Y. O. (2001). Responses of agronomically important crops to inoculation with *Azospirillum*. *Australian Journal of Plant Physiology*, *28*((9)), 871 – 879.

Dobbelaere, S. and Okon, Y. (2007). *The plant growth promoting effects and plant responses. In Nitrogen Fixation: Origins, Applications and Research Progress. Newton W. (Ed), Vol V: Associative and Endophytic Nitrogen-Fixing Bacteria and Cyanobacterial Asso*. (C. E. and W. E. Newton., Ed.) (p. pp 145–170.). Heidelberg: Springer,.

Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A., Vanderleyden, J. (1999). Phyto stimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. *Plant and Soil*, *212*, 155–164.

Dobereiner, J. and. Day. J. (1976). Associative symbiosis in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In Proceedings of the First International Symposium on Nitrogen Fixation. Edited by W.E. Newton and C.J. Nyman, Washington State University Press, P. In *In Proceedings of the First International Symposium on Nitrogen Fixation. Edited by W.E. Newton and C.J. Nyman, Washington State University Press, Pullman, Wash.* (pp.518–538).

Döbereiner, J. and Pedrosa, F. O. (1987). Nitrogen Fixing Bacteria In Non-Leguminous Crop Plants. Madison, WI, USA.: Science Tech Publishers,.

Donate-Correa, J., León-Barrios, M., & Pérez-Galdona, R. (2005). Screening for plant growth-promoting rhizobacteria in Chamaecytisus proliferus (tagasaste), a forage tree-shrub legume endemic to the Canary Islands. *Plant and Soil*, *Volume 266*(1-2), pp 261–272.

Dubuis, C., Keel, C., & Haas, D. (2007). Dialogues of root-colonizing biocontrol pseudomonads. In P. A. H. M. Bakker, J. M. Raaijmakers, G. Bloemberg, M. Höfte, P. Lemanceau, & B. M. Cooke (Eds.), *New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research* (p. pp 311–328). Springer Netherlands.

Eckert, B., Weber, O. B., Kirchhof, G., Halbritter, A., Stoffels, M., & Hartmann, A. (2001). *Azospirillum doebereinerae* sp . nov ., a nitrogen-fixing bacterium associated with the C 4 -grass Miscanthus. *International Journal of Systematic and Evolutionary Microbiology*, *51*(1), 17–26.

El-Mohandes, M. (1999). The use of associative diazotrophs withdifferent rates of nitrogen fertilization and compost to enhance growth and N 2 -fixation of wheat. *Bulletin of Faculty of Agriculture, University of Cairo*, *50*, 729–753.

Escoffier, S., LeMer, J., & Roger, P. (1997). Enumeration of methanotrophic bacteria in rice field soils by plating and MPN techniques: a critical approach. *Eur J Soil Biol*, *33:*, 41–51.

Fallik, E. Sarig, S. and Okon, Y. (1994). Morphology and physiology of plant roots associated with *Azospirillum*. In Y. Okun (Ed.), *In Azospirillum Plant Associations* (pp. 77–84). CRC Press, Raton,.

Felici, C., Vettori, L., Giraldi, E., Forino, L. M. C., Toffanin, A., Tagliasacchi, A. M., & Nuti, M. (2008). Single and co-inoculation of Bacillus subtilis and *Azospirillum brasilense* on Lycopersicon esculentum: Effects on plant growth and rhizosphere microbial community. *Applied Soil Ecology*, *40*(2), 260–270. doi:10.1016/j.apsoil.2008.05.002

Feng, K., Lu, H. M., Sheng, H. J., Wang, X. L., & Mao, J. (2004). Effect of organic ligands on biological availability of inorganic phosphorus in soils. *Pedosphere*, *14*(1), 85–92.

Feray, C., Volat, B., Degrange, V., & Clays-Josserand, A Montuelle, B. (1999). Assessment of three methods for detection and quantification of nitrite-oxidizing bacteria and Nitrobacter in freshwater sediments (MPN-PCR, MPN-Griess, immunofluorescence). *Microbial Ecol*, *37:*, 208–217.

Ferreira, a. S., Pires, R. R., Rabelo, P. G., Oliveira, R. C., Luz, J. M. Q., & Brito, C. H. (2013). Implications of *Azospirillum brasilense* inoculation and nutrient addition on maize in soils of the Brazilian Cerrado under greenhouse and field conditions. *Applied Soil Ecology*, *72*, 103–108. doi:10.1016/j.apsoil.2013.05.020

Fischer SE, Miguel MJ, M. G. (2003). Effect of root exudates on the exopolysaccharide composition and the lipopolysaccharide profile of *Azospirillum brasilense* Cd under saline stress. *FEMS Microbiol Lett.*, *14;*(219(1):), 53–62.

Galal, Y. G. M., El-Ghandour, I. A., Aly, S. S., Soliman, S., & Gadalla, A. (2000). Non-isotopic method for the quantification of biological nitrogen fixation and wheat production under field conditions. *Biology and Fertility of Soils*, *32*(1), 47–51.

Gallo, del M., & J, Fendrik. (1994). The rhizosphere and *Azospirillum* ; in *Azospirillum* –plant associations (ed)Y Okon). (Boca Raton:CRC Press.

Gamalero, E., Berta, G., Massa, N., Glick, B. R. and Lingua, G. (2008). Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus Gigaspora rosea positively affect cucumber plant growth. *FEMS Microbiology Ecology*, *64*(459-467).

Ganguly, T. K.; Jana, A. K.; Moitra, D. N. (1999). An evaluation of agronomic potential of *Azospirillum brasilense* and *Bacillus megaterium* in fibre-legume-cereal system in an Aeric haplaquept. *Indian Journal of Agricultural Research*, *33*(1), 35–39.

Gardener, McSpadden B. B. and Fravel, D. R. (2002). Biological control of plant pathogens: Research, commercialization, and application in the USA. *Plant Health Progress*. Retrieved from Online. doi:10.1094/PHP-2002-0510-01-RV.

Gholami, A., Shahsavani, S., Nezarat, S. (2009). The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. *Int J Biol Life Sci*, *1*(1), 35–40.

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol*, *41*, 109–117.

Glick, B. R. (2005). Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiology Letters*, *251*(1), 1–7.

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, *2012*, 963401. doi:10.6064/2012/963401

Glick, B. R., Cheng, Z., Czarny, J., & Duan, J. (2007). Promotion of plant growth by ACC deaminase-producing soil bacteria. In *New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research* (pp. 329–339).

Glick, B. R., Patten, C. L., Holguin, G., & Penrose, G. M. (1999). *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria*. London: Imperial College Press.

Goldstein, A. H. (1986). "Bacterial solubilisation of mineral phosphates: historical perspective and future prospects." *American Journal of Alternative Agriculture*, *1*, 51–57.

González, B. A. D., Chen, P., Tiscareño-López, M., & Srinivasan, R. (2002). Using Satellite and Field Data with Crop Growth Modeling to Monitor and Estimate Corn Yield in Mexico. *Crop Sci.*, *42*, 1943–1949. doi:doi:10.2135/cropsci2002.1943

Gonzalez JM. (1996). A general purpose program for obtaining most probable number tables. *J Microbiol Meth*, *26*, 215–218.

Goodridge, C., Goodridge, L., Gottfried, D., Edmonds, P., & Wyvill, J. (2003). A rapid most-probable-number-based enzyme-linked immunosorbent assay for the detection and enumeration of Salmonella typhimurium in poultry wastewater. *J Food Prot*, *66:*, 2302–2306.

Graham, P. H. (1998). Symbiotic nitrogen fixation. In D. S. et al. (Eds. . P. Hall. (Ed.), *Principles and applications of soil microbiology.* (p. pp 325–347.). Prentice Hall.

Gupta A, Gopal M, T. K. (2000). Mechanism of plant growth promotion by rhizobacteria. *Indian J Exp Biol*, *38*, 856–862.

Gyaneshwar, P., Kumar, G. N., Parekh, L. J. and Poole, P. S. (2002). Role of soil microorganisms in improving P nutrition of plants. *Plant and Soil*, *245,*, 83–93.

Haas, D., & Keel, C. (2003). "Regulation of antibiotic production in root-colonizing Pseudomonas spp. and relevance for biological control of plant disease,." *Annual Review of Phytopathology,*, *vol.41,*, pp. 117 –153,.

Hammer, Ø. Harper, D. A. T. and P. D. R. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis.Palaeontologia Electronica 4(1). *Palaeontologia Electronica*. Retrieved from http://folk.uio.no/ohammer/past

Han, S. O., & New, P. B. (1998). Variation in Nitrogen Fixing Ability among Natural Isolates of *Azospirillum. Microbial Ecology*, *36*(2193-201).

Hancock, J. F. (2004). *Plant Evolution and the Origin of Crop Species* (p. 313 pages). CABI, Science.

Hartmann, A.; Baldani, J. I. (2006). The Genus *Azospirillum. In: TheProkaryotes, Springer, New York*, *5,*, 115–140.

Hegazi, N. A., Fayez, M., Amin, G., Hamza, M. A., Abbas, M., Youssef, H., & Monib, M. (1998). "Diazotrophs associated with non-legumes grown in sandy soils",. In *The 7th International Symposium on Nitrogen Fixation With Non-Legumes,* (pp. 209–222,). Faisalabad, Pakistan: Kluwer Academic Publishers, Dordrecht, The Netherlands,.

http://data.daff.gov.au/brs/data/warehouse/pe_abares99001787/ACR11.1_Feb_REPORT.pdf) . (n.d.).

http://en.wikipedia.org/wiki/Wheat#Genetics. .

http://www.bom.gov.au/climate/cdo/about/about-airtemp-data.shtml.

http://www.bom.gov.au/climate/cdo/about/about-rain-data.s html.

http://www.bom.gov.au/climate/data.

http://www.fao.org. (n.d.). Retrieved from http://www.fao.org

http://www.fao.org/docrep/018/al999e/al999e.pdf.Food Outlook, Biannual report on global food markets. (2013). *FAO OutlookTeam, Food*. doi:http://www.fao.org/docrep/018/al999e/al999e.pdf

http://www.fas.usda.gov//psdonline.

http://www.pwc.com.au/industry/agribusiness/assets/Australian-Grains-Industry-Nov11.pdf.

Hungria, M., Campo, R. J., Souza, E. M., & Pedrosa, F. O. (2010). Inoculation with selected strains of *Azospirillum brasilense* and *A. lipoferum* improves yields of maize and wheat in Brazil. *Plant and Soil*, *331*(1-2), 413–425. doi:10.1007/s11104-009-0262-0

Innerebner, G., Knief, C., & Vorholt, J. A. (2011). "Protection of Arabidopsis thaliana against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system,." *Applied and Environmental Microbiology,*, *vol. 77, n*(no. 10,), pp. 3202–3210.

