The Enhancement of Wheat Growth after Inoculation with Free-Living Nitrogen-fixing Bacteria

Suzanne Constance Wiebkin

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Department of Soil and Water

Waite Campus

The University of Adelaide

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Summary

A wide range of microorganisms within the soil environment is capable of fixing atmospheric N_2. Diazotrophic or N_2-fixing microorganisms are generally classified as symbionts or as free-living associative organisms. Many of the free-living diazotrophic bacteria associate closely in the rhizosphere of Graminaceae and have been studied for their potential for improving plant growth.

Reduction in application of nitrogenous fertilisers has both economic and environmental benefits and the development of N_2-fixing inoculants for wheat to compensate for any lower N fertiliser application has been promoted in various countries. Selection of inoculants suitable for both host and geographic location has been the aim of many studies, because use of inoculants suitable in one region is not always successful in another. Problems encountered with the use of introduced inoculants include host specificity, persistence and competition from local species already adapted to the environment. Selection of local species for use as plant growth-promoting organisms may provide a better alternative than the introduction of an already developed commercial inoculant.

The focus of this work is the selection of bacteria for use as inoculants, to assist in early establishment of wheat in environments where winter rainfall is variable. This early establishment evokes the concept of early development of root growth. The beneficial effects on plants of mixed N_2-fixing bacteria from 3 local soils (Kapunda, Avon and Waite) were compared to identify soils from which to isolate potential inoculants. Criteria for selecting soils included evidence for associative N_2 fixation within the potentially N_2-fixing populations and stimulation of wheat roots. One of the soils (Kapunda) was selected for further study.

Experiments to identify dominant bacterial species within the mixed N_2-fixing assemblage were carried out using fatty acid profiles from fatty acid methyl ester analysis (GC-FAME). The community fatty acid profile from bacteria from the Kapunda soil source showed more diverse fatty acids compared with the profiles from

Avon and Waite soils. *Stenotrophomonas maltophilia*, a potential plant growth-promoting bacterium, contained fatty acids similar to dominant fatty acids within the community profile from the Kapunda soil source. The bacterium was selected as one of three used as potential inoculants for wheat. Molecular tools were used to characterise and group bacteria isolated from the soil into sub-groups of the Proteobacteria, identify isolates carrying N_2 fixation (*nif*) genes and distinguish between them using DNA fingerprinting techniques. The resulting information enabled the selection of reduced numbers of isolates for further testing without unnecessary duplication.

After preliminary tests, 3 bacterial isolates were selected to be co-inoculated onto wheat. The isolates were identified as *Pantoea agglomerans, Stenotrophomonas maltophilia* and *Enterobacter taylorae* (isolated from within the wheat plant). Inoculation tests were carried out with wheat growth in low nutrient sand with ammonium nitrate as a supplement. Results indicated that raised levels of plant N in the inoculated treatments could be attributed to N_2 fixation by the bacteria. The potential for these bacteria to increase wheat growth and yield now requires further testing under field conditions.

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

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Conference Articles

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Chapter 1: Introduction

1.1 General Introduction

In agricultural systems, excessive application of artificial nitrogenous fertilisers is not only expensive but has detrimental environmental consequences. In an attempt to reduce the use of fertilisers, inoculation of plants with microorganisms for use as bio-fertilisers may be a more desirable option. In the winter rainfall regions in southern Australia, non-leguminous crops such as wheat are subject to seasonal variation in soil moisture. Early establishment of root growth as a consequence of bacterial stimulation may provide the plant with an increased surface area and a better chance for coping with water stresses. Inoculation of field crops by associative N_2-fixing bacteria, in particular *Azospirillum* spp. which associate closely in the rhizosphere and within the plant, have been reported to stimulate early root branching in wheat (Michiels et al, 1989). As successful cropping is dependent, not only on soil water but also, on the availability of sufficient soil N, the promotion of biological associative N_2-fixing bacteria in the rhizosphere may enhance both water and N uptake in the plant, particularly where N limits plant growth (van Tran et al, 1994). Variations in environmental conditions may contribute to inconsistencies in plant and microbial growth. For identifying potential inoculants, the isolation of bacteria from local soils with adaptation to climatic conditions and competitive pressures may stimulate more effective plant growth promotion than inoculants introduced from elsewhere.

N_2-fixing bacteria or diazotrophs found in the soil are classified as either symbionts or as free-living microorganisms. The symbiotic relationship between rhizobia and most legumes benefit both bacteria and host and is dependent upon bacterial penetration into the plant tissue and subsequent root nodulation. Free-living N_2-fixing bacteria which associate closely with plant roots in the rhizosphere have been grouped together as associative diazotrophs (Kloepper and Beauchamp, 1992). Although these bacteria do not induce nodulation in the plant, they are found in intimate contact with the roots of non-legumes and can increase fixed N levels in the rhizosphere and stimulate plant growth.

A range of bacterial genera has been identified as Plant-Growth Promoting Rhizobacteria (PGPR; Kapulnik, 1996). In greenhouse experiments PGPR such as fluorescent pseudomonads have stimulated plant growth (Hoflich and Wiehe, 1994; Lifshitz et al, 1987; Glick et al, 1995). Fluorescent *Pseudomonas* spp. and *Bacillus* spp. have been used as inoculants in the field, but responses have been variable and mechanisms obscure (Dart, 1986). *Azospirillum* spp. have been used as inoculants for non-leguminous plants with varying levels of success both in field trials and in the glasshouse. Inoculation of host plants by *Azospirillum* spp. has led to increased plant N content (Heulin et al, 1989), stimulation of plant roots (Michiels, 1989), increased yield in rice and maize grain and increased uptake of soil water and minerals (Okon and Labandera-Gonzales, 1994). It is not currently known whether enhanced growth can be directly attributed to one of these characteristics or whether a combination of these factors is responsible. *Azospirillum* is one of the most widely researched associative bacteria (Bashan and Holguin, 1997) and has been selected as a reference in comparative growth studies throughout the thesis.

Approaches to the selection of bacteria as inoculants for plant growth promotion have been varied. Screening of bacteria for specific traits is one approach. Utilization of 1-aminocyclopropane-1-carbozylate (ACC) as a sole N source for pseudomonads is a trait linked to plant growth promotion (Glick et al, 1995). Other studies, in rice, have used the spermosphere model system to select bacteria from the rhizosphere in association with acetylene reduction asssay (ARA) to measure nitrogenase activity (Thomas-Bauzon et al, 1982; Omar et al, 1989). Nutrient-deficient medium, to repress bacteria other than *Azospirillum* has facilitated isolation of *Azospirillum lipoferum* from resulting pellicles with nitrogenase activity measured by ARA as a selection criterion (van Tran et al, 1997). PGPR strains pre-determined as increasing wheat yield have been tested in the field using shoot and root biomass as well as yield to measure plant growth promotion (Germida and Walley, 1996). A structured approach to the selection and isolation of PGPR is needed and is addressed in this thesis.

1.1.1 Aims

The aims of this study are:

- to develop an effective screening system to isolate free-living N_2-fixing bacteria from southern Australian soils,

- to select strains demonstrating the ability to enhance wheat growth as potential inoculants. These will be isolated from both the rhizosphere and from within the plant. Selection will be based on the following characteristics:

  a) the proven ability to fix N_2 as identified by $^{15}N_2$

  b) the ability to increase root growth

  c) the ability to improve plant N content

  d) the ability to colonise and persist in the rhizosphere

Chapter 2: Literature Review

2.1 Introduction

The rhizosphere is a complex dynamic interface between plant and soil where the success of microorganisms competing for niches on or in the plant root are linked with factors such as physical soil structures, climatic variations as well as the characteristics of plant exudates. Adaptability of microorganisms to local environmental conditions is necessary for their successful colonisation in the rhizosphere.

The mode of action taken by PGPR such as azospirilla to elicit a plant response is unclear. Evidence has been put forward that bacteria excrete phytohormones and nitrite into the rhizosphere, which appear to stimulate the growth of plant roots (Zimmer and Bothe, 1989). The ability to fix N_2 is well established (Klucas, 1991). The response of plants to a mechanism or series of mechanisms employed by the bacteria results in improved root growth, enhanced uptake of minerals, tolerance to water stress and changes in plant metabolism with associated increases in proton effluxes from the roots. It is possible that several mechanisms may act in concert, thus affecting a significant plant response over and above that which may be achieved by a single mechanism (Bashan and Levanony, 1990). If the enhancement of plant growth is due to more than one mechanism it is likely that several compatible inoculants may outweigh the benefits of one. This review will focus mainly on the mechanisms and proposed mode of action as currently documented in the literature for associative N_2-fixing bacteria.

2.2 N_2 fixation

Biological N_2 fixation in the rhizosphere by associative bacteria is one of the factors thought to contribute to improve N uptake in the plant.

2.2.1 Sources of N

N, comprising 78% of the atmospheric gases, is necessary for amino acid and protein production in plants. N is only available for plant uptake in the combined form as

ammonium or nitrate. As one of the major nutrients required by plants, N is often growth limiting especially when indirectly removed from the soil through agricultural cropping.

In nature, the input of N into the soil is primarily from the atmosphere. N_2 fixation through lightning accounts for 6-50%, although this source of fixation is extremely unpredictable (Hansen, 1994). Worldwide biological fixation of atmospheric N_2 accounts for 100 to 180 x 10^6 metric tons of N per year and provides twice as much N as artificial fertilisers (Hansen, 1994). The energy costs for production of biological N_2 fixation are three times more efficient than the industrial process and renewable plant residues provide the major energy source (Newbould, 1989). In addition to the high costs of production, losses of N from artificial fertilisers are also high through leaching in the soil profile. About 50% of N in the soil is lost through leaching of nitrate and through denitrification, both of which can have detrimental effects on the environment. Leaching of nitrate can pollute waterways and cause acidification of soils. Denitrification can lead to the formation of nitrous oxide. The nitrous oxide gas can reach the stratosphere and contribute to the destruction of the ozone layer (Delwyche and Bryan, 1976). Of the N_2 fixed biologically, only 10 to 20% is lost. Therefore, this is an important source of soil N.

Although symbiotic N_2 fixation in legumes accounts for 80% of the reduced N on land, net annual gains of N in non-legumes have been estimated to be as high as 100 kg/ha/year (Giller and Day, 1985). However controversy exists regarding the amount of fixed N that is actually transferred to the plant as a result of the associative interaction with diazotrophs. Between 1-10% of the bacteria in the rhizosphere are estimated to be N_2-fixing bacteria (Patriquin et al, 1983). However, in the rice rhizosphere, 40% of bacteria were found to be N_2 fixers (van Tran et al, 1996). Irrespective of the numbers of N_2-fixing bacteria in the rhizosphere, conditions must be conducive to nitrogen fixation by the bacteria and the levels of fixed N may or may not be sufficient to consider N_2 fixation as a characteristic feature necessary for a potential inoculant.

2.2.2 Conditions for nitrogenase activity

The fixation of 1kg of N_2 by bacteria requires 10 kg of carbon compounds and, without adequate supply of soluble carbon compounds to provide energy, nitrogenase activity is reduced (Giller and Day, 1985). As N_2 fixation will only occur in actively growing bacteria, the carbon source must sustain cell growth as well as that required for nitrogenase activity (Silvester and Musgrave, 1991). The amount of N_2 fixed in Australian soils by associative diazotrophs, when plant residues are retained as stubble, has been estimated in the range of 32-56 kg N ha^{-1} (Halsall et al, 1985). As the associate diazotrophs are unable to degrade starch, cellulose and carboxymethyl cellulose, cellulolytic microorganisms are necessary to provide increased carbon substrates to facilitate N_2 fixation. In a study designed to evaluate effective N_2 fixation, mixed microbial cultures were compared with a single microorganism. The amount of N_2 fixed was increased 22 fold when *Azospirillum* sp DN64 was mixed with 3 fungi and grown in sand with an N-free, C-free mineral salts medium and straw as the carbon source (Halsall, 1993). The fungi were isolated from straw-amended soil or straw from the field and selected for their ability to decompose cellulose. A synergistic response was observed.

Nitrate assimilation also requires a similar energy input to N_2 fixation (Kennedy, 1988). The supply of available energy from the plant roots may not enable populations of associative diazotrophs to colonise and grow in sufficient numbers to make a significant contribution of fixed N to the plant (Whipps and Lynch, 1983). However, some studies have demonstrated that rhizosphere bacteria have stimulated the plant to increase root exudates, which in turn have increased the available energy source (Barber and Lynch, 1977).

Fixation of N_2 is mediated through the enzyme nitrogenase, which is suppressed by interaction with oxygen. One mechanism for protection against high concentrations of oxygen is via the production of exopolysaccharides, which act as barriers against oxygen diffusion (Volpon et al, 1981). This is perhaps an evolutionary attempt to reduce contact with oxygen by creating an oxygen gradient. In addition, many of the diazotrophic bacteria (associative N_2 fixing bacteria) are micro-aerophyllic and their motility enables them to locate the appropriate partial pressure of oxygen appropriate for growth in the rhizosphere. This mechanism is known as aerotaxis.

The nitrogenase activity, responsible for catalysing the reduction of N_2 to ammonia, can be inhibited or switched off and on by a number of environmental factors. These factors include the availability of carbon, nitrate or ammonium as well as the above-mentioned response to the partial pressure of oxygen (Alexander and Zuberer, 1989).

Nitrogenase activity is measured, most commonly, through the acetylene reduction assay (ARA) by gas chromatography. By adopting ARA to measure the nitrogenase activity, the optimal partial pressure of oxygen necessary for effective nitrogenase activity was assessed. ARA measurements were highest when partial pressures between 1.3 and 2.1 kPa O_2 were maintained and became correspondingly lower when oxygen concentrations were increased (Alexander and Zuberer, 1989). ARA measurements made on hydroponic solutions with low O_2 was similar to that found around the roots grow in solid medium. When partial pressure of oxygen dropped below 1.3 kPa in an N-free medium in the root zone of maize and sorghum, nitrogenase activity was substantially reduced due to insufficient energy input from respiration (Alexander and Zuberer, 1989).

2.2.3 Methods to measure N_2 fixation

Direct and indirect methods are employed to measure amounts of N_2 fixed by bacteria. Indirect measurements such as the ARA are commonly used to quantify nitrogenase activity. Apart from N, nitrogenase can reduce other triple bonded compounds such as cyanide, nitrous oxide and acetylene (Hansen, 1994). At concentrations of 6%, C_2H_2 is a competitive inhibitor of N_2 fixation. Acetylene is reduced to ethylene, which can be measured simply and cheaply by gas chromatography with a Flame Ionisation Detector (FID) (Hardy et al, 1973). Although this method has been most commonly used, it is actually neither a measure of N_2 fixed nor of N transferred to the plant. It provides temporal information on nitrogenase activity and therefore its use is limited to assessing bacterial responses in the rhizosphere to environmental factors such as seasonal changes and pH.