Jahanian, A., Chaichi, M. R., Rezaei, K., Rezayazdi, K., & Khavazi, K. (2012). The effect of plant growth promoting rhizobacteria (pgpr) on germination and primary growth of artichoke (*Cynara scolymus*). *Int J Agri Crop Sci*, *4*, 923–929.

Jain, D.K. and D.G. Patriquin. (1984). Root hair deformation, bacterial attachment and plant growth in wheat-*Azospirillum* associations. *Appl. Environ. Microbiol.,*, *48:*, 1208–1213.

James, E. K., & Olivares, F. L. (1997). Infection and Colonization of Sugar Cane and Other Graminaceous Plants by Endophytic Diazotrophs. *Critical Reviews in Plant Sciences,*, *17*(1), 77–119.

Johnson, A., & Henriksen, S. (2009). Microplate MPN-enumeration of monocyclic- and dicyclic-aromatic hydrocarbon degraders via substrate phase-partitioning. *Biodegradation*, *20,*, 581–589.

Jolly, S., Shanta, N., Zahed, U., & Khan, M. (2010). Quantification of Heterotrophic Bacteria and *Azospirillum* from the Rhizosphere of Taro (Colocasia esculenta L. Schott.) and the Nitrogen Fixing Potential of Isolated *Azospirillum. International Journal of Botany.*, *6*(2), 117–121.

Joo, G. J., Kim, Y. M., & Lee, I.J. Song, K.S. Rhee, I. K. (2004). "Growth promotion of red pepper plug seedlings and the production of gibberellins by *Bacillus cereus*, *Bacillus macroides* and *Bacillus pumilis*." *Biotechnology Letters*, *26;*, 487–491.

Joseph, B., Patra, R. R., & Lawrence, R. (2007). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L .), International Journal of plant Production,1(2), 141-152.

Kapulnik, Y., Okon, Y., & Henis, I. (1985). Changes in root morphology of wheat caused by *Azospirillum* inoculation. *Canadian Journal of Microbiology*, *31*(10), 881–887.

Kapulnik, Y., Sarig, S., Nur, I., & Okon, Y. (1983). Effect of *Azospirillum* inoculation on yield of field-grown wheat. *Canadian Journal of Microbiology*, *29,*(8), 895–899. doi:10.1139/m83-145

Kapulnik, Y., Sarig, S., Nur, I., Okon, Y., Kigel, J., & Henis, Y. (1981). Yield Increases in Summer Cereal Crops in Israeli Fields Inoculated with *Azospirillume*. *Experimental Agriculture*, *17*(2), 179–187. doi:doi:10.1017/S0014479700011431.

Katupitiya, S., Millet, J., Vesk, M., Viccars, L., Zeman, Lidong, Z.,Kennedy, I. R. (1995). A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. *Applied and Environmental Microbiology*, *61*(5), 1987–95. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=167461&tool=pmcentrez&rendertype=abstract

Kennedy I R. (1991). *Acid Soil and Acid Rain* . (p. 254). Research Studies Press/ Wiley. Chichester, UK.

Kennedy, I. (2004). Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? *Soil Biology and Biochemistry*, *36*(8), 1229–1244. doi:10.1016/j.soilbio.2004.04.006

Kennedy, I. R., Pereg-Gerk, L. L., Wood, C., Deaker, R., Gilchrist, K., & Katupitiya, S. (1997). Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. *Plant and Soil*, *194*(1-2), 65–79.

Kennedy, I.R., Cocking, E. C. (1997). *Biological Nitrogen Fixation: The Global Challenge & Future Needs.* (p. 83). Sydney (Australia): SUNFix Press, University of Sydney.

Kennedy, I.R., Tchan, Y. (1992). Biological nitrogen fixation in nonleguminous field crops: recent advances. *Plant and Soil*, *141,*, 93–118.

Keyeo, F., Shah, O. N. A., & Amir, H. G. (2011). The effects of nitrogen fixation activity and phytohormone production of diazotroph inpromoting growth of rice seedlings. *Biotechnology*, *10*(3), 267–273. doi:10.3923/bitech.2011.267.273

Khalid, Arshad, M., & Zahir, Z. (2004). Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology*, *96*(3), 473–480. doi:10.1046/j.1365-2672.2003.02161.x

Khalid, A., Tahir, S., Arshad, M. and Zahir, Z. A. (2004). Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere and non-rhizosphere soils. Australian Journal of Soil Research. *Australian Journal of Soil Research*, (42), 921–926.

Khammas KM, Ageron E, Grimont PA, Kaiser P. (1989). *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soiL. *Research in Microbiology*, *140*(9), 679–693.

Kim, J., & Rees, D. C. (1994). Nitrogenase and biological nitrogen fixation. *Biochemirty*, *33*(2), pp 389–397. doi:DOI: 10.1021/bi00168a001

Kim, S., Lowman, S., Hou, G., Nowak, J., Flinn, B., & Mei, C. (2012). Growth promotion and colonization of switchgrass (*Panicum virgatum*) cv. Alamo by bacterial endophyte

Burkholderia phytofirmans strain PsJN. *Biotechnology for Biofuels*, *5*(1), 37. doi:10.1186/1754-6834-5-37

Kloepper, J. W. (1994). Plant Growth Promoting Rhizobacteria (other systems). In Y. Okon (Ed.), *Azospirillum/plant associations* (pp. 111–118). CRC press, Boca Raton,Florida,USA.

Kloepper, J. W., N.Schroth, & Miller, T. . (1980). Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant development and yield. *Phytopathology*, *70*, 1078–1082.

Kloepper, J.W. and C.J. Beauchamp. (1992). A review of issues related to measuring of plant roots by bacteria. *Can. J. Microbiol.*, *38*, 1219–1232.

Kloepper, J.W., R. L. and R. M. Z. (1989). Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol.,*, *7*, 39–44.

Kloepper, J.W., J. Leong, M. T. and M. N. S. (1980). Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature*, *286*, 885–886.

Kloepper, J.W., Zablotowick, R.M., Tipping, E.M., Lifshitz, R. (1991). Plant growth promotion mediated by bacterial rhizosphere colonizers. In P. B. (Eds. . In: Keister, D.L., Cregan (Ed.), *The Rhizosphere and Plant Growth.* (pp. pp. 315–326.). Dordrecht,Netherlands: Kluwer Academic Publishers,.

Knauth, S., Hurek, T., Brar, D., & Reinhold-hurek, B. (2005). Influence of different Oryza cultivars on expression of nifH gene pools in roots of rice, *7*, 1725–1733. doi:10.1111/j.1462-2920.2005.00841.x

Kolb, W., & Martin, P. (1988). Influence of nitrogen on the number of N2-fixing and total bacteria in the rhizosphere. *Soil. Biol. Biochem.*, *20*, 221.

Kuai, L., & Arjun A. Nair, A. M. F. P. (2001). Rapid and Simple Method for the Most-Probable-Number Estimation of Arsenic-Reducing Bacteria,. Applied and Environmental Microbiology., *67*(7), 3168–3173.

Kundu, B. S., & Gaur, A. C. (1980). Establishment of nitrogen-fixing and phosphate-solubilising bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop. *Plant and Soil*, , *Volume 5*(Issue 2-3,), pp 223–230.

Lambrecht M, Okon Y, Vande Broek A, V. J. (2000). Indole-3-acetic acid: a reciprocal signalling molecule in bacteria-plant interactions. *Trends Microbiol.*, *8*(7), 298–300.

Lemaux, P. G. (1999). Plant growth regulators and biotechnology,. In *Western Plant Growt h Regulator Society meeting,*. Anaheim, CA, USA.

Lerner, A., Herschkovitz, Y., Baudoin, E., Nazaret, S., Moenne-Loccoz, Y., Okon, Y., & Jurkevitch, E. (2006). Effect of *Azospirillum brasilense* inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis. *Soil Biology and Biochemistry,*, *38*(6), 1212–1218.

Liljeroth, E., Baath., E., Mathiasson, I., & Lundborg, T. (1990). Root exudation and rhizosphere bacterial abudance of barley (*Hordeum vulgare* L.) in relation to nitrogen fertilization and root growth. *Plant and Soil,*, *127,*, 81,.

Liu L, Kloepper JW, T. S. (1995). Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. *Phytopathology*, *85,*, 695–698.

Liu, W., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA. *Applied and Environmental Microbiology*, *63*(11), 4516–4522.

Lobell, D. B., Ortiz-Monasterio, J. I., Gregory P. Asner, R., Naylor, osamond L., & Falcon, and W. P. (2005). Combining Field Surveys, Remote Sensing and Regression Trees to Understand Yield Variations in an Irrigated Wheat Landscape. *Agronomy Journal,*, *97,*(1), 241–249.

Lofton, J., & Brenda S. Tubana, Yumiko Kanke, Jasper Teboh, Howard Viator, and M. D. (2012). Estimating Sugarcane Yield Potential Using an In-Season Determination of Normalized Difference Vegetative Index. *Sensors (Basel)*, *12*(6), 7529–7547.

Lovell, Charles R., Piceno, Yvette M. , Quattro Joseph M., and Bagwell, C. E. (2000). Molecular Analysis of Diazotroph Diversity in the Rhizosphere of the Smooth Cordgrass, Spartina alterniflora. *Appl Environ Microbiol.*, *Sep . 66*(sep (9): 3814–3822), sep (9): 3814–3822.

Lucy, M., Reed, E., & Glick, B. R. (2004). “Applications of free living plant growth-promoting rhizobacteria,.” *Antonie van Leeuwenhoek*, *vol. 86,*(1), 1–25.

Lukow1, T., Dunfield, P., & Liesack, W. (2000). Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiology Ecology*, *32*(3), 241–247. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10858583

Magalhes, F.M. Baldani, J.I. Souto, S.M. Kuykendall, J.R. Dobereiner, J. (1983). A new acid-tolerant Azospirillum species. *An. Acad. Brasil Ciênc.,*, *55*, 417–430.

Malik, K. A., Mirza, M., Hassan, U., Mehnaz, S., Rasul, G., Haurat, J., Normand, P. (2002). The role of plant-associated beneficial bacteria in rice-wheat cropping system. In Kennedy IR & C. ATMA (Eds.), *Biofertilisers in action. Rural Industries Research and Development Corporation,* (p. pp 73–83). Canberra,.

Marti, J., Bort, J., Slafer, G. A., & Araus, J. L. (2007). Can wheat yield be assessed by early measurements of Normalized Difference Vegetation Index? *Annals of Applied Biology,*, *150*(2), 253–257.

Maurhofer M, Reimann C, Sacherer SP, Heebs S, Haas D, D. G. (1998). Salicyclic acid biosynthetic genes expressed in Pseudomonas fluorescens strain P3 improve induction of systemic resistance in tobacco against necrosis virus. *Phytopathology*, *88,*, 678–684.