Direct methods for accurately measuring the amount of fixed N transferred to the plant include the ^{15}N isotope dilution method and the exposure of the plant/microbe system to $^{15}N_2$. The ^{15}N isotope dilution method uses a known amount of ^{15}N as a fertiliser source. If used in the laboratory as the sole mineral form, N_2 fixed biologically within plants

such as legumes can be calculated by subtracting the difference between ^{15}N and ^{14}N present in the plant tissue (Hansen, 1994). The ^{15}N isotope dilution method has been used to assess the contribution of N_2-fixation to sugar cane, using *Brachiaria radicans* as a reference plant (Urquiaga et al, 1989). However, the two plants showed quite different patterns of N uptake and quantification of biologically fixed N was difficult. Exposure of the plant to $^{15}N_2$ may therefore be an appropriate method for assessing levels of biologically fixed N present in the plant. The $^{15}N_2$ method is not used as commonly as ARA, as the isotope is expensive and access to mass spectrometers for outcome measurements is limited. However, it does provide a more accurate measure of N_2 fixation in the plant. $^{15}N_2$ has been used to measure the transfer of low levels of fixed N from free-living bacteria to sorghum plants (Giller et al, 1984) and wheat (Merbach et al, 1998). Other studies using the ^{15}N isotopic dilution method found that the contribution of fixed N to rice N content was significantly higher. In rice, inoculated with either *Azospirillum* Wb3 or *Enterobacter* S1 the fixed N content comprised 46% and 41% of total plant N respectively (Mehnaz et al, 2001).

Results from assessment of the efficacy of N transfer are variable and make long-term analysis of fixed N in field crops, especially in wheat, extremely difficult. However, where the N is transferred to the plants following biological fixation, the transfer may be more effectively achieved within the plant. Here the prerequisites for nitrogenase activity may be more favourable. Within the plant, endophytic bacteria would have access to carbon solutes, reduced partial pressures of oxygen favourable for nitrogenase activity, adequate moisture and reduced competition for resources. Endophytes are frequently seen in the parenchyma of the root cortex, with access to cytoplasmic contents. The high amino acid levels of the cytoplasm is thought likely to inhibit nitrogenase activity but the intercellular spaces may contain glycoproteins which would provide a carbon source and small nitrogenous source which may support N_2-fixation by bacteria (Sprent and James, 1995). There is also a suggestion that the bacteria may be able to utilise products of host defence as metabolic substrates. Endophytes have also been found in the xylem of species such as sugar cane and sorghum as well as in wheat and rice and this would provide transport for relocation (Sprent and James, 1995). Although, theoretically, the potential exists for endophytes to make a positive contribution of fixed N to the plant, it is uncertain whether there are sufficient numbers of endophytes present within the plant, to account for that potential.

2.2.4 Genera of bacteria which fix N$_2$

Free-living N$_2$-fixing bacteria include species such as *Enterobacter cloacae* and *Klebsiella oxytoca* (Mirza et al, 2001), well-studied bacteria such as *Azotobacter* spp. (De Luca et al, 1996) and *Azospirillum* sp. (Chotte et al, 2002; Mehnaz et al, 2001). Other diazotrophic bacteria found in non-leguminous crops have been identified as *Acetobacter diazotrophicus, Herbaspirillum scropedicae* and *Herbaspirillum rubrisubalbicans* (Dobereiner et al, 1995). Nitrogenase activity also occurs in free-living bacteria such as *Burkholderia vietnamiensis, Enterobacter cloacae* as well as *Azospirillum lipoferum* (van Tran et al, 1996). Amongst the most widely studied N$_2$-fixing bacteria are *Azospirillum* spp. Although the bacterium was first described over 70 years ago (Beijerinck, 1925) it was not until 38 years later that it was described to fix atmospheric N$_2$. When assessment of biological N$_2$ fixation was facilitated by improved methods of analysis, such as acetylene reduction, it became evident that there was a relationship between non-legumes and N$_2$-fixing bacteria. *Pantoea agglomerans* also able to fix nitrogen is also frequently identified in the wheat rhizosphere (Merbach et al, 1998). Strains of *Azospirillum brasilense* and *Azospirillum lipoferum* have been most commonly used in physiological and genetic studies and as a result *Azospirillum* is often adopted as a model system for studying the genetics of associative N$_2$-fixing bacteria (Bashan and Holguin, 1997). Strains *Azospirillum lipoferum* 596, isolated from irrigated wheat in eastern Australia near Griffith (New and Kennedy, 1989) and *Azospirillum brasilense* Sp7 (ATCC 29145) have been used as reference strains in this thesis.

2.3 Stimulation of plant roots

Increases in grain yield, uptake of minerals and water, changes in root length and early stimulation of germination are the most frequently reported features of plants following inoculation with diazotrophic bacteria.

2.3.1 Role of root hairs

Increased branching in the lateral roots and a large number of root hairs increases the surface area in the roots and enhances the uptake of water and solutes by the plant (Bowen and Rovira, 1969). The root hairs are elongated epidermal cells that vary in

length from 80 to 1500 μm and with a diameter between 5 to 20 μm (Ewens and Leigh, 1985). The cell size is dependent on both microbial influences and environmental factors such as pH, water potential and soil texture. Investigations carried out on the role of root hairs in relation to water transport, found that a gradient of turgor pressures was mainly responsible for water uptake (Jones et al, 1983). It is the turgor pressure, rather than the membranes of root hairs, which is actively involved in water transport into the plant (Jones et al, 1983). While turgor pressure is directly linked to osmolarity of cell contents, the low turgor pressure in the root hair cells is grossly associated with a gradient, which increases with proximity to the cells of the cortex. Observations of improved water uptake following inoculation with free-living diazotrophic bacteria led to a study to test the water uptake in dryland sorghum following inoculation with *Azospirillum brasilense* Cd (Sarig et al, 1988). Sorghum was grown in the field where moisture was derived solely from stored winter rains. The water potential of inoculated plants was consistently higher than the controls and despite the increased leaf area and associated evapotranspirational demand on the root system. The inoculated plants were significantly less water stressed than controls. The mechanisms responsible for the improved water status of the plants are unclear. The bacteria may have promoted a deeper rooting system thus providing better access to soil moisture. Early plant growth and avoidance of water stress appears to have been one of the major differences between the two treatments.

Nutrient transport in the root hairs appears to be controlled by a H^+ extrusion pump in the plasmalemma. Protons are intimately involved in nutrient transport and are transferred between cellular compartments and surroundings (Felle, 1987). The acidification of the rhizosphere as a consequence of proton efflux through the membranes of the root cells has been put forward as a mechanism for facilitating mineral uptake in plants (Gardner et al, 1982). Observations of increased mineral uptake in association with *Azospirillum* were tested for a possible effect by the bacteria on root membrane potential. Wheat seedlings were inoculated with *Azospirillum brasilense* Cd and grown in hydroponic conditions where pH changes were monitored. Quantitative analysis of protons from the roots was also assessed by titrating the nutrient solution with NaOH (0.05M) back to the original pH at the commencement of the experiment (Bashan et al, 1989). The results showed that both inoculated seeds and seedlings increased proton efflux from the roots. When alternative bacteria such *as Pseudomonas*,

Bacillus and *Azotobacter* spp. were used as inoculants, there was no increase in proton efflux or in root surface area. It appears that *Azospirillum* is capable of affecting the metabolism of plant cells. A current suggestion is that the bacteria may excrete signal molecules, which are transported across the plant cell wall. Internal receptors may then respond to these signals by initiating changes in plant metabolism (Bashan and Holguin, 1997).

2.3.2 Bacterial influence on lateral root growth

Lateral roots emerge at constant distances behind the tip of the root and appear to emerge in acropetal (proceeding towards the apex) sequence. Although exogenously applied auxins, which are transported into the roots appear to stimulate lateral root emergence, cytokinins and absciscic acid are inhibitory and gibberellins do not appear to have any effect on lateral root development (Charlton, 1996). Although at present there is no evidence to support the notion that endogenous plant hormones are responsible for initiating lateral root emergence, cytokinins and abscisic acid have been found in the root tip. It is possible that gradients of plant hormones may be involved.

Like root hairs, lateral root emergence contributes to the overall surface area of the roots. In wheat, the upper roots, laterals and root hairs are responsible for the majority of water and nutrient uptake to the plant. The deeper roots play a very minor part in water and nutrient uptake (Bole, 1977).

Inoculation of plant root PGPR bacteria such as *Pseudomonas cepacia* R85 and *Pseudomonas putida* have demonstrated a direct influence on plant roots, in particular increasing the total length of the root system (de Freitas and Germida, 1990). Inoculation of wheat roots with *Pseudomonas* sp., *Azotobacter chrococcum, Bacillus megaterium, Bacillus subtilis, Azospirillum* sp. and *Klebsiella pneumoniae* can increase root length 72 hours after inoculation (Kapulnik et al, 1985). However, only *Azospirillum* was able to significantly enhance the total root area in 20-day old plants. Other studies have reported root stimulation by bacteria leading to alteration in root growth and function. Changes observed in the root include proliferation of root hairs, increase in surface area, increases in lateral branching and a more developed root system (Okon and Kapulnik, 1986). In drought conditions in Israel, which are similar to a low moisture status in southern Australia, wheat inoculation trials have been carried

out over many years (using *Azospirillum* as an inoculant). *Azospirillum brasilense* Cd and a locally adapted strain *Azospirillum brasilense* Cd1 were used as inoculants. Where rainfall was 350 mm with early high temperatures and water stress, experimental plants were taller than uninoculated controls. Increases in grain due mainly to increased numbers of fertile tillers, increased grain yield, increased shoot dry weights and increased total plant N, all significantly higher than controls (Kapulnik *et al*, 1983).

2.3.3 Phytohormones

Studies have been carried out to test the involvement of auxins produced by bacteria such as *Azospirillum* spp. in initiating changes in root morphology (Bashan and Holguin, 1997). In addition to *Azospirillum* spp., species such as *Enterobacter cloacae* strains (NCIMB11461 and NCIMB11463), *Enterobacter agglomerans* strains (333 and 339), *Pantoea agglomerans* IMET11328 and *Klebsiella aerogenes* DSM681 converted trytophan into indole acetic acid (IAA) (Zimmer et al, 1994). Bacteria isolated from stems and roots of sugar cane were also identified as producing IAA, which was further, enhanced in medium when trytophan was added (Mirza et al, 2001). Increased root and shoot growth of sugar cane was observed following re-inoculation, but it is unclear whether phytohormones played a direct role in growth promotion. Similarly IAA producing isolates obtained from the roots and shoots increased root growth (Mehnaz et al, 2001). The release of phytohormones into the rhizosphere by bacteria may influence plant metabolism, which, in turn, may control the release of root exudates (Martens and Frankenberger, 1994). Increased root exudates would therefore benefit the rhizosphere bacteria. Conversely, inhibition of plant growth in field bindweed (*Convolvulus arvensis* L.) has been observed following the production of bacterial phytohormones (IAA) by bacteria such as *Enterobacter taylorae* when in association with L-trytophan (Sarwar and Kremer, 1995). However, it does seem likely that IAA is implicated in plant growth-promotion by associative bacteria but there is no evidence at present to suggest that plays a major role in the interaction. Growth enhancement has been observed in many different plant species and phytohormones are unlikely to be the sole stimulant in every case.

2.4 Selection of locally adapted bacteria

Examples of plant growth enhancement following bacterial inoculation are documented in the literature, but plant responses to the bacterial strains used as inoculants can vary. When comparing basic growth parameters of bacteria isolated from wheat and rice rhizospheres, maximum growth rates in N-free medium ranged between 0.1 1/hour to 0.2 1/hour while carbon substrate affinity (Ks-values) showed 40 fold differences in optimal conditions (Ueckert and Fendrik, 1995). Similarly, when N_2-fixing characteristics were assessed with ARA on strains isolated from seven regions in New South Wales in eastern Australia, differences in nitrogenase activity in the bacteria in each region ranged between 13 to 100 nmol C_2H_4 mg protein/hour (Han and New, 1996).

The importance of using locally adapted strains as inoculants is demonstrated in Israeli field trials of inoculated wheat, where the local strain stimulated shoot dry weight and grain yield significantly more than the reference strain (Kapulnik et al, 1983). Adaptation to a location, soil type, soil nutrient or host genotype may bring competitive advantages to the strain having previously interacted successfully with rhizosphere colonisers. For example the comparison of bacterial communities from 2 soils demonstrated that one of the communities was well adapted to nickel, which was thought to influence soil structure (Hery et al, 2003). Adaptation by bacteria to local soil conditions was also described after sampling soils at various distances from an old growth forest (Belotte et al, 2003). Bacterial isolates were cultured in media prepared from soils from which they had been isolated and in general growth was poor. However some of the isolates grew 50% better in medium supplied with soil from which they had been originally isolated. The ability to colonise and persist in the rhizosphere in sufficient numbers to evoke a plant response may be improved with a local strain. Successful colonisation, however, may depend in part on the method chosen for inoculation.

2.5 Colonisation

The ability of a potential inoculant to persist and colonise is crucial for success. Factors such as competitiveness, the physical structure of soil and the ability to migrate towards and attach to plant roots play a major role in bacterial colonisation of the roots. N_2-fixing bacteria have frequently been isolated from cereals and grasses, maize and rice. Until recently, it was generally accepted that bacteria such as *Azospirillum* spp. colonised specific host plants, particularly cereals (Bashan and Levanony, 1990). Although host specificity has been documented, a review has proposed that *Azospirillum* spp. appear to be capable of colonising most plants (Bashan and Holguin, 1997). Sixty four plant species are documented as supporting colonisation by *Azospirillum* spp. and the bacteria have been isolated from the rhizosphere of both C3 (rice and wheat) and C4 (sugar cane, maize and sorghum) plants. Other N_2-fixing bacteria such as *Pantoea agglomerans* and *Rahnella aquatilis* were isolated from the rhizosphere of wheat and maize grown in two distinct French soils. The strains from both wheat and maize were similar to each other (Berge et al, 1991).

Despite the ability to colonise a broad range of plants, specificity exists between some plant genotypes and *Azospirillum* spp. Studies carried out using *Azospirillum* spp. as inoculants for Argentinian and Brazilian maize gentoytpes generally showed significant increases in grain yield and total N content (Garcia de Salome and Dobereiner, 1996). However there was no growth response in one Argentinian maize genotype, indicating that there was specificity between the inoculant strains and plant genotype.

Bacterial strains also demonstrate different N_2-fixing efficiencies in association with various rice cultivars. In Bangladesh, *Azospirillum lipoferum* MRB16 was the most effective N_2-fixer isolated from rice cv. Nizershail and in Egyptian soils, *Azospirillum brasilense* NO40 was the dominant strain isolated from rice cv. Giza 171 (Heulin et al, 1989). The spermosphere model, which utilises seed exudates as the sole carbon source for bacteria, has been used as a selective system for the isolation of N_2-fixing bacteria. This system, used in conjunction with ARA has enabled researchers to select the most effective N_2-fixing strains from the rhizosphere of rice (Thomas-Bauzon et al, 1982). Selection of strains isolated in this system resulted in improved yields in inoculated rice.

2.5.1 Competition

Antibiosis may provide bacteria with a competitive advantage for colonising the plant rhizosphere. For example:

- *Burkholderia cepacia* strains isolated from the rhizosphere of maize inhibited *Fusarium* spp. in *in vitro* experiments (Bevivino et al, 1998). When the same bacteria were inoculated onto maize seeds grown in the field soil from which they were originally isolated, four of the strains successfully promoted plant growth whilst competing with native microbia.

- Fluorescent *Pseudomonas* spp. are known to produce secondary metabolites (Budzikiewicz, 1993). *Pseudomonas fluorescens* strain CH33 suppressed *Pythium aphanidermatum* root rot in cucumbers (Moulin et al, 1994).

- Non-fluorescent *Pseudomonas* strains such as *Pseudomonas* sp. strain AN5 protected wheat against *Gaeumannomyces graminis* var *tritici* in pot trials (Nayudu et al, 1994).

- Bacteria such as *Azospirillum* spp. have also been described as producing bacteriocins (Bashan and Holguin, 1997).

Little is known about the interaction of the bacteriocins within the microbial environment. Because *Azospirillum* spp. do not constitute a major component of the rhizosphere populations, competitive advantages within indigenous microbial communities are difficult to predict (Balandreau, 1986). *Azospirillum brasilense* BS52 and *Aoarcus* BH72 were grown in culture together to assess competitiveness. In immobilised culture, *Azospirillum* was the better competitor (Ueckert et al, 1992). The suggestions for improved viability included the ability to produce exopolysaccharides, which may be linked to plant recognition as well as an antagonistic role.

Competition with native microorganisms in the rhizosphere following inoculation has been assessed by a variety of methods. If competitiveness is to be monitored, traditional methods such as plate count, selective media and direct microscopy do not enable identification of bacteria at a strain level. However, the use of immuno-methods and molecular techniques for monitoring colonisation and competitive interactions, enable identification of strains to be confidently carried out. This is of particular value in the

endorhizosphere, where mode of entry and preference of colonisation sites are of interest for promoting possible benefits to plant growth.

2.5.2 Soil physical factors

The microbial community structure is influenced by soil physical factors such as the size, shape and stability of soil particles and the size, shape and continuity of the air pores between the particles. The role that soil structure plays on the dynamics of soil bacteria has been studied by comparing the relationship between the free-living N_2-fixing bacteria, total heterotrophic bacteria and aggregate size fractionation in a sandy soil (Chotte et al, 2002). Active N_2-fixation by bacteria was favoured in the coarse soil fractions while the majority of N_2-fixing bacteria were located in the dispersible clay fraction. The partial pressure of oxygen within the coarse fractions may have contributed to an environment appropriate for nitrogenase activity. Other studies also indicate that soil type influences the composition of microbial communities (Gelsomino et al, 1999; Kreitz and Anderson, 1997).

The water content in soil impacts on the growth of soil bacteria and where rainfall is variable, the drying and re-wetting of soils can influence the composition of the bacterial communities (Fierer et al, 2003). The structure of the bacterial communities from 2 soils receiving similar rainfalls but with different histories of drying and re-wetting were subjected to various drying-re-wetting cycles in the laboratory. The bacterial community adapted to drying and re-wetting soils remained stable whilst the structure of the second community unused to the rewetting cycles was significantly altered.

Despite the ability to cope with water stress and salinity, *Azospirillum* spp. do not persist well in rough sand and calcium carbonate (Bashan et al, 1995). The inability of the bacteria to bind to the plant in sandy conditions or due to the poor motility of bacteria in sand where water films tend not to be continuous may be responsible. Fibrillar binding by the bacteria to the plant is more readily achieved in clay soils as opposed to sandy textures. In addition survival in some soils has also been linked with pH (New and Kennedy, 1989). Data from a survey carried out in eastern Australia to identify locations and species of *Azospirillum*, showed that the bacteria were most commonly found in soils with pH ranging from 5 to 6.6 and rarely below pH 4.5. The

rates of survival and persistence of other bacteria such as the fluorescent pseudomonads are affected by changes in soil physical factors. For example the addition of bentonite into a loamy sand soil increased the survival rate of *Pseudomonas fluorescens* (Heinjen et al, 1992; 1993). Others studies have shown that in finely textured soils bacteria survive better following the addition of specific clays (England et al, 1993).

2.5.3 Chemotaxis

Chemotactic response of some bacteria in the rhizosphere towards root exudates may be a basic factor during colonisation. Such a response towards root exudates by *Pseudomonas fluorescens* is assisted by flagella-driven motility, which can provide the bacteria with a competitive advantage during colonisation of the roots (de Weert et al, 2002). The ability to detect and act on stimulus gradients is due to a cluster of receptors (Shi and Duke, 1998; Taylor and Zhulin, 1998), but the most successful motile response may depend on the shape of the bacteria, in particular rod-like shapes (Dusenberry, 1998). Co-operation within motile bacterial colonies may be employed by cell to cell signalling in response to both chemorepulsion and chemoattraction (Benjacob et al, 1998). The location of chemoreceptors, in particular at the cell poles, may also facilitate response time, with transfer of signalling information among chemoreceptors in *Bacillus subtillus* (Lamana et al, 2002).

An experiment designed to test the chemotactic response of *Azospirillum brasilense* and *Azospirillum lipoferum* isolated from different C_3 and C_4 plants showed that the strains showed marked preferences for root exudates from wheat or maize, irrespective of species (Fedi et al, 1992). The data indicated that although four of eight strains (including *Azospirillum brasilense* and *Azospirillum lipoferum*) were isolated originally from the rhizosphere of maize, they were preferentially attracted to wheat. Other strains responded equally towards exudates from both wheat and maize. The authors indicate that the attraction does not appear to be a specific host-dependent response but a general chemotactic behaviour by *Azospirillum*. It may be useful to pre-test the chemotactic response of a proposed inoculant strain to a wheat genotype as part of the selection process.

2.5.4 Attachment

The attachment of bacteria to the plant must be secure to prevent dislodgment and removal from the root exudates, which provide the carbon source. Many bacteria are known to produce fibrillar structures which anchor them to the root surface ensuring that the growing tip transports them as it travels through the soil (Umali-Garcia et al, 1980). Other bacteria in turn bind to the fibrils or plant cell surface, resulting in large aggregates of bacteria bound to the cell surface of the plant (Matthysse, 1992). The mechanism for the adhesion of bacteria to the plant roots may be mediated by wheat germ agglutinin, (WGA) a plant lectin found on wheat embryos and root tips of wheat plants. If binding does occur between the cell surface carbohydrates of the bacteria and plant lectins, this may provide a pathway for transmission of information from the plant to the bacterium (Karpati et al, 1995).

2.6 Inoculation

The requirements for the establishment of a bacterium on or in the roots include a concentration of inoculant that is viable, the determination of the most effective time of application to the seed or seedling and an effective mode of delivery.

2.6.1 Inoculant concentration

Bacterial cell concentration is critical for the establishment of an inoculant and can have a detrimental effect on plants if too high. *Azospirillum brasilense* (1 ml) was used as an inoculant for each of 5 wheat seedlings, 72 hours after germination at a concentration of 10^5 to 10^6 colony-forming units (cfu/plant) (Kapulnik et al, 1985). Higher bacteria concentrations at 10^8 cfu/plant inhibited plant growth. A study using PGPR inoculants *Pseudomonas aeruginosa* R80, *Pseudomonas fluorescens* R92 and *Pseudomonas putida* R104 required a higher cell concentration of 10^7 to 10^8 cfu/seed, to achieve increased yields in wheat (Germida and Walley, 1996). In the field, concentration of inoculant will also depend on environmental conditions.

2.6.2 Inoculation without carriers

Traditional methods for inoculation have relied on chemotactic responses by the bacteria towards the root exudates followed by attachment. In a recent method

Azospirillum has been introduced into the seeds prior to germination (Creus et al, 1996). Surface-sterilised seeds were soaked for 3 hours in water, followed by 3 hours in inoculum. Seeds were checked at intervals for up to 33 days for bacterial numbers. *Azospirillum brasilense* was introduced to seeds at a concentration of 4.02×10^7 bacteria/g of seeds. When tested at regular intervals, *Azospirillum* inoculant numbers remained constant at 3.7×10^6 cells/g of seeds. Eleven days after inoculation, the number of *Azospirillum* was similar whether plants were inoculated on the seed or the root. This technique may eliminate the need for an inoculant carrier. This method has only been tried for *Azospirillum brasilense*, but it is known that *Azospirillum brasilense* and *Azospirillum lipoferum* are able to encyst and form flocs when environmental conditions are harsh (Sadasivan and Neyra, 1985). This characteristic may be expressed by both species when seeds are dried following inoculation. The position of the inoculum in relation to the plant may influence the success of inoculation. In maize, *Burkholderia ambifaria* MCI 7 promoted plant growth when the inoculum was applied onto the seed but when applied directly to the soil poor growth was observed (Ciccillo et al, 2002).

In general, the use of carriers to deliver inoculants to either the seed or plant allows preparation in advance of planting and the likelihood that the inoculum will persist for a defined period of time.

2.6.3 Inoculant carriers

A variety of carriers have been tested for use with inoculants. Carriers such as granular and ground peat, vermiculite, talcum powder, basalt granules and bentonite have been tested with the *Azospirillum* inoculant. (Fallik and Okon, 1995). Although the peat carriers proved to be the most successful, viable counts of *Azospirillum brasilense* declined over 6 months from 10^{10} cfu/g peat to 10^5 to 10^6 cfu/g peat. When freshly cut peat was used containing 10^8 to 10^9 cfu/g peat, yields in greenhouse experiments improved for both foxtail millet (*Setaria italica*) and maize (*Zea mays*). There was a significant increase in yield from plants inoculated with *Azospirillum brasilense* where growth in the fermentor had encouraged the formation of 40% poly-β-hydroybutyrate (PHB) granules as a consequence of eliminating a source of N in the last stages of culture. The PHB granules can be utilised by the bacteria as a source of carbon and cells containing PHB are able to resist environmental stresses such as desiccation, heat and

starvation, more readily than bacteria that are not associated with stored granules (Fallik and Okon, 1995).

Azospirillum lipoferum CRT1 has been used as an inoculant in association with a microgranulate based on peat and registered under the trade name AZOGREEN® (Fages, 1992). Microgranulates are chemical carriers such as calcium carbonate, sand and marble powder. The physical properties of the carrier enable delivery of the inoculant mechanically into the furrow next to the seed. As an inoculant for cereals, a microgranulate based on an alginate matrix has also been successfully applied (Berge et al, 1990). More recent inoculant carriers include alginate microbeads as substrate for *Azospirillum brasilense,* which can be applied to either wet or dry seeds successfully (Bashan et al, 2002). Whether an inoculant is incorporated into a carrier, fertiliser or directly onto the seed, laboratory conditions do not mimic field conditions and inoculation must therefore be tailored to meet local environmental conditions.

2.6.4 Time of inoculation

The optimal time for inoculation has been tested both in hydroponic and in soil experiments (Bashan, 1985). The results indicated that inoculation shortly after imbibition (initial absorption of water by the seed) was most effective for the stimulation of foliage and root development. Surface-sterilised seeds (100) were inoculated by dipping (slightly covering) them into bacterial suspensions (3 l) for 12 hours. Counts of 10^5 to 10^6 cfu/ml proved to be optimal for significant growth increases while lower concentrations of 10^2 to 10^4 were less effective. If inoculation was performed at a later stage of plant growth, colonisation of inoculants decreased with age of the plant. Shortly after emergence, 83% of bacteria were able to colonise the plant. When inoculation occurred at the two-leaf stage of plant growth, only 28% of the bacteria were able to achieve colonisation (Bashan, 1985). When four successive inoculations were carried out over time, significant increases in root surface area and leaf dry weight were recorded in wheat plants. However, within 20 days bacterial counts returned to the initial count (Bashan, 1985). Time of inoculation is, therefore, a relevant factor, perhaps explaining why many field trials return inconsistent results.

2.6.5 Co-inoculation

The application of mixed cultures as inoculants for plants has resulted in improved as well as synergistic plant responses. Dual inoculation using *Azospirillum brasilense* Cdr and VAM fungus *Glomus* sp. strain A6 resulted in a significant increase in root dry weight compared with single inoculants in association with wheat (Gori and Favilli, 1995). Similarly, a synergistic response was achieved when *Azospirillum lipoferum* and *Phialphora radicola*, an avirulent root parasite of grasses and cereals, were inoculated onto wheat plants (Flouri et al, 1995). In a mixed culture inoculation of *Azospirillum* Cd, Sp7 and Cd-1 at 10^6 cfu/ml, a 70% increase in root elongation occurred. This increase in root growth exceeded those induced by pure cultures where increases of only 7% to 65% were noted (Kapulnik et al, 1985). Application of endophytes as dual inoculants also increased yield and percentage of protein in maize after two bacteria, *Klebsiella oxytoca* VN13 and *Xanthomonas maltophilia* VN12 were introduced directly to seeds as dual inoculants (Kozyrovska et al, 1996). Although the recent use of mixed cultures for inoculating a variety of plants such as sorghum, barley, wheat and legumes have resulted in reports of enhanced plant growth, some studies have reported inhibition of plant growth (Bashan and Holguin, 1997). In other studies, single inoculants promoted growth *in Sorghum bicolor* while a mixture of three bacteria (*Burkholderia cepacia* strain PHP7, *Pseudomonas fluorescens* A23/T3c and *Enterobacte*r sp. strain BB23/T4d) elicited no growth response (Chiarini et al, 1998). Mixed inocula *Azospirillum brasilense, Azotobacter chroococcum, Bacillus polymyxa* and *Enterobacter cloacae* showed varied benefits to wheat depending on length of time after planting with no benefits at maturity (de Freitas, 2000). However, the combination of microorganisms for use as inoculants may reduce the effects of inhibitory metabolites or interact beneficially with each other through mechanisms both physical and biochemical. This area of research has attracted attention recently and co-inoculation may prove to be of more benefit to the plant than a single inoculant, if the right combination of microorganisms is applied to the right plant.