Mavrodi, D. V., Blankenfeldt, W. and Thomashow, L. S. (2006). Phenazine compounds in fluorescent Pseudomonas spp.: Biosynthesis and regulation. *Annual Review of Phytopathology*, *44,*, 417–445.

Mehnaz, S., Weselowski, B., & Lazarovits, G. (2007). *Azospirillum zeae* sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of *Zea mays*. *International Journal of Systematic and Evolutionary Microbiology*, *57*(Pt 12), 2805–9. doi:10.1099/ijs.0.65128-0

Mercado-Blanco, J. and Bakker, P. A. H. M. (2007). "Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection." *Antonie van Leeuwenhoek*, *92;*, 367–389.

Merrington G, Winder L, Parkinson R, R. M. (2002). *"Agricultural Pollution:Environmental Problems and Practical Solutions."* London and New York: Spon Press:

Michiels, K., De Troch, P., Onyeocha, I., Van Gool, A., Elmerich, C., & Vanderleyden, J. (1989). Plasmid localization and mapping of two *Azospirillum brasilense* loci that affect exopolysaccharide synthesis. *Plasmid*, *21*(2), 142–146. doi:10.1016/0147-619X(89)90058-9

Michiels, K. W., Croes, C. L., & Vanderleyden, J. (1991). Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *Journal of General Microbiology*, *137*(9), 2241–2246. doi:10.1099/00221287-137-9-2241

Miller, J. H. (1972). *Experiments in molecular genetics Bacterial genetics - E. coli*. (Jeffrey H. Miller, Ed.) (3, illustr., p. 466). Cold Spring Harbor Laboratory, University of Michigan.

Miller, L. D., Russell, M. H. and Alexandre, G. (2009). Diversity in bacterial chemotactic responses and niche adaptation. *Advances in Applied Microbiology*, *66,*, 53–75.

Mullen, M. D. (2005). "Phosphorus and other elements."),. In Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G., Zuberer, D.A. (Ed.), *Principles and applications of soil microbiology* (pp. pp. 463–488,). New Jersey, USA.: Pearson Education,.

Naiman, A. D., Latrónico, A., & García de Salamone, I. E. (2009). Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens:* Impact on the production and culturable rhizosphere microflora. *European Journal of Soil Biology*, *45*(1), 44–51. doi:10.1016/j.ejsobi.2008.11.001

Nascimento, F. X., Brígido, C., Glick, B. R., Oliveira, S., & Alho, L. (2012). "Mesorhizobium ciceri LMS-1 expressing an exogenous ACC deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints,." *Letters in Applied Microbiology,, vol. 55*, , pp. 15–21,.

Nelson, L. M. (2004). Plant Growth Promoting Rhizobacteria (PGPR): Prospects for New Inoculants. *Crop Management*. doi:10.1094/CM-2004-0301-05-RV

Nielsen, J. L., Juretschko, S., Wagner, M., & Nielsen, P. H. (2002). Abundance and Phylogenetic Affiliation of Iron Reducers in Activated Sludge as Assessed by

Fluorescence In Situ Hybridization and Microautoradiography. Applied and Environmental Microbiology*, S*, *68*(9), 4629–4636.

O'Sullivan, D. J., & O'Gara, F. (1992). "Traits of Fuorescent Pseudomonas spp. involved in suppression of plant root pathogens,. *" Microbiological Reviews,*, *vol. 56,*(no. 4), pp. 662–676,.

Okada, T., Sugahara, K., Masayuki, H., Masahiro, S., Miura, & Watanabe, and Y. (2008). The Relationship between Wheat Yield and the Vegetation Indices Using Image Data from Aerial Photographs in the Kanto Region of Japan. In World Conference on Agricultural information and It. IAALD AFITA WCCA.

Okon, Y. (1985). *Azospirillum* as a potential inoculant for agriculture. *Trends in Biotechnology*, *3*(9), 223–228. doi:10.1016/0167-7799(85)90012-5

Okon, Y. (1994). *Azospirillum/plant associations,* (p. pp. 175.). Boca Raton, FL.: CRC Press,.

Okon, Y., Albrecht, S. L., & Burris, R. H. (1977). Methods for Growing *Spirillum lipoferum* and for Counting It in Pure Culture and in Association with Plants. *Applied and Environmental Microbiology*, *33*(1), 85–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=170579&tool=pmcentrez&rendertype=abstract

Okon, Y., & Itzigsohn, R. (1995). The development of *Azospirillum* as a commercial inoculant for improving crop yields. *Biotechnol Adv.*, *13*(3), 415–424.

Okon, Y., & Kapulnik, Y. (1986). Development and function of *Azospirillum*- inoculated roots. *Plant and Soil*, *90*, 3–16.

Okon, Y., Labandera-Gonzalez, & A, C. (1994). Agronomic applications of *Azospirillum*:an evaluation of 20 years worldwide field inoculaion. *Soil Biology and Biochemistry*, *26*(12), 1591–1601. Retrieved from http://www.sciencedirect.com/science/article/pii/0038071794903115

Okon, Y. and Kapulnik, Y. (1986). Development and function of *Azospirillum*-inoculated roots. *Plant and Soil,*, *90*, :3–16.

Okon, Y. and Vanderleyden, J. (1997). Root-associated *Azospirillum* species can stimulate plants. *ASM News*, *63*, 366–370.

Ona, O., Smets, I., Gysegom, P., Bernaerts, K., Impe, J. V., Prinsen, E., & Vanderleyden, J. (2003). The effect of pH on indole-3-acetic acid (IAA) biosynthesis of *Azospirillum brasilense* sp7. *Symbiosis*, *35*, 199–208.

Ona, O., Van Impe, J., Prinsen, E. and Vanderleyden, J. (2005). Growth and indole-3- acetic acid biosynthesis of *Azospirillum brasilense* Sp245 is environmentally controlled. *FEMS Microbiology Letters*, *246,*, 125–132.

Osborn, a M., Moore, E. R., & Timmis, K. N. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environmental Microbiology*, *2*(1), 39–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11243261

Osborne CA, Rees GN, Bernstein Y, J. P. (2006). (2006). New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. *Appl Environ Microbiol*, *72:*(2), 1270–8.

Ozturk, A., Caglar, O., & Sahin, F. (2003). Yield response of wheat and barley to inoculation of plant growth promoting rhizobacteria at various levels of nitrogen fertilization. *Journal of Plant Nutrition and Soil Science*, *166,*(2), 262–266. doi:DOI: 10.1002/jpln.200390038

Panda, S. S., Ames, D. P., & Panigrahi, S. (2010). Application of Vegetation Indices for Agricultural Crop Yield Prediction Using Neural Network Techniques; *Remote Sensing*, *2,*, 673–696; doi:doi:10.3390/rs2030673

Panwar, J. D. S. ., & Singh, O. (2000). Response of *Azospirillum* and *Bacillus* on growth and yield of wheat under field conditions. *Indian Journal of Plant Physiology*, *5*(1), 108–110.

Pati, B. R., Sengupta, S., & Chandra, A. K. (1995). "Impact of selected phyllospheric diazotrophs on the growth of wheat seedlings and assay of the growth substances produced by the diazotrophs,." *Microbiological Research,*, *vol. 150,*(no. 2), pp. 121–127,.

Patriquin, D. G., Döbereiner, J., & Jain, D. K. (1983). Sites and processes of association between diazotrophs and grasses. *Canadian Journal of Microbiology,*, *29*(8), 900–915.

Patten, C. L., & Glick, B. R. (1996). "Role of Pseudomonas putida indoleacetic acid in development of the host plant root system,." *Applied and Environmental Microbiology,*, *vol. 68,*(no. 8,), pp. 3795–3801,.

Paul, E. A,. and Clark, F. E. (1989). *Soil microbiology and biochemistry*. (F. E. C. E.A. Paul, Ed.). San Diego Calif.: Academic Press,.

Peng, G., Wang, H., Zhang, G., Hou, W., Liu, Y., Wang, E. T., & Tan, Z. (2006). *Azospirillum melinis* sp. nov., a group of diazotrophs isolated from tropical molasses grass. *International Journal of Systematic and Evolutionary Microbiology*, *56*(Pt 6), 1263–71. doi:10.1099/ijs.0.64025-0

Peoples, M. B., Herridge, D. F., & Ladha, J. K. (1995). Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production? *Plant and Soil*, *174,*(1-2), 3–28.

Pereg-Gerk, L., Gilchrist, K., & Kennedy, I. R. (2000). Mutants with Enhanced Nitrogenase Activity in Hydroponic *Azospirillum brasilense*-Wheat Associations *Appl Environ Microbiol*, *66*(5), 2175–2184. doi:10.1128/AEM.66.5.2175-2184.2000.Updated

Pereyra, M. a., González, R. L., Creus, C. M., & Barassi, C. a. (2007). Root colonization vs. seedling growth, in two Azospirillum -inoculated wheat species. *Cereal Research Communications*, *35*(4), 1621–1629. doi:10.1556/CRC.35.2007.4.9

Pieterse, C. M. J., Leon-Reyes, A., Ent, S. vander, & Wees, S. C. M. van. (2009). “Networking by small-molecule hormones in plant immunity,.” *Nature Chemical Biology,*, *vol. 5,*(no. 5,), pp. 308–316,.

Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., & Monrozier, L. J. (2001). Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties. *Applied*

and Environmental Microbiology, *67*(5), 2255–62. Retrieved from http://aem.asm.org/cgi/content/abstract/67/5/2255

Raaijmakers, J. M., & Maria Vlami, J. T. de S. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek*, *Volume 81,*(Issue 1-4), pp 537–547.

Raaijmakers, J. M., Van der Sluis, I., Koster, M., Bakker, P. A. H. M., Weisbeek, P. J. and Schippers, B. (1995). Utilization of heterologous siderophores and rhizosphere competence of fluorescent Pseudomonas spp. *Canadian Journal of Microbiology*, *41,*, 126–135.

Rahim, KA. (2002). Biofertilizer in Malaysia agriculture: Perception, demand and promotion. In *"FNCA Joint Workshop on Mutation Breeding and Biofertilizer."* Beijing, China.

Raymond, J., Siefert, J. L., Staples, C. R., & Blankenship E., R. (2004). The Natural History of Nitrogen Fixation. *Mol Biol Evol*, *21*(3), 541–554.

Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Ley, J. D. E. (1987). *Azospirillum halopraeferens* sp. nov., a Nitrogen-Fixing Organism Associated with Roots of Kallar Grass (*Leptochloa fusca* (L.) Kunth). *International Journal of Systematic and Evolutionary Microbiology*, *37*(1), 43–51.