2.7 Summary

Inoculation of field grown wheat with N_2-fixing bacteria has improved grain yield but inconsistencies in plant responses occur following subsequent inoculation and application of the inoculant as reviewed by Bashan and Holguin, (1997). Unpredictable outcomes are therefore common. Inconsistencies may be due to differences in inoculation techniques as well as seasonal conditions. Apart from climatic considerations, the selection of bacterial strains to actively improve early plant growth is dependent on plant genotype, soil texture and the ability to out-compete native microorganisms. The selection and application of locally adapted bacteria as inoculants for wheat may therefore sustain competitive environmental pressures more successfully than introduced bacteria and therefore provide more effective plant growth stimulation. Many of the mechanisms thought to be associated with the stimulation of plant growth have been described, but it is unclear whether they represent major factors or whether they have a small part to play in the whole. The identification of bacteria for use as inoculants for wheat in southern Australia to promote early establishment and enhance plant growth has been limited by a lack of suitable screening methods for Australian conditions. In the wheat growing areas, particularly in South Australia, crops are seeded to benefit from winter rains. The break of the season, demonstrated by effective rainfall, is variable. As discussed previously (2.4, 2.5.2) soil physical conditions including water content, can influence the structure of the microbial communities. Bacteria adapted to stresses such as low rainfall may survive in the soils for longer periods than bacterial inoculants introduced from elsewhere.

It is necessary to develop methods to facilitate selection and isolation of locally adapted strains as potential inoculants. In investigating these methods the selection of bacterial isolates from local soils has yielded a small population of potential N_2-fixing species. The aims of the current research project therefore address these issues.

Chapter 3: General Materials and Methods

3.1 Introduction

The following chapter describes methods used more commonly throughout this work. Methods relevant to individual experiments are specifically described within the following chapters.

3.2 Materials and Methods

3.2.1 Preparation of bacteria for use as reference strains and inoculants

3.2.1.1 Reference strains

The strains were used for reference in molecular studies and in plant experiments are tabulated in Table 3.1.

TABLE 3.1 Reference strains used in thesis

Name	Host	Location	source
Azospirillum brasilense Sp7 (ATCC 29145)	*Digitaria decumbens*	Brazil	A. Hartmann, Germany
Azospirillum lipoferum 596	*Triticum aestivum*	Griffith, New South Wales	P.New
Azospirillum lipoferum C10	*Triticum aestivum*	Camden, New South Wales	P. New
Rhizobium leguminosarum WSM 409	*Trifolium subterraneum*	North Olbia Sardinia	R. Ballard
Bradyrhizobium japonicum WU425	*Ornithopus compressus*		ALIRU
Sinorhizobium meliloti RRI 1.28	*Medicago sativa L.*	Rutherglen, Vic. Australia	R.Ballard

ATTCC = American Type Culture Collection, Rockville, Maryland.
AIRCS = Australian Legume Inoculants Research Unit
P.New = Dept. Microbiology, University of Sydney, (New and Kennedy, 1989).
R.Ballard = SARDI, Adelaide, South Australia

After receipt of the reference strains sub-culturing on nutrient agar was carried out to obtain single colonies and identification was confirmed using GC-FAME analysis (3.2.4). When needed strains were taken from frozen stocks (-80°C).

3.2.1.2 Preparation of reference strains as inoculants

Reference strains were grown aseptically in tryptic soy broth (TSB, Appendix 4) (5ml) and shaken (180 rpm) in an incubator overnight (28° C). Centrifugation (4,000 g) for 5 min was carried out, the medium decanted and the pellet was washed with phosphate buffered saline (PBS, Appendix 3) (5 ml), centrifuged a second time and finally resuspended in PBS (5 ml). The optical density (OD) was adjusted to OD_{550} =1 (10^9 cfu/ml) and the concentration of the bacteria diluted to 10^6 cfu/ml in phosphate buffer (0.05 M).

3.2.1.3 Purification and storage of isolates

Bacterial reference strains and bacteria isolated from semi-selective media were sub-cultured onto nutrient agar (NA, Appendix 4) to confirm purity. Bacteria were stored on NA agar slopes at 4°C and frozen in cryo-vials at -80°C. The cultures were prepared for freezing by adding broth culture (1 ml) to sterile glycerol (300 µl) contained in cryo-vials. These vials were then snap-frozen by plunging them into liquid N_2.

3.2.1.4 Isolation of soil rhizosphere bacteria

Bacteria were isolated from the plant macerate/rhizosphere soil suspension (3.2.1.6, henceforth referred to as macerate suspension) from plants grown in pots in soils obtained from Kapunda, Avon and Waite. The bacteria were plated onto four carbon source (4CS), phosphate azelaic acid tryptamine (PCAT) and semi-solid N-free (Nfb) medium (Appendix 4) to select N_2-fixing bacteria. The macerate suspension was used to inoculate wheat grown in N-free semi-solid medium in the spermosphere model system. Twenty isolates from each medium and soil source were selected.

[N.B. *Plant macerate/rhizosphere soil suspension is referred to as "macerate suspension" throughout the thesis.*]

3.2.1.5 Isolation of bacteria from the rhizosphere and within roots and stems of wheat grown in N-free medium in the spermosphere model system

Bacteria were isolated from the wheat rhizosphere of plants and from within the roots and stems after removing plants aseptically from the spermosphere tubes. Roots were macerated in a sterile mortar and pestle with phosphate buffer (Appendix 3). Aliquots were plated onto N-free semi-selective medium (4CS, PCAT and Nfb, Appendix 4) to select N_2-fixing bacteria.

Bacteria were isolated from within the roots. Roots were surface-sterilised with Chloramine T 1% w/v H_2O (15 min) and washed five times in sterile distilled water (Okon et al, 1977). Sections of roots (2 cm) were cut aseptically and the cut ends sealed with melted dental wax. Dental wax was used to contain bacteria within the root and ensure that external surface-sterilisation was effective. Roots were rolled onto NA to check for contamination and then placed into tubes containing semi-solid Nfb, to check for external contamination by microaerophilic bacteria. Surface-contaminated roots were discarded. Roots were then macerated in phosphate buffer (500 μl).

The method used for surface-sterilising roots was not used for isolating bacteria from within the stems as all bacteria within the stems were killed using that method. Surface-sterilisation was carried out by immersing the stems in cetylpyridinium chloride 1% in NaCl (0.05 M) for five minutes (Wiebkin, 1995, unpublished). Preliminary studies using cetylpyridinium chloride were carried out to confirm sterilisation procedures. Stems were dried in the laminar flow cabinet (5 min) to dehydrate and lyse surface bacteria and were rolled onto NA to check for effectiveness of surface sterilisation. Stems were then cut aseptically to lengths of 2 cm. Stem samples shown to have external contamination were subsequently discarded. Stems were macerated in a sterile mortar and pestle in phosphate buffer (500 μl) and aliquots (10 μl) of macerate were added to semi-solid Nfb medium to isolate microaerophilic bacteria such as *Bacillus*, *Pseudomonas* or *Azospirillum* spp. as indicated by a rising white pellicle in the medium. Bacteria from the pellicle were then plated onto solid Nfb agar plates for purification. Aliquots of macerate were also plated onto 4CS and PCAT medium to select a range of N_2-fixing bacteria.

Twenty isolates (derived from Kapunda, Avon and Waite soil sources) from either the rhizosphere or from within the roots and stems of plants grown in the spermosphere model system were selected from each medium and purified and stored (3.2.1.3).

3.2.1.6 Preparation of plant macerate and soil rhizosphere suspensions as source of N₂-fixing communities

To compare the growth in plants grown in association with communities of bacteria from Kapunda, Avon and Waite soils, wheat was first grown as a host for the microbial communities in each of the soils in a controlled environment cabinet for 4 weeks 3.2.3.3). Three plants from each soil source were gently removed from the soil and roots shaken to retain only the closely adhering soil, considered as rhizosphere soil. The roots were then vigorously shaken and roots (4 g) and stems (1 g) were macerated in a sterile mortar and pestle and mixed with rhizosphere soil (5 g). Maceration was carried out to release endophytic bacteria as well as bacteria bound to root surfaces. The macerate suspension was diluted with sterile 0.05 M phosphate buffer (90 ml) and dispersed on an orbital shaker (190 rpm) for 1 hour. After a 30 min period for settlement the supernatant suspension was decanted. Pre-germinated seeds (surface-sterilised) were immersed for 1 hour in the decanted suspension (20 ml) from each soil source. Heat-killed controls of decanted suspensions from each soil source were prepared by autoclaving (121 °C) for 30 min.

3.2.1.7 Mixed N₂-fixing bacteria from wheat roots grown in N-free medium used as suspension for re-inoculating wheat

Two plants grown in the spermosphere model system for 6 weeks (after inoculation with macerate suspension) were removed aseptically from the spermosphere tubes. Roots were macerated in phosphate buffer (1 ml) centrifuged at 10,000 g for 3 min washed and the pellet resuspended in phosphate buffer before being used to inoculate wheat.

3.2.1.8 Estimation of bacterial numbers in plants and soil

To determine numbers of bacteria in the macerate suspensions from Kapunda, Avon and Waite, dilutions of the suspensions were prepared with phosphate buffer 0.05 M as a diluent. Serial 10-fold dilutions were carried out in a microtitre plate.

Estimation of bacterial numbers from wheat roots and stems grown in spermosphere tubes was carried out by macerating the tissue in a sterile mortar and pestle with added phosphate buffer (1 ml). Serial 10-fold dilutions of the macerates were then prepared. Aliquots from each dilution (10 μl x 3 replicates) were applied to agar plates containing either PCAT, a medium semi-selective for *Burkholderia cepacia*, 4CS medium to select a broad range of N_2-fixing bacteria and Nfb medium with congo red, (Appendix 4) to select *Azospirillum* spp. Replicate drops from the suspensions were also applied to NA (nutrient agar) which was used to estimate overall cultivable bacterial numbers. Spread plating was also carried out on all three selective media (100 μl x 3 replicates) using appropriate dilutions to enable accurate counting of colonies on the plate (generally 10^{-5} dilution). Bacterial colonies on the agar plates were counted after 48 hours of incubation at 28°C using a Gallenkamp colony counter. The colony forming units (cfu) were expressed as cfu/ml macerate suspension or cfu/g dry weight of soil.

3.2.2 Soils used for isolating N_2-fixing bacteria

3.2.2.1 Soil characteristics

Soil samples were collected from long-term field trials both under continuous wheat and cultivation. These soils had a range of pH and textures and were used as sources of N_2-fixing bacteria. The field trials were based in South Australia at Kapunda, Avon and the University of Adelaide, Waite Campus. It had been noted that soil N levels following the growth of wheat grown at both Avon and Waite Campus sites prior to 1996, had been 40 kg/ha higher than was expected (Dr. D. Roget, CSIRO, Land and Water pers. com.). As neither soil had received nitrogenous supplements, it was postulated that N_2-fixing bacteria were perhaps contributing to elevated soil N levels, suggesting that these soils may provide potential inoculants for enhancing the growth of wheat.

Avon (34° 14'S, 138° 18'E) is located 70 km to the north west of Adelaide. The soil from Avon is described as a solonized brown earth (Stace *et al*, 1968) or a mixed Palexeralf (Soil Survey Staff, 1975). The soil composition is 16% clay, 7% silt and 77% sand, pH is 6.2, (1:5 soil to water) and pH 5.4 (1:5 soil to 10 mM $CaCl_2$). Soil was taken from six direct-drilled plots, which had been sown with wheat since 1978.

Kapunda, situated 75km north east of Adelaide (34° 21'S, 138° 18'E) has a sodic red-brown earth (Stace *et al*, 1968), which is described as a fine mixed thermic Calcic Natrixeralf (Soil Survey Staff, 1990), pH 6.0 (1:5 soil to water) and pH 5.3 (1:5 soil to 10 mM $CaCl_2$). The soil composition is 15% clay, 25% silt and 60% sand. The plots at Kapunda had also been direct-drilled and soil was taken from a continuous wheat trial (plot 14).

Soil from the long-term field trial at the Waite Campus of the University of Adelaide (described as a red-brown earth, Calcic Luvisol, pH 5.0 (1:5 soil to water) and pH 5.3 (1:5 soil to 10 mM $CaCl_2$). Soil was sampled from a plot sown with continuous wheat (plot 17).

3.2.2.2 Soil pH

Soil (20 g) was mixed with water (100 ml) and the resulting soil suspension was shaken vigorously for 1 hour at 190 rpm on an orbital shaker. The suspension was then allowed to settle for 30 min prior to measuring the pH. Soil pH was also measured by adding 5ml $CaCl_2$ (0.21 M) to 100 ml of the soil suspension, mixing well and allowed to settle. The pH was measured to the nearest 0.01 unit.

3.2.2.3 Soil water potential

For experimental purposes soil water potential was standardised in soils from Kapunda, Avon and Waite. The soils were used to grow host wheat plants for isolation of rhizosphere bacteria (potential inoculants) or the spermosphere model system (3.2.3.4). The method used to assess soil water potential was the suction plate method (Papendick and Campbell, 1978).

3.2.3 Conditions for wheat growth in association with rhizobacteria

3.2.3.1 Wheat variety

Wheat (*Triticum aestivum* var. 'Stiletto') was grown in association with rhizobacteria from Kapunda, Avon and Waite soils. Stiletto had been previously grown at both Kapunda and Avon trial sites and as some associative bacteria are known to be host specific (Bashan and Holguin, 1997) and was selected for experimental use. The

average weight for Stiletto seeds was 36 mg per seed. Seeds were weighed prior to use and selected according to weight (36 mg ± 1mg).

3.2.3.2 Surface-sterilisation of wheat seed

Prior to use, seeds were surface-sterilised by soaking in 95% ethanol for one minute to dehydrate the seed coat. They were then vigorously shaken in 3% sodium hypochlorite for 5 min followed by 5 changes of sterile reverse osmosis (RO) water. Sterilisation was confirmed by incubating seeds on Petri dishes containing NA, for 48 hours at 25°C and discarding any seeds showing evidence of microbial growth.

3.2.3.3 Conditions for growth of wheat in pots as host for rhizobacteria

Wheat was grown in soil collected from Kapunda, Avon and Waite to provide a macerate suspension for inoculating wheat grown in the spermosphere model system. The seeds were planted 48 hours after germination and were grown from 4 to 6 weeks in plastic non-draining pots (300 ml). As water availability differs between soil types, soil water potential was assessed for Kapunda, Avon and Waite soils to standardise the availability of water to the plants. Soil water in each pot was maintained at a soil water potential of -10 kPa. Pots were watered to weight 3 times a week with RO water in a controlled environment cabinet with a 15°C with a 12 hour photoperiod, a relative humidity 60-70% and with irradiance of 240 $\mu E\ m^{-2}\ s^{-1}$. Plastic beads (25 g) were placed on the surface of the pots to reduce evaporation of water.