Remans, R., Ramaekers, L., Schelkens, S., Hernandez, G., Garcia, A., Reyes, J. L., & Nancy Mendez, Vidalina Toscano, Miguel Mulling, L. G. (2008). Effect of *Rhizobium–Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus*

vulgaris L. genotypes cultivated across different environments in Cuba. *Plant and Soil*, *312,*(1-2), 25–37.

Reynolds, M., Dreccer, F., & Trethowan, R. (2007). Drought-adaptive traits derived from wheat wild relatives and landraces. *Journal of Experimental Botany*, *58*(2), 177–86. doi:10.1093/jxb/erl250

Richards, R. A., Rebetzke, G. J., Condon, A. G., & Herwaarden, A. F. van. (2002). Breeding Opportunities for Increasing the Efficiency of Water Use and Crop Yield in Temperate Cereals. *Crop Sci.*, *42*, 111–121.

Richardson AE, Hadobas PA, Hayes JE, O'Hara CP, S. R. (2001). Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil microorganisms. *Plant Soil*, *229*, 47–56.

Rodrigues, E. P., Santos, L., Rodrigues, A. L., Martinez de Oliveira, V. L. D. B., & Kátia Regina dos Santos Teixeira, Segundo Urquiaga, V. M. R. (2008). *Azospirillum amazonense* inoculation: effects on growth, yield and N2 fixation of rice (Oryza sativa L.). *Plant and Soil*, *302*(1-2), 249–261.

Rodríguez, H., & Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, *17*(4-5), 319–39. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14538133

Roper, M. M., & Ladha, J. K. (1995). Biological N2 fixation by heterotrophic and phototrophic bacteria in association with straw. *Plant and Soil*, *174*, pp 211–224.

Saharan, B. S., & Nehra, V. (2011). Plant Growth Promoting Rhizobacteria : A Critical Review, *2011*, 1–30.

Salamone, G. de, Inés, E., Funes, M., Juan, Di Salvo, L. P., Escobar-Ortega, J. S., Fernandez-Scavino, A. (2012). Inoculation of paddy rice with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact of plant genotypes on rhizosphere microbial communities and field crop production. *Applied Soil Ecology*, *61*, 196–204. doi:10.1016/j.apsoil.2011.12.012

Salamone, I. E. G., Hynes, R. K., & Nelson, L. M. (2005). "Role of cytokinins in plant growth promotion by rhizosphere bacteria,",. In Z. A. Siddiqui (Ed.), *PGPR: Biocontrol and Biofertilization* (pp. Ed., pp. 173–195, Springer, Amsterdam, The Netherl). Amsterdam, The Netherlands,: Springer,.

Saleem, M., Arshad, M., Hussain, S., & Bhatti, A. S. (2007). Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *J.Indian Microbiol. Biotechnol.*, *34*, pp. 635–648.

Salomone, I. G. de, & Döbereiner, J. (1996). Maize genotype effects on the response to Azospirillum inoculation. *Biology and Fertility of Soils*, *Volume 21,*(Issue 3,), pp 193–196.

Saubidet, M. I., & Barneix, A. J. (1998). Growth stimulation and nitrogen supply to wheat plants inoculated with *Azospirillum brasilense. Journal of Plant Nutrition.*, *21*(12), 2565–2577.

Saubidet, M. I., Fatta, N., & Barneix, A. J. (2002). The effect of inoculation with *Azospirillum brasilense* on growth and nitrogen utilization by wheat plants. *Plant and Soil*, *245*(2), 215–222.

Schippers, B., Bakker, A. W. and Bakker, P. A. H. M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology*, *25*, 339–358.

Schloter, M., Hartmann, A. (1998). Endophytic and surface colonization of wheat roots (*Triticum aestivum*) by different *Azospirillum brasilense* strains studies with strain-specific monoclonal antibodies; *Symbiosis*, *25,*, 159–179.

Schultze, M., Kondorosi, E. F., Ratet, P., Buire, M. & Kondorosi, A. (1994). Cell and molecular biology of Rhizobium-plant interactions. *Zntern Rev Cytol*, *156,*, 1–75.

Seo, K., Valentin-Bon, I., & Brackett, R. (2006). Detection and enumeration of *Salmonella enteritidis* in homemade ice cream associated with an outbreak: comparison of conventional and real-time PCR methods. *J.Food Prot. Mar;*, *69*(3), 639–643.

Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H, K. E. (2001). Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Appl Environ Microbiol.*, *Sep;67*((9), :4215–24.

Shaharoona, B., Arshad, M., & Zahir, Z. A. (2006). “Effect of plant growth promoting rhizobacteria containing ACC-deaminase on maize (*Zea mays* L.) growth under axenic

conditions and on nodulation in mung bean (*Vigna radiata* L.),." *Letters in Applied Microbiology,*, *vol. 42,*(2), pp. 155–159,.

Shaharoona, B., Naveed, M., Arshad, M., & Zahir, Z. a. (2008). Fertilizer-dependent efficiency of Pseudomonads for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). *Applied Microbiology and Biotechnology*, *79*(1), 147–55. doi:10.1007/s00253-008-1419-0

Sharma, S. K., Johri, B. N., Ramesh, A., Joshi, O. P., & Prasad, S. V. S. (2011). Selection of plant growth-promoting Pseudomonas spp. that enhanced productivity of soybean-wheat cropping system in central India. *J. Microbiol. Biotechnol.,*, *21*, pp. 1127–1142.

Siddiqui, Z. A. (2005). "PGPR: Prospective biocontrol agents of plant pathogens." In Z. A. Siddiqui (Ed.), *PGPG: Biocontrol and Biofertilisation*. Dordrecht, The Netherlands.: Springer,.

Sikora, R. A. (1988). Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. Med. ... 1988. 1 75. *Med. Fac. Landbouw*, *2:*, 867–878.

Simon, R., Priefer, U., & Puhler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nature Biotechnol*, *1:*, 784–791.

Skvortsov, I. M., & Ignatov, V. V. (1998). Extracellular polysaccharides and polysaccharide-containing biopolymers from *Azospirillum* species: properties and the possible role in interaction with plant roots. *FEMS Microbiology Letters*, *165*, 223–229.

Smalla, K., Oros-Sichler, M., Milling, A., Heuer, H., Baumgarte, S., Becker, R., … Tebbe, C. C. (2007). Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: do the different methods provide similar results? *Journal of Microbiological Methods*, *69*(3), 470–9. doi:10.1016/j.mimet.2007.02.014

Smith, C. J., B. S. Danilowicz, A. K. Clear, F. J. Costello, B. Wilson, and W. G. M. (2005). T-Align, a web-based tool for comparison of multiple terminal restriction fragment length polymorphism profiles. *FEMS Microbiology Ecology*, *54:*, 375–380.

Soares, R. A., & Luiz Fernando W. Roesch, Geancarlo Zanatta, Flávio A. de Oliveira Camargo, L. M. P. P. (2006). Occurrence and distribution of nitrogen fixing bacterial community associated with oat (Avena sativa) assessed by molecular and microbiological techniques. *Applied Soil Ecology*, *33*(3), 221–234.

Somers, E., Vanderleyden, J., Srinivasan, M. (2004). Rhizosphere bacterial signalling: a love parade beneath our feet. *Crit. Rev.Microbiol.*, *30,*, 205–240.

Spaepen, S., Dobbelaere, S., Croonenborghs, A., & Vanderleyden, J. (2008). Effects of *Azospirillum brasilense* indole-3-acetic acid production on inoculated wheat plants. *Plant and Soil*, *312*(1-2), 15–23. doi:10.1007/s11104-008-9560-1

Spaepen, S., Vanderleyden, J., & Okon, Y. (2009). Chapter 7 Plant Growth-Promoting Actions of Rhizobacteria. *Advances in Botanical Research*, *51*, 283–320. doi:10.1016/S0065-2296(09)51007-5

Stancheva, I., Dimitrov, I., Kaloyanova, N., Dimitrova, A., & Angelov, M. (1992). Effects of inoculation with *Azospirillum brasilense* on photosynthetic enzyme activities and grain yield in maize. *Agronomy Journal,*, *12*, 319–324.

Steenhoudt, O., & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiology Reviews*, *24*(4), 487–506. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10978548

Sun, Y., & Liu, Y. (2013). Investigating of yeast species in wine fermentation using terminal restriction fragment length polymorphism method. *Food Microbiology*. doi:10.1016/j.fm.2013.09.001

Sutton, S. (2010). The Most Probable Number Method and Its Uses in Enumeration, Qualification, and Validation. *Journal of Validation Technology*, *microbiolo*, 35–38.

Suzuki, S., He, Y., & Oyaizu, H. (2003). Indole-3-Acetic Acid Production in *Pseudomonas fluorescens* HP72 and Its Association with Suppression of Creeping Bentgrass Brown Patch. *Current Microbiology*, *Volume 47,*(Issue 2,), pp 0138–0143.

Swędrzyńska, D., & Sawicka, D. Ċ. Ĕ. (2000). Effect of Inoculation with *Azospirillum brasilense* on Development and Yielding of Winter Wheat and Oat under Different Cultivation Conditions, *9*(5), 423–428.

Tan, Z., Hurek, T., & Reinhold-hurek, B. (2003). Brief report Effect of N-fertilization , plant genotype and environmental conditions on nifH gene pools in roots of rice, *5*, 1009–1015.

Tarrand, J. J., Krieg, N. R., & Dobereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Canadian Journal of Microbiology,*, *24,*, 967–980.

Tchan, Y. T., & Kennedy, I. R. 1. (1989). Possible N2-fixing root nodule induced in non-legumes. *Agric. Sci.*, *2*, 57–59.

TienT. M., Gaskins, M. H. and D. H. H. (1979). Plant Growth Substances Produced by *Azospirillum brasilense* and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L. *Appl Environ Microbiol.*, *37*(5), 1016–1024.

Trabelsi, D., & Alessio Mengoni, H. B. A. & R. M. (2011). E¡ectofon-¢eld inoculation of *Phaseolus vulgaris* with rhizobia on soil bacterial communities. *FEMS Microbiol Ecol*, *77*, 211–222.

Trabelsi, D., Ben Ammar, H., Mengoni, A., & Mhamdi, R. (2012). Appraisal of the crop-rotation effect of rhizobial inoculation on potato cropping systems in relation to soil

bacterial communities. *Soil Biology and Biochemistry*, *54*, 1–6. doi:10.1016/j.soilbio.2012.05.013

Trabelsi, D., & Mhamdi, R. (2013). Microbial inoculants and their impact on soil microbial communities: a review. *BioMed Research International*, *2013*, 863240. doi:10.1155/2013/863240

Trethowan, R. M., & Mujeeb-Kazi, A. (2008). Novel Germplasm Resources for Improving Environmental Stress Tolerance of Hexaploid Wheat. *Crop Sci.*, *Vol. 48*(No. 4,), p. 1255–1265.

Trethowan, R. M., Reynolds, M. P., Ortiz-Monasterio, J. I., & Ortiz., R. (2007). The genetic basis of the on-going Green Revolution in wheat production. *Plant Breeding Reviews,*, *28:*, 38–58.

University Of California, S. B. (2001). Increased Use Of Chemicals In Agriculture Worldwide Seen As Major Environmental Threat. ScienceDaily. Retrieved July 11, 2014 from www.sciencedaily.com/releases/2001/04/010415223052.htm. *ScienceDaily.* doi:www.sciencedaily.com/releases/2001/04/010415223052.htm

Van Loon, L., Bakker, P., & Pieterse, C. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Review in Phytopathology*, *36,*, 453–483.

Veresoglou, S. D., & Menexes, G. (2010). Impact of inoculation with *Azospirillum* spp. on growth properties and seed yield of wheat: a meta-analysis of studies in the ISI Web of

Science from 1981 to 2008. *Plant and Soil*, *337*(1-2), 469–480. doi:10.1007/s11104-010-0543-7

Verhagen, B., Glazebrook, J., Zhu, T., Chang, H., van Loon, L., & Pieterse, C. (2004). The transcriptome of rhizobacteria-induced systemic resistance in arabidopsis. *Mol Plant Microbe Interact.*, *17*(8), 895–908.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil*, *255,*, 571–586.

Walker, T.S., Bais, H.P., Grotewold, E., and Vivanco, J. M. (2003). "Root exudation and rhizosphere biology." *Plant Physiology*, *132*, 44–51.

Wani, P. A., & Khan, M. S. (2010). *Bacillus* species enhance growth parameters of chickpea (*Cicer arietinum* L.) in chromium stressed soils. *Food and Chemical Toxicology*, *48*(11), 3262–3267.

Weiss, J. V, Emerson, D., Backer, S., & Megonigal, J. (2003). Enumeration of Fe (II)-oxidizing and Fe(III)-reducing bacteria in the root zone of wetland plants: implications for a rhizosphere iron cycle. *Biogeochemistry*, *64:*, 77–96.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, *52*(suppl 1), 487–511. doi:10.1093/jexbot/52.suppl_1.487

Wohlsen, T., Bates, J., Vesey, G., Robinson, W. A., & Katouli, M. (2006). Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. *Letters in Applied Microbiology,*, *42*(4), 350–356.

Wood, C., Islam, N., Ritchie, R., & Kennedy, I. (2001). A simplified model for assessing critical parameters during associative 15N2 fixation between *Azospirillum* and wheat. *Australian Journal of Plant Physiology*, *28*, 969–974.

Woomer, P., Bennett, J., & Yost, R. (1990). Overcoming the Inflexibility of Most-Probable-Number Procedures. *Agron. J.*, *82:*, 349–353. Retrieved from doi:10.2134/agronj1990.00021962008200020035x

Wu, L., Ma, K., & Lu, Y. (2009). Prevalence of Betaproteobacterial Sequences in nifH Gene Pools Associated with Roots of Modern Rice Cultivars, 58–68. doi:10.1007/s00248-008-9403-x

Xie, A., & Yokota, C. H. (2005). *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant Oryza sativa. *Int. J. Syst. Evol. Microbiol.,*, *55*, 1435–1438.

Yardin MR, Kennedy IR, T. J. (2000). Development of high quality carrier materials for field delivery of key microorganisms used as bio-fertilisers and bio-pesticides. *Radiation Phys Chem*, *57*, 565–568.

Yegorenkova, I. V., Konnova, S. A., Sachuk, V. N., & Ignatov, V. V. (2001). *Azospirillum brasilense* colonisation of wheat roots and the role of lectin–carbohydrate interactions in bacterial adsorption and root-hair deformation. *Plant Soil,*, *231*(275-282).

Zahir, Z. A., Arshad, M., & Frankenberger, W. T. (2003). Plant Growth Promoting Rhizobacteria: Applications and Perspectives In Agriculture. *Advances in Agronomy*, *81*, 97–168. doi:10.1016/S0065-2113(03)81003-9

Zahir, Z. A., Shah, M. K., Naveed, M., & Akhter, M. J. (2010). Substrate-dependent auxin production by Rhizobium phaseoli improves the growth and yield of *Vigna radiata* L. under salt stress conditions. *J. Microbiol. Biotechnol.,*, *20*, 1288–1294.

Zahir, Z.A., Arshad, M., and Frankenberger, W. T. (2004). "Plant growth promoting rhizobacteria: Applications and perspectives in agriculture." *Advances in Agronomy*, *81;*, 97–168.

Zehnder, G., Kloepper, J., Yao, C., & Wei, G. (1997). Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera: Chrysomelidae) by plant growth promoting rhizobacteria. *J. Econ. Entomol.*, *90,*, 391–396.

Zeman, A., Tchan, Y., Elmerich, C., & Kennedy,IR. (1992). Nitrogenase activity in wheat seedlings bearing para-nodules induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and inoculated with *Azospirillum*. *Research in Microbiology Institut Pasteur : Elsevier*, *143:*, 847±855.

Zhang, B. G., Reffye, P. De, Liu, L., Kang, M. Z., & Li, B. G. (2003). Analysis and modeling of the root system architecture of winter wheat seedling. In *International Symposium on Plant Growth Modeling, Simulation, Visualization and their Applications - PMA'03* (pp. 321–328).

Zhang F, Dashti N, Hynes RK, S. D. (1996). Plant growth-promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.]. Nodulation and fixation at suboptimal root zone temperatures. *Ann Bot*, *7:*, 453–459.

Zhang R, Thiyagarajan V, Q. P. (2008). Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. *FEMS Microbiol Ecol.*, *Jul;65*((1):), 169–78. Retrieved from doi: 10.1111/j.1574-6941.2008.00493.x. Epub 2008 May 22.

Zimmer, W., Roeben, K., & H. Bothe. (1988). An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum*. *Planta*, *Volume 176*(Issue 3,), pp 333–342.

Appendix

Appendix A

Composition of media, buffers and solutions.

Media for hydroponic solution

Use 0.2 mL of each of the following solutions per litre of water

$MnSO_4$. 4H $_2O$	0.02M
Mg SO_4. $7H_2O$	0. 3M
K_2SO_4	0.4M
KH_2PO_4	0.2M
$CaCl_2$	0.2M
Iron chelate (add 0.4mL/L)	50g/L
Trace elements*	0.2mL

* Trace elements

$Na_2MoO_4.2H_2O$	0.2g
H_3BO_3	0.28g
$CuSO_45H_2O$	0.008g
$ZnSO_4.7H_2O$	0.024g
Reverse Osmosis water	1L

Adjust PH to 6.8 with 0.2 M K_2HPO_4 solution.

Luria Bertani (LB) broth

Bacto tryptone	10g
NaCl	5g
Yeast extract	5g
Distilled water	1L

Adjust PH to 6.8 with 0.2 M K_2HPO_4 solution

Luria Bertani Agar

Bacto tryptone	10g
NaCl	5g
Yeast extract	5g
Distilled water	1L
Agar	15g

Adjust PH to 6.8 with 0.2 M K_2HPO_4 solution

Yeast Mannitol agar

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast Extract	0.5 g
Distilled Water	1.0 liter
Agar	15 g

- Adjust pH to 6.8 with 0.1 N NaOH

Nutrient agar

Dehydrated nutrient broth (Difco ™)	8 g
Agar	15 g
water to	1L

Nutrient broth

Dehydrated nutrient broth (Difco ™)	8 g
Water to	1 L

Nfb Agar media (***Azospirillum*** **selective media**)

L-malic acid	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g

$CaCl_2$	0.02 g
Agar	15g
Fe-EDTA (1.64% solution)	4ml
Trace elements solution *	2ml
Congo red solution (0.25%w/v in water)	15ml
Vitamin #solution	1ml

Semi solid nitrogen free broth medium (Nfb)

L-malic acid	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$CaCl_2$	0.02 g
Agar	2g
Fe-EDTA (1.64% solution)	4ml
Trace elements solution *	2ml

Bromothymol blue(0.5% w/v in 0.2 M KOH solution)	15ml
Vitamin #solution	1ml

*Trace elements stock solution

$Na_2MoO_4.2H_2O$	0.2 g
$MnSO_4.H_2O$	0.235 g
H_3BO_3	0.28 g
$CuSO_4.5H_2O$	0.008 g
$ZnSO_4.7H_2O$	0.024 g
water to	1 L

#Vitamin stock solutions

Biotin	0.01 g
Pyridoxin	0.02 g
water to	20 mL

Diluted from stock, 50 times and added 1 mL/L medium

pH was adjusted to 6.8 with KOH, the solution should be green in colour and autoclaved at 121°C for 15 minutes)

Buffer

Saline solution (0.85%)

NaCl	8.5 g
Water to	1 L

Z buffer

KCL	0.75g
$MgSO_4.7H_2O$	0.246g
Na_2HPO_4(anhydrous)	21.489g
$NaH_2PO_4.\ 2\ H_2O$	6.24g

Adjusted pH 7.4 with $NaH_2PO_4.\ 2\ H_2O$ / Na_2HP

Appendix B

Table 1: Mean Maximum Temperature (degrees Celsius) NARRABRI AIRPORT AWS (2002-2010), Station Number: 054038 NSW

Opened: 2001 · Status: Open · Latitude: 30.32°S · Longitude: 149.83°E · Elevation: 229 m (http://www.bom.gov.au/climate/cdo/about/about-airtemp-data.shtml.)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2002	34	32.8	32.4	28.2	22.8	19.1	19.4	21.8	25.2	30.9	34.7	33.2
2003	36.3	33.5	29.5	24	22.5	18.8	17.2	19.2	25	25.3	30.2	34.8
2004	33.9	34.5	30.5	28.6	21.9	19	18.1	20.1	23.1	28	29.9	30.6
2005	33.9	34.6	30.6	30	22.4	18.5	17.8	19.3	22.3	28.2	30	34.4
2006	36.3	34.8	31.7	26.7	22.7	19.1	18.2	20.8	25.5	29.8	32.5	32.5
2007	35.7	34.8	31.9	28	23.7	15.3	16.4	20.7	24.7	30.4	28.5	30.4
2008	31.8	29.9	30.3	25	23.1	19.8	17.6	18.2	23.3	28.2	28.5	31.6
2009	34.6	33.4	31.6	25.8	22.3	18.5	17.8	23.1	24.6	28	35.4	33.6
2010	34.2	31.7	29.6	26.7	22.2	17.6	17.6	17.2	22	25.1	27.4	30.5
2011	34	34.9	30.9	26.6	21	18.1	17.5	20.5	23.6	25.3	31	28
Avg. temp	34.47	33.49	30.9	26.96	22.46	18.38	17.76	20.09	23.93	27.92	30.81	31.96