3.2.3.4 Growth of inoculated wheat in modified spermosphere model

The spermosphere model system (Thomas-Bauzon et al, 1982), in which bacteria rely on a carbon source from seed exudates, was modified to grow plants in the dark and light for a defined period of time. Glass tubes (13 cm x 1.6 cm) containing 4CS semi-solid mineral medium (10 ml) without C and N supplements, to stimulate the growth of the N_2-fixing bacterial community were used to grow wheat seedlings previously immersed in plant macerate suspension 48 hours after germination (3.2.1.6) under sterile conditions. Whatman No1 filter paper (3 cm x 1 cm) was folded into thirds and placed on the surface of the medium to prevent the germinating wheat seed from becoming submerged in the medium. Alternatively, a hole was cut in the bottom of an Eppendorf centrifuge tube (1.5 ml), the lid removed and the tube wedged into position above the medium (Bothe et al, 1992). The germinating seedling was placed inside the

Eppendorf tube, which provided support for the seedling and enabled the roots to enter into the medium through the hole in the bottom of the tube. Sterility was maintained by plugging the neck of the tubes with non-absorbent cotton wool and aluminium foil (Fig.1), or by inverting larger glass tubes (14 cm x 3 cm) over the top of the smaller tubes. The lower third of the tubes was covered with aluminium foil to exclude light and encourage the entry of the roots into the medium. The plants were grown in a controlled environment with a 12 hour photoperiod, temperature at 15°C, relative humidity at 60-70% and with irradiance of 240 $\mu E\ m^{-2}\ s^{-1}$ for periods between 28 days and 31 days.

cotton wool

seedling

roots

Whatman paper support

semi-solid medium
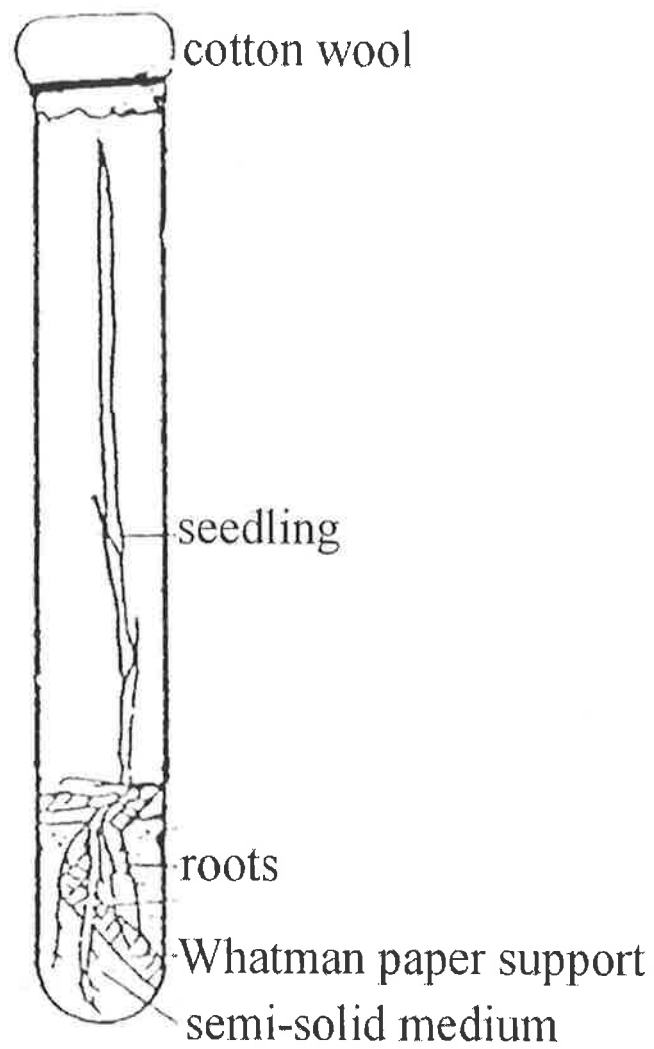
Fig.3.1 Modified spermosphere model system

3.2.4 GC-FAME studies

3.2.4.1 Gas Chromatography of Fatty Acid Methyl Esters (GC-FAME) for identification of soil bacteria

The procedure and preparation of cultures for the Microbial Identification System version 3.8 (MIS, MIDI Inc[R]. Newark, Delaware, 1994) was carried out as recommended by the manufacturer (Appendix 2). The samples were analysed by gas chromatography (Hewlett-Packard model 5890) to obtain fatty acid methyl ester profiles. An Ultra 2 column (25 m x 0.2 mm, cross -linked 5%) was used with H_2 as the carrier-gas (30 ml/min) in association with a flame ionisation detector.

Fatty acid methyl ester profiles were compared with the Sherlock TSBA aerobe library version 3.8 (manufacturer as above) and a computer generated similarity index was constructed to compare the GC-FAME profile from the sample with the GC-FAME database. The index ranged from 0 – 1.0. The likely identity of the sample was based on the similarity of GC-FAME profile in the database and sample GC-FAME profile (0.6 similarity was regarded as similar to the database for the purposes of comparison in this thesis). GC-FAME analyses were repeated to confirm the reproducibility.

3.2.4.2 Sand, porous and semi-solid media used to grow wheat for GC-FAME analysis

Sand, porous and semi-solid media were prepared as growth substrates for inoculated wheat to select the substrate providing optimal growth conditions for the provision of sufficient bacteria for GC-FAME analysis. GC-FAME analysis was used to compare fatty acid profiles of bacterial communities from Kapunda, Avon and Waite soils. Wheat was inoculated with macerate suspensions (3.2.1.4)

Sand

Low nutrient sand was washed (x 5) with RO water. Measurement of available nitrate in the sand was determined (3.2.6.3) to confirm that the sand was nitrate-free. The sand was dried at 40°C for 3 days, weighed into each pot (217 g) and autoclaved at 121°C for 20 min on 3 consecutive days. Nutrient solution (C and N-free) derived from N-free 4CS medium (Appendix 4) was autoclaved and added to the sand and mixed by shaking vigorously 20 times prior to potting. The modified 4CS nutrient solution for each pot

consisted of the following: supersalts (12.5 ml as in 4CS medium, Appendix 4) phosphate buffer (3.75 ml), Difco yeast extract (0.025 g) and RO water (11.75 ml), pH 6.8.

Porous agar

Porous agar medium was prepared from 4CS medium with no added carbon source (Appendix 4), with the addition of Difco Bacto agar, (2.5%), pH 6.8. The medium was autoclaved and cooled to 50°C, after which 60 ml was withdrawn up into sterile disposable syringes (Barrett-Lennard and Dracup, 1987)). The nozzles of the syringes were covered with sterile foil, supported at a 90° angle and the agar left to solidify in the laminar flow cabinet. The syringe was then secured in the top of a retort stand clamp and pressure was applied to the syringe to dispense small blocks of agar into sterile pots. Porous agar (120 ml) was added to each pot. The volume and concentration of nutrients were comparable to those in the pots containing sand.

Semi-solid agar

Semi-solid modified 4CS medium (without added carbon source) was prepared with the addition of 1.75 g agar per litre. Two inoculated seeds were placed on a supportive plastic perforated grid, which was placed over the semi-solid medium and secured with cotton thread ties to the neck of the autoclavable pots to prevent the seed sinking into the semi-solid medium. Nutrient content was similar to the sand and porous media.

3.2.5 Nutrients used for wheat inoculation experiments to test plant N content

To prepare wheat for comparative growth studies including N content and concentration, the plants were inoculated with either a mixture of rhizobacteria from the spermosphere or three bacteria used as co-inoculants isolated from the rhizosphere of plants grown in the modified spermosphere system in N-free semi-solid medium. Two plants were grown in each pot containing Laffer sand (1 kg) supplemented with nutrients as described below.

Sieved Laffer sand was washed (x 3) with RO water, dried and weighed into 1 kg samples. Sand was autoclaved at 121°C for 1 hour on 3 consecutive days. It was dried

and nutrients mixed into sand before potting. The pH was adjusted to 7.0 with calcium carbonate. After potting, water content was maintained at 12%.

The following nutrients (recommended by Dr. N. Howes, SARDI, South Australia) prepared in RO water for mixing into the sand were added as described

The following were added to RO water (5 ml) and mixed in 1 kg dry sand:

K_2SO4 (120 mg); $MgSO_4.7H_2O$ (90 mg); $CuSO_4.5H_2O$ (10 mg); $MnSO_4.4H_2O$ (7 mg); $ZnSO_4.7H_2O$ (2 mg); $CoSO_4.7H_2O$ (1 mg); H_3BO_3 (1 mg); $H_2MoO_4.H2O$ (0.5 mg); $NiO_4.6H_2O$ (0.15 mg).

The following was added to RO water (4 ml) and mixed in 1 kg dry sand:

$FeSO_4.7H_2O$ (1.4 mg)

The following was added to RO water (2 ml) and added to dry sand:

KH_2PO_4 (150 mg).

3.2.6 Quantification of plant N

3.2.6.1 Kjeldahl method

The quantification of total plant N was carried out using the micro Kjeldahl method (Bremner and Mulvaney, 1982) (Appendix1). Three replicates of each sample were tested. Calculation of N was as follows in Table 3.2.

Table 3.2 Calculation of total plant N

µg N/ml = [standard(10mg) x dilution x peak of sample on autoanalyser printout chart(mm)] ÷ peak height of standard (mm) = [A]
Digest volume = 50ml
Plant weight = 13mg (used for N estimation)
µgN in 50ml digest.... = [A] x 50
µgN/ g.. = [A] x (50 / 0.013g) x 1g
µgN/total plant weight= [A] x (50 / 0.013g) x g plant DW

3.2.6.2 Total combustion gas chromatography

Roots and shoots were finely ground. The powder was dried at 60°C overnight and aliquots weighed to 4 decimal points prior to analysis. This method was used to measure plant N to a high degree of accuracy after plant inoculation experiments. The procedure was carried out by Plant Science, University of Adelaide (Appendix1).

3.2.6.3 Quantification of available nitrate in soils

Available soil nitrate was extracted with 2M KCl (Page et al, 1982). Soils from Kapunda, Avon and Waite were oven-dried at 105°C for 24 hours. KCl (2 M, 100 ml) and oven-dried soil (10g x 3 replicates) were placed in a 250 ml Schott bottle on an orbital shaker for one hour. The soil suspension was then allowed to settle and 1:5 dilutions were prepared for analysis on the autoanalyser. The analysis and calibration of the autoanalyser were carried out by Mr. C. Rivers (Dept Soil and Water, University of Adelaide, Appendix1). Calculations were as follows in Table 3.3.

Table 3.3 Calculation for availability of nitrate in soils

µg N/ml = [standard (2.5mg) x dilution x peak of sample (mm) on autoanalyser chart] ÷ peak height of standard = [A]	
Extraction volume= 10ml KCl	
Soil weight = 10 g	
µg N in 100ml = [A] X 100 ÷ 10 = 10 X [A]	

3.2.7 Preparation of $^{15}N_2$ for assessing bacterial N_2 fixation

$^{15}N_2$ was prepared (supervision by Dr. M. Amato, CSIRO Land and Water, pers.com.) for use in experiments to verify the occurrence of bacterial N_2 fixation in the rhizosphere of wheat. Apparatus for preparing $^{15}N_2$ is shown in Fig. 3.2. Two litres of $^{15}N_2$ was generated from a reaction of ammonium sulphate ($[^{15}N] H_4)_2 SO_4$ (enriched with 10% ^{15}N, Amersham) with lithium hypobromite, (LiBrOH). Lithium hydroxide LiOH (88 g) was added to distilled water (800 ml) in a 1 litre bottle and dissolved by heating. The LiOH solution was then placed on ice and chilled bromine (27 ml) was added. Then, $([^{15}N] H_4)_2 SO_4$ (8 g) was added to a round-bottomed flask which was placed under vacuum. The LiBrOH was introduced into the flask and cooled by

immersing the base of the flask in liquid N_2. The trap was simultaneously cooled by insertion of the trap tube into liquid N_2. The flask was slowly thawed and N gas from the reaction of $([^{15}N] H_4)_2 SO_4$ and LiBrOH was collected in the wine cask bladder, which had been placed under vacuum to eliminate air. The three-way tap prevented contamination of the $^{15}N_2$ with air. The $^{15}N_2$ gas was stored in the wine cask bladder until used (Fig.3.2).
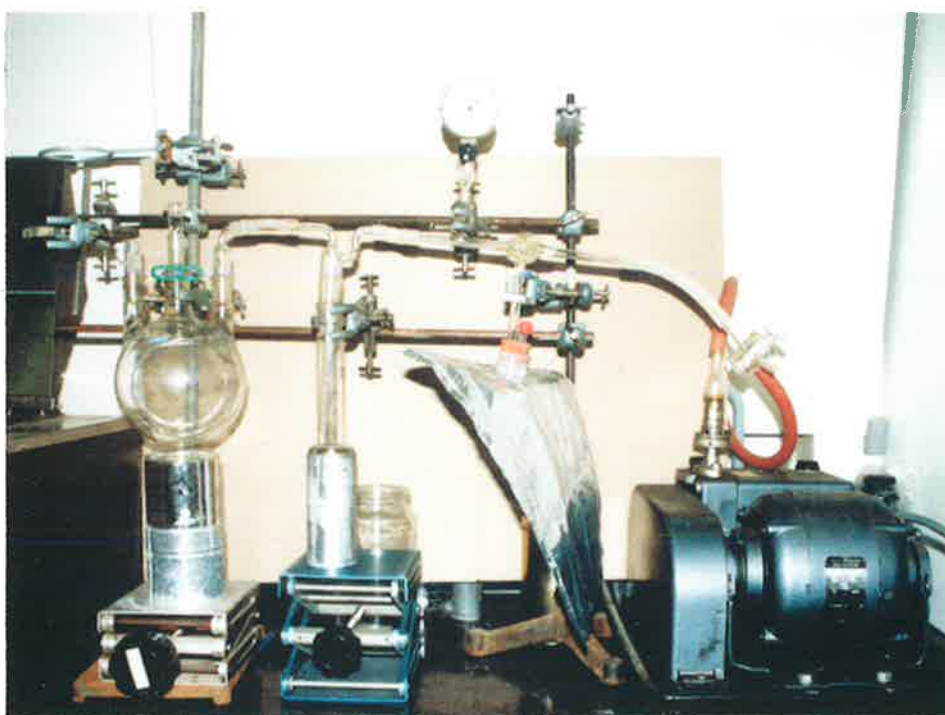
Fig. 3.2 Apparatus used to generate $^{15}N_2$

3.2.8 Molecular studies

3.2.8.1 Nucleic acid preparation

To extract nucleic acid for hybridisation studies to group-specific probes, bacteria were taken from frozen stocks and grown on agar plates on dextrose yeast glutamic acid medium (DYGS, Appendix 4) and streaked to single colonies. They were then grown in DYGS broth (5 ml) overnight on a shaker at 28°C. Bacterial broth suspensions (200 µl) were seeded into fresh DYGS broth (5 ml) and shaken for a further 2 hours at 28°C until the suspensions turned milky.