Table 2: Monthly Rainfall (millimetres) NARRABRI AIRPORT AWS (2002-2010), Station Number: 054038 · State: NSW ·

Opened: 2001 · Status: Open · Latitude: 30.32°S · Longitude: 149.83°E · Elevation: 229 m (http://www.bom.gov.au/climate/cdo/about/about-rain-data.s html.)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2002	49.4	66.6	38.2	1.2	3	39	1	1.8	27	3.6	34	62.6
2003	13.6	77	44.4	92.4	0.2	63	47.4	56.6	0.4	49.8	54.6	33.6
2004	196	51	62	18.2	18.8	13.6	35.2	34.6	502	538	110.6	247.4
2005	34.6	39.6	22	3.6	24.2	186.4	19	212	82.2	53.2	107	141.8
2006	56.8	133.2	6.2	34.4	0	35.6	66	7.8	16.6	10.2	40	18.6
2007	45.8	54.6	42	14.4	57.4	94.6	2.4	32.4	1.6	57.6	46.4	103
2008	80.4	70.8	4.8	14.6	3.4	412	40.4	31.4	56.8	43.4	134.2	97
2009	19.8	105.2	13.2	13.2	38.4	19.2	17.2	38	29.2	41	86	130
2010	82.6	129	51	57	36	212	68.6	48.2	46.6	48.4	67.6	26.2
2011	416	25.2	30	9.4	38.8	23.4	0.6	44	80.8	7.6	200	47.8
Avg rainfall	99.5	75.22	31.38	25.84	22.02	109.88	29.78	50.68	84.32	85.28	88.04	90.8

Table 3: Experimental design used for field experiments at Narrabri in 2010

	4 Sp7		3 Control		5 Sp7-S		2 Sp7-S		1 Sp7		2 Control
	1 Sp7		1 Control		4 Sp7-S		5 Sp7-S		4 Sp7		3 Control
	2 Sp7		2 Control		1 Sp7-S s		4 Sp7-S		2 Sp7		4 Control
	5 Sp7		5 Control		3 Sp7-S		1 Sp7-S		5 Sp7		5 Control
	3 Sp7		4 Control		2 Sp7-S		3 Sp7-S		3 Sp7		1 Control
	2 Sp7		3 Sp7-S		2 Control		5 Sp7		4 Sp7-S		2 Control
	3 Sp7		2 Sp7-S		5 Control		4 Sp7		2 Sp7-S		1 Control
	5 Sp7		4 Sp7-S		3 Control		2 Sp7		1 Sp7-S		4 Control
	4 Sp7		5 Sp7-S		1 Control		1 Sp7		3 Sp7-S		3 Control
	1 Sp7		1 Sp7-S		4 Control		3 Sp7		5 Sp7-S		5 Control
	2 Control		5 Sp7-S		3 Sp7		1 Sp7		4 Sp7-S		2 Control
	1 Control		1 Sp7-S		1 Sp7		2 Sp7		5 Sp7-S		4 Control
	4 Control		4 Sp7-S		4`Sp7		3 Sp7		3 Sp7-S		1 Control
	3 Control		3 Sp7-S		2 Sp7		4 Sp7		1 Sp7-S		5 Control
	5 Control		2 Sp7-S		5 Sp7		5 Sp7		2 Sp7-S		3 Control
	2 Sp7-S		2 Sp7		3 Control		3 Sp7-S		2 Control		2 Sp7
	5 Sp7-S		1 Sp7		2 Control		2 Sp7-S		4 Control		1 Sp7
	1 Sp7-S		4 Sp7		5 Control		4 Sp7-S		5 Control		5 Sp7
	4 Sp7-S		5 Sp7		1 Control		1 Sp7-S		3 Control		3 Sp7
	3 Sp7-S		3 Sp7		4 Control		5 Sp7-S		1 Control		4 Sp7
	Environment 1						Environment 2				

Grey colour indicates buffer zone

Table 4.a: Experimental design used in for field trials at Narrabri in 2011: low N

BUFFER	5	2	3	4	1	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFEI	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFEI	BUFFER
BUFFER	3	5	2	4	1	BUFFER	3	4	2	1	5	BUFFER	2	5	1	4	3	BUFFER
BUFFER	5	3	1	2	4	BUFFER	3	5	2	1	4	BUFFER	1	2	3	5	4	BUFFER
BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFEI	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFEI	BUFFER
BUFFER	5	3	4	1	2	BUFFER	4	5	3	2	1	BUFFER	4	2	3	5	1	BUFFER
BUFFER	3	1	4	5	2	BUFFER	5	1	4	2	3	BUFFER	2	1	4	5	3	BUFFER
BUFFER	1	5	2	3	4	BUFFER	4	5	1	3	2	BUFFER	1	4	2	3	5	BUFFER

Table 4.b: Experimental design used for field trials at Narrabri in 2011: High N

BUFFER	2	4	1	5	3	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER
BUFFER	2	4	1	5	3	BUFFER	3	4	1	2	5	BUFFER	4	5	1	2	3	BUFFER
BUFFER	2	4	3	5	1	BUFFER	4	3	1	2	5	BUFFER	5	1	3	4	2	BUFFER
BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER	BUFFER
BUFFER	3	4	2	5	1	BUFFER	2	4	5	3	1	BUFFER	5	4	1	3	2	BUFFER
BUFFER	2	4	5	1	3	BUFFER	3	1	5	4	2	BUFFER	2	1	4	3	5	BUFFER
BUFFER	3	2	4	5	1	BUFFER	4	5	3	1	2	BUFFER	2	4	1	3	5	BUFFER

Colour indicating the replication in the design, Number indicating genotypes (1-5)

Treatment 1	Uninoculated
2	Sp7
3	Sp7-S
4	Sp245

1	EGA GREGORY
2	CBRD/KAUZ//KASO2
3	CROC_1/AE.SQUARROSA (205)//KAUZ/3/2*METSO
4	SOKOLL
5	KRICHAUFF

Table 5: Means of different traits assessed in the field, 2010

Treatments	Genotypes	Plant ht at 43 DAS	Plant ht at 103 DAS	Plant ht at 133 DAS	Dry wt 43DAs	Dry wt 103 DAS	Dry wt 133	Chl 43 DAS	Chl 103DAS	Chl 133DAs
control	1	24.56	78.05	107.7	0.2553	8.563	19.64	38.13	47.57	42.05
control	2	24.31	63.23	99.9	0.2337	7.429	19.12	37.12	49	41.56
control	3	25.87	88.75	113.5	0.3217	9.203	19.77	37.74	38.74	42.09
control	4	25.62	89.55	109	0.3363	11.246	26.33	39.85	48.13	42.85
control	5	24.69	67.73	120.8	0.2344	11.766	28.54	38.19	46.81	40.62
Sp7	1	24.38	77.58	112.1	0.2878	9.317	23.08	37.33	45.19	41.72
Sp7	2	23.5	67.88	99.2	0.2789	7.69	19.46	36.57	47.96	42.62
Sp7	3	23.88	90.58	116.9	0.2897	8.63	23.14	36.45	38.92	41.13
Sp7	4	26.5	92.6	109.1	0.335	11.008	22.95	41.46	46.72	42.72
Sp7	5	22.88	72.75	118.7	0.2701	10.017	27.27	37.41	46.62	40.84
Sp7-S	1	21.8	73.3	114.1	0.3215	9.538	23.18	38.78	44.91	41.34
Sp7-S	2	21.56	69.4	101.2	0.2055	8.215	19.18	38.36	46.27	42.51
Sp7-S	3	23.25	90.78	116.1	0.3279	12.202	23.4	38.07	37.36	41.44
Sp7-S	4	27	89.73	107.4	0.2857	12.325	18.81	39.65	46.76	42.86
Sp7-S	5	22.25	73	119.1	0.2604	11.372	24.55	39.84	46.49	41.9

Table 5: Means of different traits assessed in the field, 2010 (continued)

Treatments	genotypes	NDVI index	Tiller No	1000grain wt	yield /ha2010	MPN at 43 DAS	MPN at 103 DAS	MPN at 133 DAS
control	1	0.7694	5.725	39.15	5.014	6.504	7.428	8.573
control	2	0.7052	6.475	39.24	5.184	0	7.95	8.782
control	3	0.6782	5.25	42.39	5.111	7.876	8.089	8.57
control	4	0.7566	4.725	43.94	5.013	7.892	7.504	8.322
control	5	0.6952	5.95	39.96	4.911	8.733	7.669	8.652
Sp7	1	0.7962	5.55	38.51	4.9	7.814	8.643	7.826
Sp7	2	0.8078	6.25	34.06	5.055	7.829	9.043	8.151
Sp7	3	0.8275	4.5	46.57	5.332	8.477	8.715	8.255
Sp7	4	0.8084	5.225	49.42	4.967	8.456	7.991	8.604
Sp7	5	0.8168	5.544	40.11	5.034	8.822	8.399	8.469
Sp7-S	1	0.7911	5.775	40.38	5.083	7.367	8.693	8.395
Sp7-S	2	0.7354	7.525	37.23	5.085	8.466	8.964	8.385
Sp7-S	3	0.7756	5.4	48.94	5.799	8.52	8.327	8.812
Sp7-S	4	0.7963	5.775	49.4	5.142	8.893	8.093	8.919
Sp7-S	5	0.776	6.55	39.84	4.919	9.002	8.774	8.312

Table 6: Means of different traits measured in the field in 2011

Treatments	genotypes	Plant ht at 43 DAS	Plant ht at 103 DAS	Plant ht at 133 DAS	Dry wt 43DAs	Dry wt 103 DAS	Dry wt 133	Relative chl 43DAS	RChl 103 Das	Rchl 133 DAS
control	1	20.51	86.2	99	0.2697	24.28	36.41	46.48	49.55	48.15
control	2	15.21	78.03	92	0.2573	21.25	28.06	43.73	51.7	47.42
control	3	24.71	76.2	96.37	0.3406	17.49	31.31	45.05	47.77	46.09
control	4	21.83	74.03	99.37	0.3783	23.1	35.28	47.1	48.43	48.84
control	5	20.55	75.2	99.75	0.3075	20.45	30.76	45.59	47.68	45.9
Sp-245	1	17.11	78.47	96.12	0.2493	19.32	35.95	47.11	50.88	42.85
Sp-245	2	16.99	76.51	82.12	0.2747	22.03	44.46	48.24	52.85	43.31
Sp-245	3	20.7	74.68	95.75	0.3305	20.36	25.04	43.6	47.07	42.62
Sp-245	4	23.9	76.34	100.13	0.3705	15.44	39.15	49.76	48.73	52.68
Sp-245	5	19.57	77.34	96.88	0.2906	22.73	23.95	45.78	50.15	47.09
Sp7	1	22.46	75.87	97.37	0.2741	20.58	36.9	49.93	51.12	51.04
Sp7	2	19.46	72.87	87.77	0.25	19.11	43.98	45.52	50.1	46.79
Sp7	3	22.37	87.7	91.14	0.3436	26.46	40.05	43.22	48.72	43.09
Sp7	4	22.5	89.03	92.75	0.3785	24.06	37.16	46.15	50.15	43.26
Sp7	5	20.62	78.37	96.75	0.314	19.88	31.34	43.47	46.8	46.2
Sp7-S	1	21.61	72.14	94.25	0.2953	16.52	29.06	46.7	48.37	44.96
Sp7-S	2	15.37	70.6	92.75	0.2924	16.99	28.3	49.07	50.88	49.85
Sp7-S	3	24.09	75.43	96.75	0.3225	16.33	35.9	45.43	46.67	44.61
Sp7-S	4	21.58	78	105.63	0.3551	23.22	33.62	43.97	51.18	41.71
Sp7-S	5	21.44	88.1	96.25	0.3293	21.58	26.51	43.33	49.7	41.46

Table 6: Means of different traits measured in 2011 in field trials (continued)

Treatments	genotypes	NDVI index	Tiller no	1000 grain wt	havest index	yield /ha2011	MPN at 43 DAS	MPN at 103 DAS	MPN at 133 DAS
control	1	0.7438	5.333	49.95	0.3859	4.071	4.055	7.927	8.71
control	2	0.6702	8.667	46.41	0.4385	3.743	1.859	7.871	8.435
control	3	0.7882	7.667	51.32	0.4537	4.591	3.935	6.915	8.704
control	4	0.7218	8	54.84	0.4278	4.175	2.069	8.31	8.136
control	5	0.8027	6.167	46.19	0.3329	3.528	2.323	7.495	8.806
Sp-245	1	0.6754	7.333	46.94	0.3966	4.066	5.463	7.216	8.686
Sp-245	2	0.6533	7.667	40.16	0.4041	3.852	7.201	7.233	8.346
Sp-245	3	0.7713	11	50.4	0.4035	4.558	5.54	8.422	8.808
Sp-245	4	0.6844	6.167	56.58	0.4026	4.16	5.86	8.717	8.49
Sp-245	5	0.7601	7	48.29	0.3328	3.688	6.449	7.051	8.546
Sp7	1	0.6971	7	37.83	0.3834	3.912	6.828	7.109	8.698
Sp7	2	0.6776	7.833	46.09	0.4	3.757	7.351	8.838	8.663
Sp7	3	0.7519	7.667	52.67	0.4007	4.548	7.432	8.057	8.942
Sp7	4	0.6718	9	54.28	0.3823	4.072	7.006	7.914	8.704
Sp7	5	0.7832	7	46.42	0.3335	3.594	7.152	8.722	8.226
Sp7-S	1	0.6577	6.333	49.05	0.4017	4.038	6.192	7.569	8.475
Sp7-S	2	0.6538	7.333	44.22	0.4311	3.844	6.693	7.837	8.376
Sp7-S	3	0.7855	6.333	53.43	0.4472	4.592	7.187	7.631	7.768
Sp7-S	4	0.6625	7.5	55.66	0.401	4.023	6.632	7.462	8.039
Sp7-S	5	0.7973	7	47.87	0.319	3.719	7.08	8.337	8.433

Table 7: Combined mean data of different traits measured in field trials, 2010 and 2011

			Plant height cm			Dry weight g			Relative chlorophyll					
Treatments	Genotypes	yield t/ha	43 DAS	103 DAS	133 DAS	43 DAS	103 DAS	133 DAS	43 DAS	103 DAS	133 DAS	NDVI index	Tiller no	TKW
control	1	4.542	22.535	82.125	103.35	0.262	16.421	28.025	42.30	48.56	45.1	0.756	5.529	44.55
control	2	4.463	19.76	70.63	95.95	0.245	14.339	23.59	40.42	50.35	44.49	0.687	7.571	42.825
control	3	4.851	25.29	82.475	104.93	0.331	13.346	25.54	41.39	43.255	44.09	0.733	6.458	46.855
control	4	4.594	23.725	81.79	104.18	0.357	17.173	30.805	43.47	48.28	45.845	0.739	6.362	49.39
control	5	4.219	22.62	71.465	110.27	0.270	16.108	29.65	41.89	47.245	43.26	0.748	6.058	43.075
Sp7	1	4.406	23.42	76.725	104.73	0.280	14.948	29.99	43.63	48.155	46.38	0.746	6.275	38.17
Sp7	2	4.406	21.48	70.375	93.485	0.264	13.4	31.72	41.04	49.03	44.705	0.742	7.041	40.075
Sp7	3	4.94	23.125	89.14	104.02	0.316	17.545	31.595	39.83	43.82	42.11	0.789	6.083	49.62
Sp7	4	4.5195	24.5	90.815	100.92	0.3567	17.534	30.055	43.80	48.435	42.99	0.7401	7.1125	51.85
Sp7	5	4.314	21.75	75.56	107.72	0.2920	14.948	29.305	40.44	46.71	43.52	0.8	6.272	43.265
Sp7-S	1	4.5605	21.705	72.72	104.17	0.3084	13.029	26.12	42.74	46.64	43.15	0.7244	6.054	44.715
Sp7-S	2	4.4645	18.465	70	96.975	0.2489	12.602	23.74	43.71	48.575	46.18	0.6946	7.429	40.725
Sp7-S	3	5.1955	23.67	83.105	106.42	0.3252	14.266	29.65	41.75	42.015	43.025	0.7805	5.8665	51.185
Sp7-S	4	4.5825	24.29	83.865	106.515	0.3204	17.7725	26.215	41.81	48.97	42.285	0.7294	6.6375	52.53
Sp7-S	5	4.319	21.845	80.55	107.675	0.29485	16.476	25.53	41.585	48.095	41.68	0.78665	6.775	43.855

Table 8.a: Correlations among variables in the field with control inoculation

CHL 43 DAS	1	-	-					
CHL 133DAS	2	0.2071						
CHL 133DAS	3	0.5568	-0.0849	-				
DRY WT 43DAS	4	0.6324	-0.4841	0.8091	-			
DRY WT 103 DAS	5	0.7153	-0.0003	-0.1148	0.2902	-		
DRY WT 133	6	0.6347	0.2473	-0.2643	0.0654	0.9527	-	
PLANT HT AT 43 DAS	7	0.501	-0.6966	0.5838	0.9449*	0.3878	0.1401	
PLANT HT AT 103 DAS	8	0.6233	-0.5319	0.7907	0.9528*	0.2821	0.0148	
PLANT HT AT 133 DAS	9	0.2467	-0.4114	-0.4338	0.0825	0.7938	0.6794	
TILLER NO	10	-0.8269	0.339	-0.7364	-0.9347*	-0.5277	-0.3063	
YIELD HA2010	11	-0.5432	-0.1656	0.2984	0.0692	-0.838	-0.8102	
%1000GRAIN WT	12	0.7244	-0.3618	0.6704	0.9471*	0.5004	0.3316	
NDVI INDEX	13	0.5717	0.5938	0.545	0.1187	0.0132	0.0176	
MPN AT 43 DAS	14	0.6014	-0.4158	0.0717	0.4705	0.7986	0.5933	
MPN AT 103 DAS	15	-0.6544	-0.6232	-0.2027	0.0405	-0.3904	-0.4165	
MPN AT 133 DAS	16	-0.9401	0.0631	-0.7423	-0.8302	-0.5677	-0.4007	
		1	2	3	4	5	6	

PLANT HT AT 43 DAS	7	-										
PLANT HT AT 103 DAS	8	0.9059*	-									
PLANT HT AT 133 DAS	9	0.3316	0.1759	-								
TILLER NO	10	-0.8748	-0.9484*	-0.2777	-							
YIELD HA2010	11	-0.0112	-0.067	-0.7833	0.2705	-						
%1000GRAIN WT	12	0.9154*	0.8377	0.1948	-0.9100*	-0.0559	-					
NDVI INDEX	13	-0.1595	0.237	-0.3387	-0.3091	-0.2524	0.0302	-				
MPN AT 43 DAS	14	0.5971	0.6066	0.8531	-0.6902	-0.7769	0.4954	0.0632	-			
MPN AT 103 DAS	15	0.2195	-0.1026	-0.0802	0.2706	0.6659	0.0284	-0.8809*	-0.3439	-		
MPN AT 133 DAS	16	-0.706	-0.849	-0.198	0.9585**	0.3744	-0.8318	-0.5285	-0.6387	0.5076	-	
		7	8	9	10	11	12	13	14	15	16	

Table 8.b: Correlations among variables in the field with Sp7 inoculation

CHL 43 DAS	1	-					
CHL 103DAS	2	0.3418	-				
CHL 133DAS	3	0.5222	0.5341	-			
DRY WT 43DAS	4	0.9007*	0.0355	0.605	-		
DRY WT 103 DAS	5	0.8409	0.1865	0.0022	0.6283	-	
DRY WT 133	6	0.0979	-0.1149	-0.7596	-0.1806	0.6077	-
PLANT HT AT 43 DAS	7	0.8861*	0.0865	0.6391	0.9854**	0.6143	-0.2174
PLANT HT AT 103 DAS	8	0.5617	-0.5815	0.0461	0.7712	0.5168	0.1024
PLANT HT AT 133 DAS	9	-0.0622	-0.5563	-0.871	-0.1474	0.4412	0.8841
TILLER NO	10	-0.1147	0.8752	0.4584	-0.3205	-0.2982	-0.3582
YIELD HA2010	11	-0.4512	-0.8038	-0.4168	-0.1999	-0.4218	-0.0335
%1000GRAIN WT	12	0.6461	-0.4735	-0.0305	0.7515	0.6653	0.2947
NDVI INDEX	13	-0.2415	-0.6469	-0.5169	-0.1482	-0.1097	0.2877
MPN AT 43 DAS	14	0.2569	-0.2271	-0.5461	0.0699	0.5664	0.7943
MPN AT 103 DAS	15	-0.8834*	-0.1311	-0.0625	-0.7108	-0.9872	-0.5394
MPN AT 133 DAS	16	0.6116	0.1288	0.063	0.4458	0.5937	0.3677
		1	2	3	4	5	6

PLANT HT AT 43 DAS	7	-									
PLANT HT AT 103 DAS	8	0.7156	-								
PLANT HT AT 133 DAS	9	-0.1873	0.3648	-							
TILLER NO	10	-0.2495	-0.8488**	-0.7034	-						
YIELD HA2010	11	-0.3194	0.3259	0.2881	-0.6227	-					
%1000GRAIN WT	12	0.671	0.9617	0.4614	-0.8218	0.2919	-				
NDVI INDEX	13	-0.3012	0.3427	0.4683	-0.6283	0.9074	0.4243	-			
MPN AT 43 DAS	14	-0.0579	0.3608	0.7182	-0.5199	0.342	0.5807	0.693	-		
MPN AT 103 DAS	15	-0.6776	-0.6095	-0.4013	0.3627	0.3231	-0.7536	0.0182	-0.5958	-	
MPN AT 133 DAS	16	0.3093	0.3913	0.1868	-0.2405	0.1571	0.6039	0.5002	0.796	-0.6761	-
		7	8	9	10	11	12	13	14	15	16