The bacterial suspensions (1.5 ml x 2) were centrifuged in sterile Eppendorf tubes (2 ml) for 15 min at 10,000 g and the supernatants discarded. The bacterial pellets were mixed with Tris HCl / EDTA buffer (TE) pH 8.0 (400 µl). TE buffer was prepared with Tris-HCl 10 mM pH 8.0, 0.1 mM EDTA pH 8.0 and 10% SDS (40 µl). Phenol (400 µl) was then added and vortexed (30 sec). The tubes were placed in a metal rack and immediately boiled for 2 min. Following cooling to room temperature, the tubes were centrifuged at 10,000 g for 15 min. The top aqueous layers were retained. Chloroform/isoamyl alcohol 24:1 (400 µl) was then added. Centrifugation was repeated twice at 10,000 g (10 min). The nucleic acids were precipitated by the addition of 5 M potassium acetate (one tenth volume) and absolute alcohol (two and a half volumes) and mixed gently and placed at -20°C overnight.

Nucleic acids were recovered by centrifugation at 10,000 g for 15 min (4°C) and washing in 70% ethanol and the dried pellet was dissolved in TE buffer (100-200 µl). The yield from nucleic acid extractions was determined by gel electrophoresis on a 1% agarose gel run in Tris-acetate-EDTA buffer (TAE, 100 mm Tris, 125 mM sodium acetate, 1 mM EDTA, pH 8.0).

3.2.8.2 Hybridisation of nucleic acid to group specific oligonucleotide probes

Hybridisation was carried out according to Kirchhof and Hartmann, (1992). MOPS buffer (30 µl Appendix 3) and nucleic acid (10 µl) were then gently mixed and applied to a Hybond-N+ filter (9 X 12 cm, Amersham Pharmacia Biotech) using a dot blot apparatus (Bio Rad). After transfer of samples the fixed membranes were placed in

hybridisation bottles and pre-washed in 0.1 x SSC, 0.5% SDS for 1 hour at 37°C. The membranes were incubated at 60°C (1 hour) in pre-hybridisation buffer containing 5 x SSC (20 x SSC: 3 M NaCl, 0.3 M trisodium citrate, diluted 1/4), 5 x modified Denhardt's solution (100 x modified Denhardt's solution: 2% polyvinylpyrrolidone, 2% Ficoll 400, 2% polyethylene glycol 8000, diluted 1/20) and 1% sarkosyl (15 ml).

The sequences for group-specific rRNA directed oligonucleotide probes, ALF 1b (alpha sub class), BET 42a (sub class), GAM 42a (gamma sub class), AZO (*Azospirillum*) and *nif*D (based on *nif* D sequence from *Klebsiella*, Arnold et al, 1988) were each used with the membranes (Table 3.4). Target sites for alpha, beta and gamma sub-class rRNA positions are described by Manz et al, (1992). Specific sites for the alpha subclass (ALF1b) are near to the 5' end of the 16S rRNA, position 19-35. Sites for both beta (BET42a) and gamma (GAM42a) are at helix 42 of 23S rRNA and are identical except for T at position 1037 in beta and A at the same position in the gamma sub-class organisms. The AZO probe was directed at a highly conserved region of 23S rRNA (Kirchhof and Hartmann. 1992). Probes were labelled with [^{32}P] using a 3'end labelling kit (Amersham). The labelling reaction was carried out at 37°C according to the manufacturer's recommendations. After incubation, the labelling reaction was added to the pre-hybridisation buffer in the bottles and incubated overnight with the membranes in a rotating oven set at the melting temperatures of the oligonucleotide probes. The melting temperatures (T_m) of the oligonucleotides were as follows: ALF1b= 50°C, BET42a=47°C, GAM42a=47°C (Manz et al, 1992), AZO=48°C (Kirchhof and Hartmann, 1992), *nif*D=56.8°C (Kirchhof, G., unpublished).

TABLE 3.4 Sequences and target sites for oligonucleotide probes ALF 1b, BET 42a, GAM 42a, AZO, *nif*D used to hybridise to rRNA or DNA from bacterial isolates

Probe	sequence	Target site rRNA position
ALF1b	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16S, 19-35
BET42a	5'-GCCTTCCCACTTCGTTT-3'	23S, 1027-1043
GAM42a	5'-GCCTTCCCACATCGTTT-3'	23S, 1027-1043
AZO	5'-GGGGCT(A/G)TTTCC(C/T)GG-3'	23S
*nif*D	5'-GACATCGTNTTCGGCGGCGA-3"	

Target sites for rRNA position (Manz et al, 1992, Kirchhof and Hartmann, 1992).

Membranes were then washed in 2x SSC / 0.1% SDS at room temperature (20 min). This was followed by a further wash at hybridisation temperature (5 min).

The membrane was then air dried and exposed to a phosphor-imaging screen overnight. Scanning of the membrane was carried out by a phosphor imager and quantified using Storm Phosphor Image software (Molecular Dynamics). The volume, background value and area of the image were analysed and an average value (volume-background/area) used to confirm positive hybridisation. Hybridisation was recorded as positive with average values of 800 to 1999 and strongly positive with average values greater than 2000.

3.2.8.3 Southern blot of *nif* amplicates

The products from *nif*D and *nif*H PCR (25 µl, 5.4.2.2.1) were mixed with six times concentrated loading buffer (4 µl) (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O) and electrophoresed on a 1.5% agarose gel. Each gel was stained with ethidium bromide and then visualised under UV light. The gel was depurinated in 0.25 M HCl and shaken gently for 10 min before denaturation was carried out for 20 min in denaturation buffer (0.4 M NaOH, 0.6 M NaCl) to convert the DNA to the single stranded form. The gel was then neutralised by shaking gently twice for 15 min in neutralisation buffer (1.5 M NaCl, 0.5 M Tris HCl, pH 7.5).

Southern blots were prepared to transfer the DNA to Hybond N+ membranes. The method followed was similar to Sambrook et al (1989). After the blotting was completed, the membranes were fixed for 30 min by placing on 3 Whatman filter papers that had been soaked in 0.4 M NaOH. The membranes were then rinsed for 1 min in 5 x SSC and air-dried. They were then stored between filter papers at 4ºC until hybridisation was carried out.

3.2.8.4 Hybridisation of *nif* probes to Southern blot

The membrane was pre-hybridised in the following reagents: MQ H_2O (2 ml), 5 x HSB (3 M NaCl, 100 mM PIPES, 25 mM Na_2EDTA, pH 6.8), Denhardt's III solution (2 ml), 10% dextran sulphate (4 ml), which were mixed together and incubated at 65°C for 15 min. Salmon sperm (5 µg/ml, 500 µl) was incubated at 100ºC for 15 min then chilled and added to the pre-hybridisation mixture. The membrane was soaked in 2 x SSC, placed into the hybridisation bottle with the pre-hybridisation buffer and incubated in a

rotating hybridisation oven at 65°C for 4 hours. The probe was then added to the pre-hybridisation buffer and incubated in a rotating oven overnight at 65°C. Probes used included *nif*D and *nif*H, which were generated from excised gel bands after *nif*D and *nif*H amplification products were electrophoresed on 1% agarose gel (5.4.2.3).

The probe was labelled with $[^{32}P]$ using a Megaprime labelling kit (Amersham) as directed by the manufacturer and incubated at 37°C for 20 min. After pre-spinning the column to elute residual buffer, the probe was then dispensed into a spin column (Pharmacia Micro-Spin). Centrifugation was carried out for 20 sec and the probe was collected in a catch tube. Salmon sperm DNA 5 µg/ml (200 µl) was added to the probe, which was heated at 100°C for 5 min, chilled and then added to the pre-hybridisation buffer. Incubation was carried out at 65°C overnight.

The membrane was washed in 2 x SSC, 0.1% SDS at 65°C for 20 min. A second wash was carried out using 1 x SSC, 0.1% SDS again at 65°C for 20 min. After discarding the second solution, 0.5 x SSC, 0.1% SDS was used as a third wash at 65°C for a further 20 min.

3.2.8.5 Preparation of cell lysates from bacteria used in PCR

A loopful of a freshly growing agar culture was scraped into an Eppendorf tube containing MQ H$_2$0 (500 µl), vortexed and boiled (10 min). The tube was then centrifuged at 10,000 g (15 min) and supernatant (1 µl) used for PCR. This method was used for *nif*D and InterLINE PCR.

A second method for preparing cell lysates was used for *nif*H PCR. Cultures in NB (5 ml) were grown overnight in a shaking incubator at 28°C. Centrifugation was then carried out at 10,000 g (3 min). The bacterial pellet was washed in MQ H$_2$0 (2 ml), centrifuged at 10,000 g for 3 min, resuspended in MQ H$_2$0 (2 ml) and the cell concentration adjusted to an optical density (OD$_{600}$ = 1-1.5). Cell lysates (100 µl), Tris 10 mM pH 8.0 (100 µl) and proteinase K (5 mg/ml, 2.5 µl) were incubated in an Eppendorf tube on a heating block at 55°C for 6 hours. Proteinase K was inactivated at 100°C (10 min) and the cell lysates stored at -20°C.

3.2.9 Statistical analysis

Analysis of variance (ANOVA) using the computer software package Statistix vs. 7 and Tukey's honestly significant difference (HSD) was used to analyse all data unless otherwise stated.

Chapter 4: Selection of a Soil Source for the Isolation of Potential Inoculants

4.1 Introduction

The focus of experiments described in this chapter was to select one of three soils as a source of N_2-fixing bacteria, which could be used as potential inoculants for growth promotion of wheat (*Triticum aestivum* var. Stiletto). N_2-fixing bacteria were isolated from three South Australian soils (Kapunda, Avon and Waite) with the expectation that adaptation to local soils and climate would increase the likelihood of successful colonisation and persistence by selected bacteria, to a greater extent than inoculants introduced from elsewhere. The ultimate choice of the soil source was based on measured growth responses of wheat to follow inoculation with communities of N_2-fixing bacteria from each of the three soils. It was hypothesised that the presence of biologically fixed N_2 within the wheat tissue and significant root elongation were measurable characteristics which could be used to differentiate responses by plants inoculated with communities of bacteria from each of the soil sources.

Inoculated wheat, grown in an N-free medium, was exposed to an atmosphere enriched with $^{15}N_2$. Mass spectrometry was then used to detect the presence of ^{15}N within the plant tissue. Where this was detected, microbial fixation of N within the rhizosphere can be presumed.

In addition to the detection of N_2 fixation, the length of inoculated wheat roots was compared with those in control plants. A variety of factors influence the development of roots. Low levels of available nitrate may trigger plants to increase the length of roots per unit shoot weight in order to compensate for low nutrient influx (Jungk 1996). Lateral root formation is stimulated by the localised supply of nitrate (Drew et al 1973; Drew, 1975; Sattelmacher and Thoms, 1989). Increased branching of the lateral roots may also occur in response to phytohormone production by the rhizosphere bacteria (Zimmer and Bothe 1989). This issue was not the focus of this study. To gain an

understanding of conditions required to produce an inoculation effect in wheat, experiments were conducted both with and without nitrate supplement. It was expected that the addition of nitrate would suppress N_2 fixation in the rhizosphere bacteria and would also provide an N source for the plants. Differences between treatments with and without added N would permit direct effects of bacteria on root growth to be distinguished from effects mediated by N_2 fixation.

Further studies were carried out to determine whether communities of N_2-fixing bacteria were capable of stimulating wheat roots in the early stage of root development where seed reserves provided the sole nutrient supply.

4.1.1 Aims

To select one of three soils as a source of N_2-fixing bacteria for potential inoculation of wheat (*Triticum aestivum* var. Stiletto) by:

- comparing the effectiveness of communities of N_2-fixing bacteria in fixing N_2 by measuring the transfer of fixed N_2 from the rhizosphere into the inoculated plant following exposure to $^{15}N_2$, to determine the effectiveness of communities of N_2-fixing bacteria to fix N_2 (section 4.2),

- comparing increases in the length of plant roots after inoculation with each of three communities of N_2-fixing bacteria when grown in minimal medium under N-free conditions (section 4.3), when grown with and without nitrate supplements (section 4.4) and when grown with the seed source as the sole nutrients (section 4.5).

4.2 Detection of Fixed N_2 in Inoculated Plants Exposed to $^{15}N_2$

4.2.1 Materials and methods

4.2.1.1 Preparation of wheat for exposure to $^{15}N_2$

Wheat seedlings (*Triticum aestivum* var Stiletto) (3.2.3.1) were surface-sterilised (3.2.3.2) and immersed for 2 hours in an inoculant plant macerate/rhizosphere soil suspension (20 ml) derived from Kapunda, Avon or Waite soils (3.2.2). N.B. This will be referred to throughout the thesis as "macerate suspension". Heat-killed macerate suspensions from each of the 3 soils were used to inoculate control plants. A reference bacterial strain *Azospirillum* sp. C10 from Dr. P. New (Dept. Microbiology, University of Sydney) was selected for its high nitrogenase activity when grown in association with wheat and included in the experiment as a comparative reference strain. Preparation of the reference strain as an inoculant is described in section 3.2.1.1. Soil characteristics, pH, soil water potential and soil nitrate measurements are described in sections 3.2.2.1, 3.2.2.2, 3.2.2.3 and Appendix 1 respectively. Two seedlings were then aseptically transferred into spermosphere tubes (3.2.3.4) and grown in a C-and N-free semi-solid medium as described in section Appendix 4. The tubes were then placed in the growth cabinet as a completely randomised design and maintained at 15° C for 3 weeks. One of the duplicate plants from each tube was used to ascertain ^{15}N uptake from bacterial N_2 fixation and the second plant was used for measurement of total plant N. There were 8 treatments, each replicated 6 times.