Table 8.c: Correlations among variables in the field with Sp7-S inoculation

CHL 43 DAS	1	-					
CHL 103DAS	2	0.6864	-				
CHL 133DAS	3	0.3953	0.6052	-			
DRY WT 43DAS	4	-0.12	-0.6076	-0.6442	-		
DRY WT 103 DAS	5	0.3715	-0.3704	-0.0027	0.5717	-	
DRY WT 133	6	0.0315	-0.3996	-0.8564	0.4897	0.227	-
PLANT HT AT 43 DAS	7	0.4265	0.08	0.5775	0.239	0.7056	-0.4887
PLANT HT AT 103 DAS	8	-0.0488	-0.5619	0.0772	0.6006	0.8429	-0.1162
PLANT HT AT 133 DAS	9	0.2435	-0.3839	-0.7225	0.6398	0.5389	0.9331*
TILLER NO	10	0.0033	0.5649	0.446	-0.9671	-0.7321	-0.3469
YIELD HA 2010	11	-0.684	-0.9586**	-0.3737	0.5498	0.4228	0.1381
%1000 GRAIN WT	12	0.017	-0.5082	0.0854	0.627	0.8573	-0.1154
NDVI INDEX	13	0.4817	-0.0467	-0.2042	0.7854	0.6763	0.2619
MPN AT 43 DAS	14	0.4524	0.1149	0.5875	-0.3945	0.5195	-0.1801
MPN AT 103 DAS	15	-0.1742	0.3225	-0.1867	-0.6039	-0.839	0.1976
MPN AT 133 DAS	16	-0.0685	-0.418	0.2862	0.4511	0.7096	-0.381
		1	2	3	4	5	6

PLANT HT AT 43 DAS	7	-									
PLANT HT AT 103 DAS	8	0.7782	-								
PLANT HT AT 133 DAS	9	-0.1441	0.1652	-							
TILLER NO	10	-0.4699	-0.7641	-0.5726	-						
YIELD HA2010	11	0.1405	0.71	0.1757	-0.5649	-					
%1000GRAIN WT	12	0.8115	0.9958***	0.1826	-0.7927	0.6558	-				
NDVI INDEX	13	0.5731	0.5329	0.5404	-0.8449	0.0464	0.6019	-			
MPN AT 43 DAS	14	0.4578	0.3142	-0.0149	0.1958	-0.0158	0.292	-0.1299	-		
MPN AT 103 DAS	15	-0.8986	-0.9525	-0.1324	0.7831	-0.4864	-0.9751**	-0.7033	-0.2652	-	
MPN AT 133 DAS	16	0.8538	0.9614	-0.1024	-0.6364	0.6262	0.9592	0.4434	0.2982	-0.9446*	-
		7	8	9	10	11	12	13	14	15	16

Table 9: Correlation chart for the 1st experiment of plants at different stages of development in controlled conditions

Total Root length	**1**	**-**	**10DAT**			**-**	**20DAT**			**-**	**30DAT**		
Root surface area	2	0.84***	-			0.61**	-			0.89***	-		
Avg Diameter	3	-0.2228	0.3212	-		0.1532	0.86***	-		0.56*	0.87***	-	
Root volume	4	0.56*	0.91***	0.66***	-	-0.2986	0.55*	0.87***	-	0	0.0228	0.50*	-
		1	2	3	4	1	2	3	4	1	2	3	4

Note: DAT = Days after transfer

Table 10.a: Correlations among variables measured in the growth chamber (control treatment)

Plant height	1	-								
Dry weight shoot	2	0.44	-							
Dry weight root	3	0.001	0.29	-						
Fresh weight root	4	0.37	0.39	0.32	-					
Total root length	5	0.21	0.19	0.52**	0.31	-				
Root surface	6	0.17	0.28	0.76***	0.47*	0.84***	-			
Root volume	7	0.05	0.31	0.81***	0.47*	0.49**	0.87***	-		
Root diameter	8	-0.19	0.14	0.20	0.04	-0.56	-0.06	0.41*	-	
SPAD	9	0.36	0.35	-0.10	0.39	0.001	-0.05	-0.07	-0.07	-
		1	2	3	4	5	6	7	8	9

Table10.b: Correlations among variables measured in the growth chamber (Sp-7 treatment)

Plant height	1	-										
Dry weight shoot	2	0.25	-									
Dry weight root	3	0.45*	0.65***	-								
Fresh weight root	4	0.19	0.21	0.39	-							
Root length	5	0.27	0.58**	0.64***	0.43*	-						
Root Surface	6	0.43*	0.60**	0.76***	0.51**	0.93***	-					
Root volume	7	0.54**	0.52**	0.78***	0.50**	0.69***	0.90***	-				
Root diameter	8	0.22	-0.14	0.008	0.042	-0.539	-0.223	0.2	-			
SPAD	9	0.11	-0.25	-0.06	0.182	-0.076	-0.139	-0.19	-0.09	-		
CFU/ml	10	-0.27	0.10	-0.34	0.149	0.057	-0.144	-0.37	-0.44*	-0.07	-	
CFU/gm	11	-0.28	0.06	-0.39	0.013	0.011	-0.205	-0.44*	-0.47*	-0.06	0.98***	-
CFU/cm2	12	-0.30	0.04	-0.40	0.095	-0.006	-0.211	-0.43*	-0.43*	-0.04	0.99***	0.99***	-
		1	2	3	4	5	6	7	8	9	10	11	12

Table10.c: Correlations among variables measured in the growth chamber (Sp7-S treatment)

Plant height	1	-										
Dry weight shoot	2	0.3466	-									
Dry weight root	3	0.358	0.2321	-								
Fresh weight root	4	-0.0007	0.1022	0.48*	-							
Total root length	5	0.1855	0.2164	0.64***	0.57**	-						
Root surface	6	0.2888	0.3293	0.76***	0.66***	0.94***	-					
Root volume	7	0.3527	0.40*	0.78***	0.68***	0.79***	0.94***	-				
Root diameter	8	0.3362	0.40*	0.3355	0.21	-0.1314	0.1892	0.478*	-			
SPAD	9	-0.1516	-0.148	-0.1413	0.1853	-0.0355	-0.0376	-0.039	-0.038	-		
CFU/ml	10	-0.2739	-0.112	-0.2301	0.0159	-0.3302	-0.3409	-0.305	-0.031	0.3812	-	
CFU/gm	11	-0.3169	-0.105	-0.3117	-0.049	-0.41*	-0.42*	-0.393	-0.061	0.41*	0.98***	-
CFU/cm^2	12	-0.3251	-0.108	-0.3202	-0.0174	-0.42*	-0.43*	-0.397	-0.054	0.42*	0.98***	0.99***	-
		1	2	3	4	5	6	7	8	9	10	11	12

Table 11a. base pair (bp) size and peak height data for all the sample used in TRFLP analysis for 2010

Genotypes	Treatments	Year	Replication	sampling time	N stress	bp size	peak height
2	control	2010	1	43 DAS	E1	127	22632
2	control	2010	2	43 DAS	E1	127	18157
2	control	2010	3	43 DAS	E1	115	21918
2	control	2010	4	43 DAS	E1	88	18468
2	Sp7	2010	1	43 DAS	E1	129	8041
2	Sp7	2010	2	43 DAS	E1	128	30877
2	Sp7	2010	3	43 DAS	E1	128	2719
2	Sp7	2010	4	43 DAS	E1	128	5799
2	Sp7-S	2010	1	43 DAS	E1	128	4850
2	Sp7-S	2010	2	43 DAS	E1	128	5009
2	Sp7-S	2010	3	43 DAS	E1	128	5724
2	Sp7-S	2010	4	43 DAS	E1	129	4016
4	control	2010	1	133 DAS	E2	128	4480
4	control	2010	2	133 DAS	E2	128	3105
4	control	2010	3	133 DAS	E2	128	3408
4	control	2010	4	133 DAS	E2	127	9490
4	Sp7	2010	1	133 DAS	E2	128	2040
4	Sp7	2010	2	133 DAS	E2	128	4284
4	Sp7	2010	3	133 DAS	E2	128	1554
4	Sp7	2010	4	133 DAS	E2	128	1573
4	Sp7-S	2010	1	133 DAS	E2	128	7825
4	Sp7-S	2010	2	133 DAS	E2	128	954
4	Sp7-S	2010	3	133 DAS	E2	129	2944
4	Sp7-S	2010	4	133 DAS	E2	128	2278

Table 11b. base pair (bp) size and peak height data for all the sample used in TRFLP analysis for 2011

Genotypes	Treatments	Year	Replication	sampling time	N treatment	bp size	peak height
2	control	2011	1	43 DAS	Hi N	128	16521
2	control	2011	2	43 DAS	Hi N	127	4630
2	control	2011	3	43 DAS	Hi N	128	9193
2	sp-245	2011	1	43 DAS	Hi N	115	1157
2	sp-245	2011	2	43 DAS	Hi N	115	2825
2	sp-245	2011	3	43 DAS	Hi N	115	2855
2	Sp7	2011	1	43 DAS	Hi N	115	1193
2	Sp7	2011	2	43 DAS	Hi N	128	12350
2	Sp7	2011	3	43 DAS	Hi N	129	15197
2	Sp7-S	2011	1	43 DAS	Hi N	129	9284
2	Sp7-S	2011	2	43 DAS	Hi N	129	20059
2	Sp7-S	2011	3	43 DAS	Hi N	128	11441
3	control	2011	1	133 DAS	low N	128	12327
3	control	2011	2	133 DAS	low N	127	22079
3	control	2011	3	133 DAS	low N	128	6062
3	sp-245	2011	1	133 DAS	low N	115	251
3	sp-245	2011	2	133 DAS	low N	115	4577
3	sp-245	2011	3	133 DAS	low N	115	986
3	Sp7	2011	1	133 DAS	low N	115	1092
3	Sp7	2011	2	133 DAS	low N	129	17160
3	Sp7	2011	3	133 DAS	low N	129	5835
3	Sp7-S	2011	1	133 DAS	low N	129	5184
3	Sp7-S	2011	2	133 DAS	low N	128	7907
3	Sp7-S	2011	3	133 DAS	low N	128	6777
3	Sp7-S	2011	4	133 DAS	low N	128	5285
3	Sp245	2011	4	133 DAS	low N	128	13780
3	control	2011	4	133 DAS	low N	87	25230