4.2.1.2 Exposure of wheat to $^{15}N_2$

Generation of $^{15}N_2$ with helium used as a diluent gas is described in section 3.2.7. In a pilot experiment, ^{15}N could be detected in the plant tissues by mass spectrometry following 3 days of plant exposure to an enriched atmosphere of $^{15}N_2$ (7%). The plants were therefore grown for 22 days in the tubes after which they were exposed for 3 days to enriched $^{15}N_2$. After applying sterile rubber Suba seals to the tubes, air (14 ml) was then withdrawn through an airtight syringe and replaced with $^{15}N_2$ enriched gas. Three days after the introduction of $^{15}N_2$ the Suba seals were removed from the tubes. The necks of the tubes were plugged with sterile cotton wool and the tubes returned to the growth cabinet for a further 5 days after which time the plants were harvested. At harvest, the plants were conventionally oven-dried overnight (65°C), dry weight

determined and the plant material finely ground. Ground plant material (4 mg) from one plant was weighed. The ground material was then placed in aluminium caps (3 mm x 5 mm), the open ends of which were squeezed shut and rolled to prevent spillage of the material. Each sample was then analysed by mass spectrometer following combustion and volatinisation of plant material within the mass spectrometer (Dr. M Amato, CSIRO Soil and Water) (Europa Scientific ANCA System). The mass spectrometer had the capability for detecting levels of ^{15}N as low as 0.010 excess (atoms %) above normal background levels. The mass spectrometer measures masses and relative concentrations of atoms and molecules. Quantification of ^{15}N from plant tissue was carried out by the mass spectrometer following the ionisation molecules of dinitrogen, allowing relative concentrations of mass 28:28:30 to be measured (mass $28:^{14}N$ ^{14}N; mass $29:^{15}N$ ^{14}N; mass $30:^{15}N$ ^{15}N). Statistical analysis was as described in section 3.2.10.

4.2.1.3 Quantification of total plant N

Total N in the second plant was determined by the Kjeldahl assay (3.2.6.1). Total seed N was also determined to assess the likelihood that wheat plants would utilise an exogenous source of N by 30 days of growth. Three groups of 10 seeds were selected for their uniformity by weighing and determining the N content. These data were used to calculate an average N content for each seed.

4.2.1.4 Estimation of bacterial numbers in the macerate suspension used to inoculate wheat prior to enrichment with $^{15}N_2$ and in the rhizosphere after harvest

Estimation of bacterial numbers in the macerate suspension used to inoculate wheat and numbers in the rhizosphere, after harvest of the wheat, was carried out as described in section 3.2.1.8.

4.2.2 Results

4.2.2.1 Growth and total N content in wheat grown for 30 days

Wheat seeds were germinated and grown for 30 days in the presence of macerate suspensions from Kapunda, Avon and Waite soils. Control macerate suspensions were heat-killed and also used as inoculants in addition to the reference inoculant. After 30 days relative growth (dry weight) in all the inoculated plants and in controls was not significantly different. Indeed, nor did their growth differ from plants inoculated with

the reference strain (Table 4.1). There was no difference between the total N content (Kjeldahl) and N concentration (mean μg/g) of all of these plants. Following germination, both the dry weights and the total N content of all plants were reduced with growth when compared with that in the seed.

TABLE 4.1 Dry weights and total N content of wheat plants after 30 days growth

Bacterial source	Inoculated		Control	
	plant DW (mg)	N per plant (ug)	plant DW (mg)	N per plant (ug)
Kapunda	32 ± 2	681 ± 44	30 ±1.7	766 ± 77
Avon	31 ±1.4	782 ± 47	28 ± 1.4	727 ± 67
Waite	28 ±1.1	591 ± 27	32 ±2.9	803 ± 99
Reference	30 ± 0.9	646 ± 28	30 ± 1.1	727 ± 67
seed	37 ± 5	807 ± 19.6		

Dry weights (DW) and plant N content from plants inoculated with macerate suspensions from Kapunda, Avon and Waite soils were compared with seed DW and N content. DW and N content of the plant were less than those of the seed. Seed DW and N content are shown at the bottom of the table. Means and standard errors of means of 6 replicates are tabulated.

4.2.2.2 ^{15}N incorporation into wheat tissues after 30 days growth

When wheat was inoculated with macerate suspensions from Kapunda, Avon and Waite soils and grown for 30 days, the incorporation of ^{15}N into tissue was not significantly different from plants grown in heat-killed controls. However, the plants inoculated with suspensions from Kapunda soil showed statistically significant elevated levels of incorporated ^{15}N when compared with levels in the plant material from Waite and Avon soils. Tukeys HSD = 0.003 ($P < 0.05$,) (Fig. 4.1).
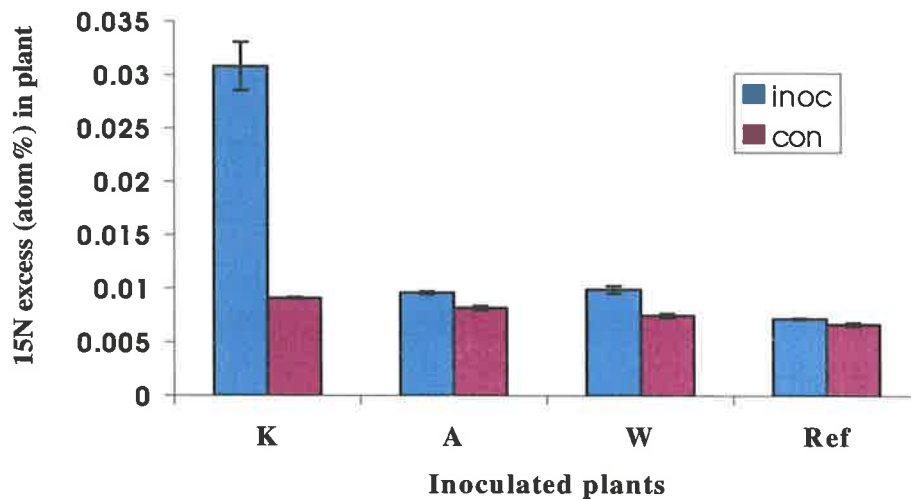
Fig. 4.1 ^{15}N **excess (atom%) in inoculated wheat plants after exposure to 7%** ^{15}N$_2$.

Uptake of ^{15}N measured by mass spectrometry. Wheat was grown in N-free semi-solid medium in spermosphere tubes and inoculated with macerate suspension from Kapunda (K), Avon (A), or Waite (W) soils or *Azospirillum* sp. C10 (Ref). Tukeys HSD = 0.003 Means and standard errors of 6 replicates.

4.2.2.3 Bacterial numbers in Kapunda, Avon and Waite soils

The macerate suspensions from Kapunda, Avon and Waite soils were incubated on N-free media, 4CS, PCAT and Nfb (Appendix 4), to determine numbers of bacteria before and after inoculation of wheat in 30-day growth experiments. The focus of this experiment was to determine the viability of N$_2$-fixing bacteria in the rhizosphere. All the media were N-free.

At the start of the experiment, the greatest numbers of bacteria in the macerate suspension ($>10^6$ cfu/ml) grew on 4CS medium, with fewer colonies growing on PCAT and Nfb media. Numbers of bacteria enumerated on 4CS were significantly higher for the Waite soil when compared with Avon and Kapunda soils (P<0.05, Tukeys HSD = 0.3021). The numbers of bacteria supported by PCAT medium from Waite were significantly greater in number than those from the Kapunda soil. (Tukeys HSD = 0.5166). There were no significant differences between inoculants from different soil sources on Nfb medium (Fig. 4.2 a). After 30 days in all the spermosphere tubes, bacterial numbers in the rhizosphere increased at least 100-fold. Estimation of numbers of bacteria from the Kapunda soil grown on 4CS, PCAT and Nfb medium and from

Avon soil on PCAT medium were less than those from Waite soil. (Tukeys HSD, 4CS = NS; PCAT = 0.7094; Nfb = 0.6094, Fig. 4.2 b). Isolates growing well on these media were purified and frozen as described (3.2.1.3).

(a)
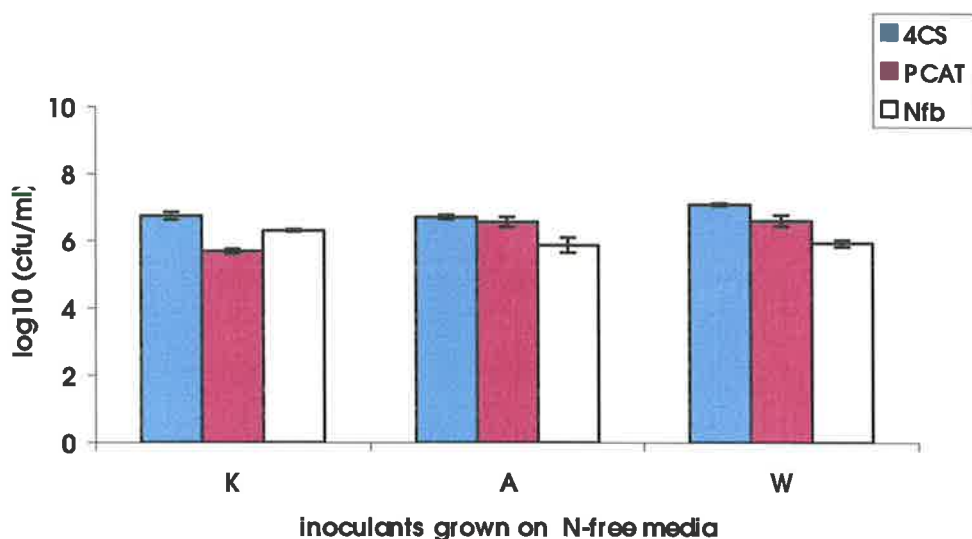
(b)
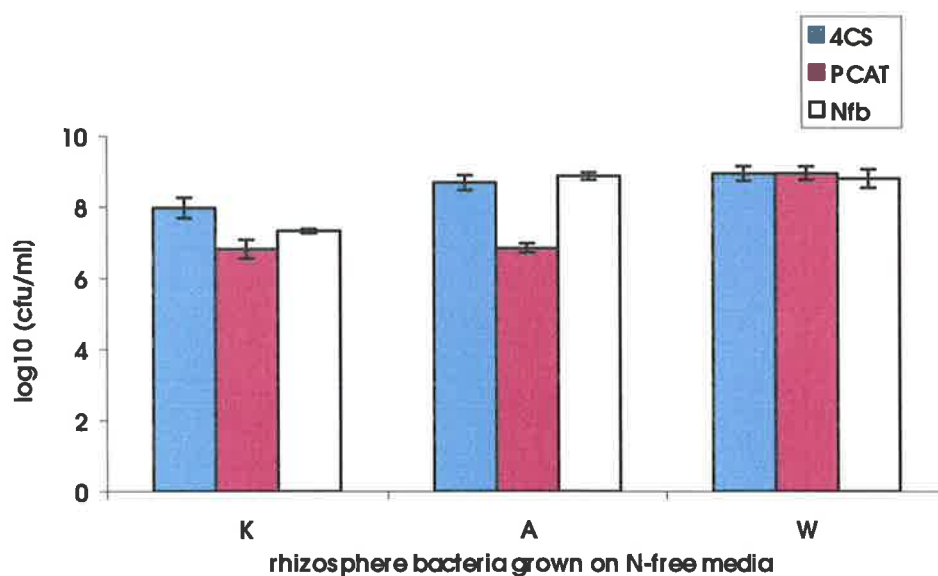
Fig. 4.2 Numbers of N$_2$-fixing bacteria in the macerate suspension used for inoculation of wheat (a) and from the wheat rhizospheres after 30 days of growth in the spermosphere tubes (b)

(a) Macerate suspensions were grown on 4CS, PCAT and Nfb N-free medium to estimate numbers of bacteria at the time of inoculation of wheat
Tukeys HSD 4CS = 0.3021; PCAT = 0.5166; Nfb = NS

(b) After 30 days of growth in the wheat rhizosphere, bacterial numbers were similarly estimated. Tukeys HSD 4CS = NS; PCAT = 0.7094;
Nfb = 0.6094. K = Kapunda, A = Avon and W = Waite.
Means and standard errors of 6 replicates.

4.3. Effect of Inoculation on Root Length

4.3.1. Materials and methods

4.3.1.1. Preparation of inoculated wheat plants for comparison of root lengths

The preparation of wheat plants grown in 3 soils for isolation of macerate suspension was essentially similar to those previously described (4.2.1.1). However, growth of the plants was extended from 4 weeks to 7 weeks to increase the bacterial density within the rhizosphere (Rovira, 1965). *Azospirillum* sp. C10 (4.2.1.1) was again included as a reference strain. In these experiments only 1 seed was inserted into each tube and grown for 31 days under controlled conditions. Growth conditions were as described in section 3.2.3.4. Inoculated wheat seeds were grown in spermosphere tubes, with 6 replicates for each of 8 treatments as elsewhere (4.2.1.1).

4.3.1.2. Numbers of bacteria in inocula and plant rhizospheres

Assessment of numbers of bacteria both in the macerate suspension from the Kapunda, Avon and Waite soils at the time of inoculation and from the wheat rhizosphere following harvest of the plants was carried out on N-free media as described in section 3.2.1.8.

Three plants were used for the isolation of bacteria from the rhizosphere and within the roots and stems as described in sections 3.2.1.5. Bacteria plated onto semi-selective N-free media 4CS, PCAT and Nfb (Appendix 4) and growing well on these media were purified and stored as described in section 3.2.1.3. Isolation of bacteria from the soil macerate suspension from the 3 soils used to inoculate wheat in the spermosphere model system is described in section 3.2.1.4.

4.3.1.3. Measurement of root length

Three of the plants from the spermosphere system were used to isolate bacteria from the roots and stems as mentioned (4.3.1.2) and the 3 remaining roots from each treatment were used to measure root length. Roots were severed from the shoots, straightened and measured with a ruler. The lengths of the seminal and crown roots were measured as well as the lateral roots.

4.3.2. Results

4.3.2.1. Estimation of numbers of bacteria in inocula and after wheat harvest

Each of the 3 macerate suspensions from Kapunda, Avon and Waite soils contained $>10^{10}$ cfu/ml at the start of experiments to measure the effect of these inocula on root growth. While the concentration fell by the end of the experiment, the numbers of bacteria from the Waite soil grown on 4CS and PCAT media were significantly higher than for bacteria from Avon and Kapunda soils (Tukeys HSD 4CD = 0.2241, PCAT = 1.0828, Nfb = NS, Fig. 4.3).
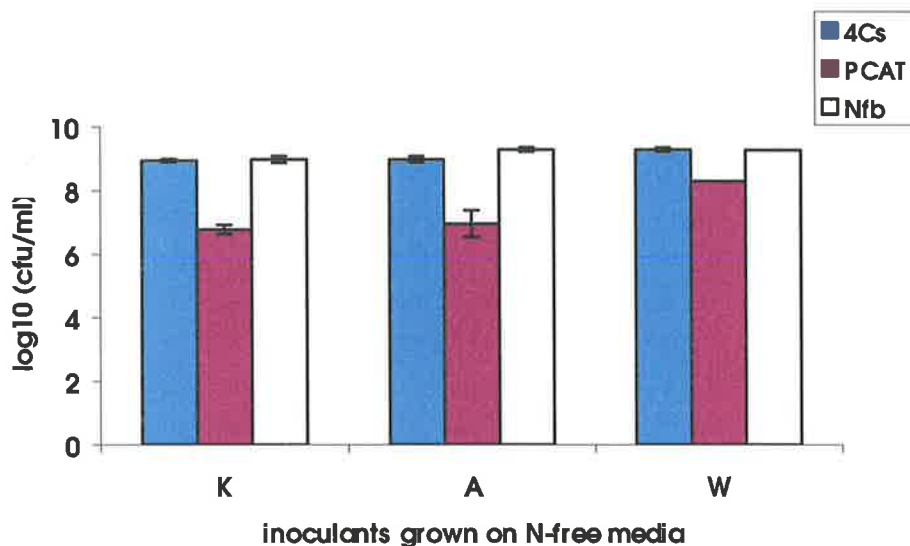
Fig. 4.3 Numbers of N_2-fixing bacteria from the wheat rhizosphere

After 31 days growth in the wheat rhizosphere in spermosphere tubes, numbers of bacteria were counted. Root macerates were plated onto the following N-free media: 4CS, PCAT and Nfb. K = Kapunda, A = Avon, W = Waite and Ref = *Azospirillum* sp. C10 inoc = inoculated, con = control. Tukeys HSD 4CS = 0.2241; PCAT = 1.0828; Nfb = NS. Means and standard errors of 3 replicates.

4.3.2.2. Root growth

When all macerate suspensions were inoculated to wheat grown for 31 days, the root growth as measured by length significantly increased over heat-killed controls. However, Kapunda and Avon inocula significantly decreased the number of seminal and crown roots per plant (Tukeys HSD = 2.1655, Fig. 4.4 a). The length of roots was increased (Tukeys HSD = 19.5, Fig. 4.4 b). (Tukeys HSD = 4.7, Fig 4.4 c). In contrast,

inoculation with *Azospirillum* sp. C10 significantly reduced the lengths of the seminal and crown roots and had no effect on lateral root length.

(a)

(b)

(c)
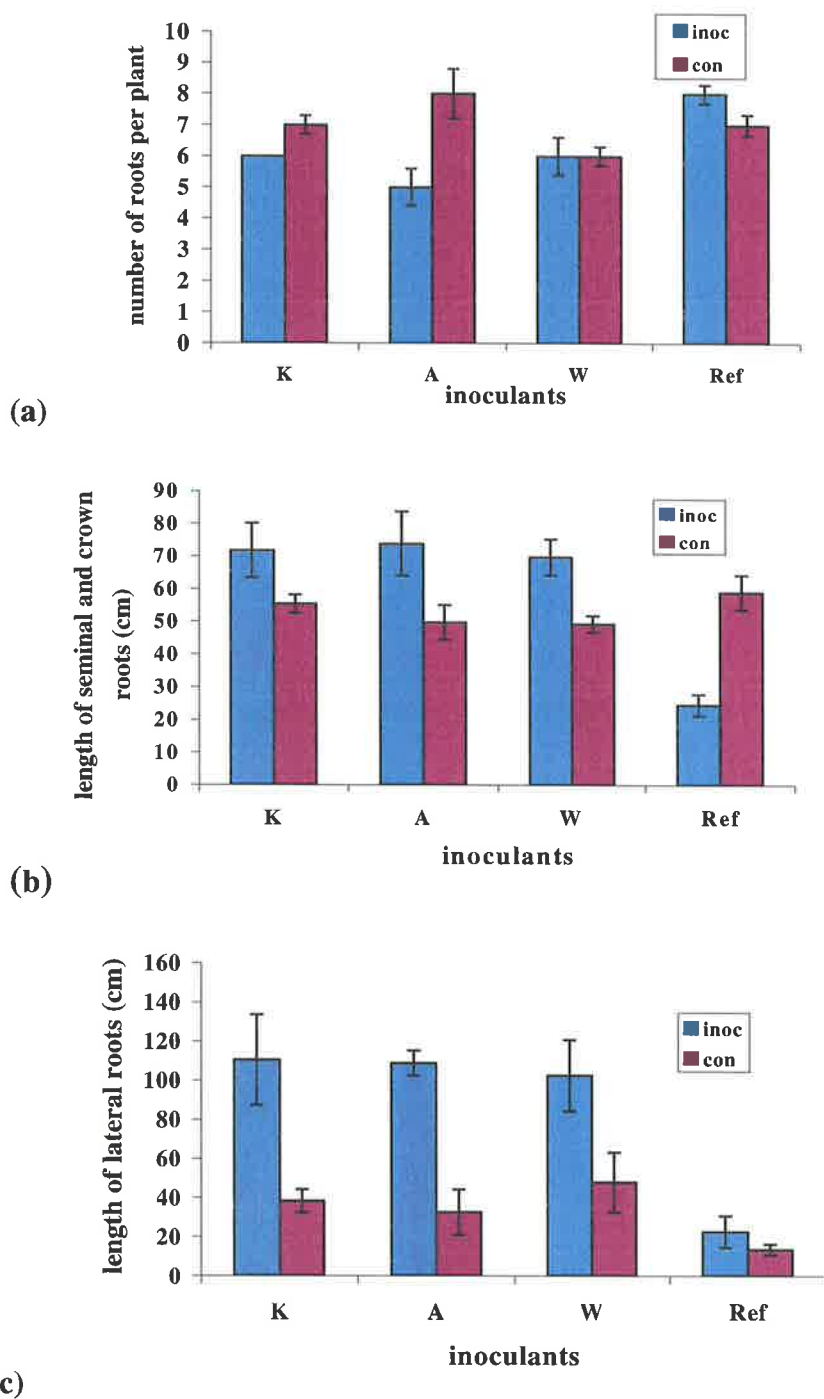
Fig. 4.4 Number of roots per plant (a), length of seminal and crown roots (b),

length of lateral roots after inoculation with macerate suspension from Kapunda,

Avon and Waite soils and reference strain *Azospirillum* sp. C10 (c).

After growth in the spermosphere tubes for 31 days, effect of inoculation on wheat roots was assessed by (a) counting the number of roots per plant, Tukeys HSD = 2.1655
(b) measuring the number of seminal and crown roots, Tukeys HSD = 19.5
(c) measuring the length of lateral roots, Tukeys HSD = 47.6.
K = Kapunda, A = Avon, W = Waite, and Ref = *Azospirillum* sp.C10, inoc = inoculated, con = control. Means and standard errors of 3 replicates.

4.4 Effects of Added Nitrate on Growth of Inoculated Wheat Roots

4.4.1 Materials and methods

4.4.1.1 Growth of inoculated wheat grown with and without addition of nitrate

Wheat seeds were grown in spermosphere tubes in semi-solid medium (10 ml) supplemented with 3.5 mM sodium nitrate (+N) or without N (-N) and grown for 28 days. These seeds were then either inoculated with macerate suspensions from plants grown for 37 days in Kapunda, Avon and Waite soils as described in section 3.2.1.6, inoculated with heat-killed suspension or left uninoculated. There were 5 replicates of each treatment.

4.4.1.2 Numbers of bacteria in macerate suspension and from the wheat rhizosphere

Bacterial numbers in the macerate suspension from Kapunda, Avon and Waite soils and from the plant rhizosphere 28 days after inoculation were counted as previously (3.2.4.1).

4.4.1.3 Measurement of root length of wheat grown with or without added nitrate

Where the effect of nitrate supplement was being determined, a Delta T scanning system was used to measure root lengths. Variations between measurements made with a ruler and with the scanner were determined.

After harvest at 28 days, roots of wheat plants were preserved in 70% ethanol and then stained with methyl violet dye in order to increase contrast (Harris and Campbell, 1989). The roots were carefully positioned on filter paper to ensure no overlap with each other (Pederson et al, 1994). An overhead transparency sheet was placed on top and the root photocopied. The photocopies were then scanned using a flatbed optical character recognition scanner, which was set at a resolution of 300 dpi. The files were saved as TIFF files. Delta-T software was then used to measure diameter, area, length, volume and range of thickness of roots (Kirchhof, 1992). Calibrations were carried out

using known thicknesses of thread. Statistical analyses were performed as described in section 3.2.10.

4.4.2 Results

4.4.2.1 Numbers of bacteria

Numbers of bacteria from the macerate suspensions used to inoculate wheat seedlings (Fig.4.5 a) were lower than in the previously described experiment, which was carried out to study the effects of inoculation on wheat root lengths (4.3.2.1). The highest numbers of bacteria grew on both 4CS and Nfb media. Numbers of bacteria from the Kapunda soil were significantly higher than from either Waite or Avon soils when grown on PCAT and Nfb media. Tukeys HSD, 4CS = 0.1131, PCAT = 0.2014, Nfb = 0.1653. After 28 days of growth with wheat in the spermosphere tubes, the number of N_2-fixing bacteria in treatments without added nitrate had increased, with the highest numbers of bacteria again being supported by 4CS and Nfb media (Fig 4.5 b). There were significantly higher numbers of bacteria from Waite soil on both 4CS and Nfb media compared with numbers of bacteria from Kapunda and Avon soil. Numbers of bacteria from heat-killed controls were significantly less than from inoculated plants. Bacteria in the rhizosphere of uninoculated and in rhizospheres with heat-killed suspensions were more than two orders of magnitude less than inoculated plants. The highest numbers of bacteria grew on 4CS medium. Tukeys HSD, 4CS = 0.2170, PCAT = 0.2048, Nfb = 0.1894. Numbers of bacteria were only assessed from plants grown with no added nitrate due to the intention to re-inoculate plants grown in N-free medium with these bacteria (4.5.1.1). Detection of bacteria in the rhizosphere of uninoculated plants, led to further studies to investigate the presence of seed borne bacteria (4.5.1.3.).

(a)

(b)
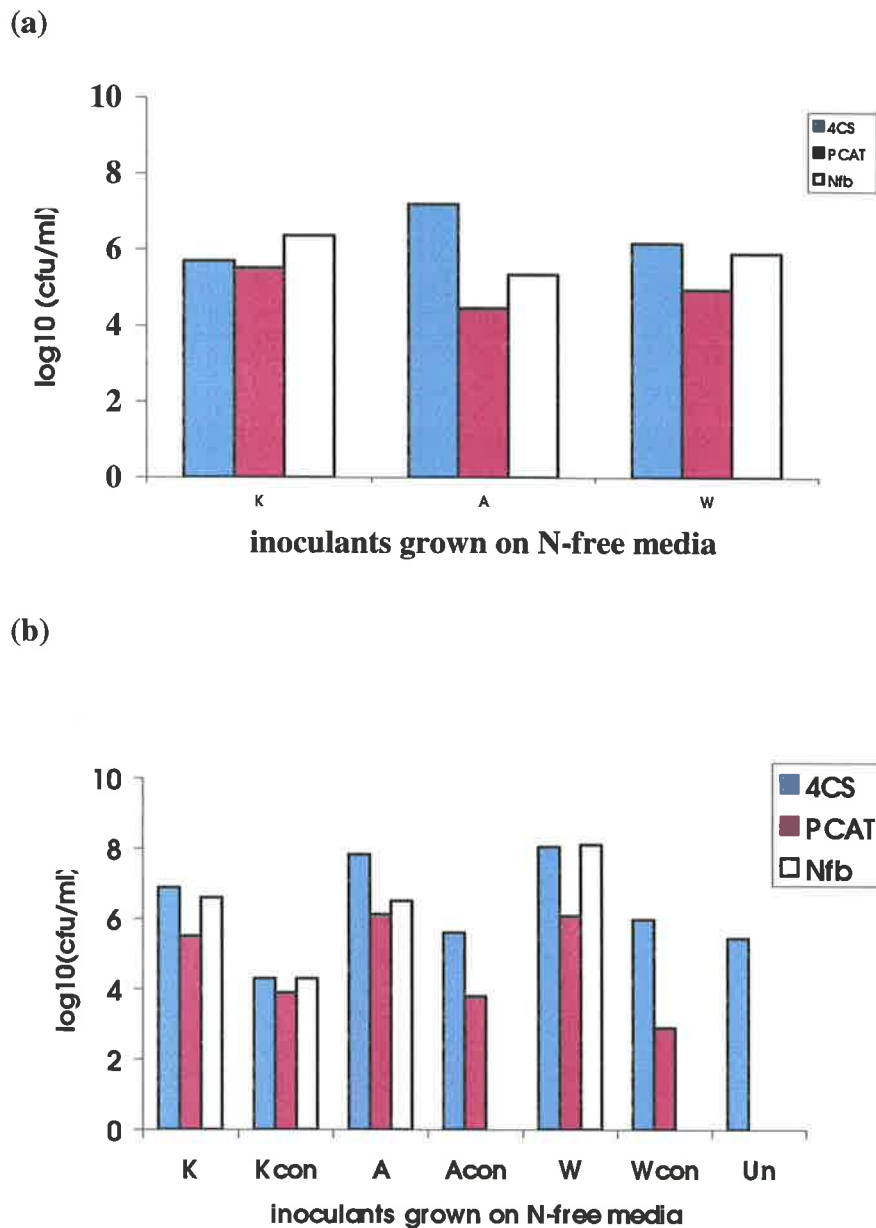
Fig. 4.5 Numbers of bacteria destined to be used to inoculate wheat in studies on the effects of nitrate on wheat root growth (a), numbers of bacteria in the rhizosphere after 28 days of growth in spermosphere tubes without added nitrate(b).

(a) Tukeys HSD 4CS = 0.1131; PCAT = 0.2014; Nfb = 0.1653
(b) Tukeys HSD 4CS = 0.2170; PCAT = 0.2048; Nfb = 0.1894

Bacteria were grown on 4CS, PCAT and Nfb, N-free media as previously described. Numbers of bacteria were counted from the rhizosphere of uninoculated and macerate suspension inoculated onto heat-killed plants.

K = Kapunda, Kcon = Kapunda control, A = Avon, Acon = Avon control, W = Waite, Wcon = Waite control, Un = Uninoculated.

4.4.2.2 Root length

Nitrate addition alone had no effect on root length of inoculated plants grown in spermosphere system at 28 days of growth. However, there was a significant increase in root length in the plants that were inoculated with the macerate suspension from Kapunda soil with added nitrate, when compared with uninoculated plants. Furthermore, the root length of wheat grown with heat-killed macerate suspensions and nitrate were not increased P<0.05 (Tukeys HSD = 22.5, Fig. 4.6). The average length of Kapunda roots was twice that of the Waite inoculated roots.
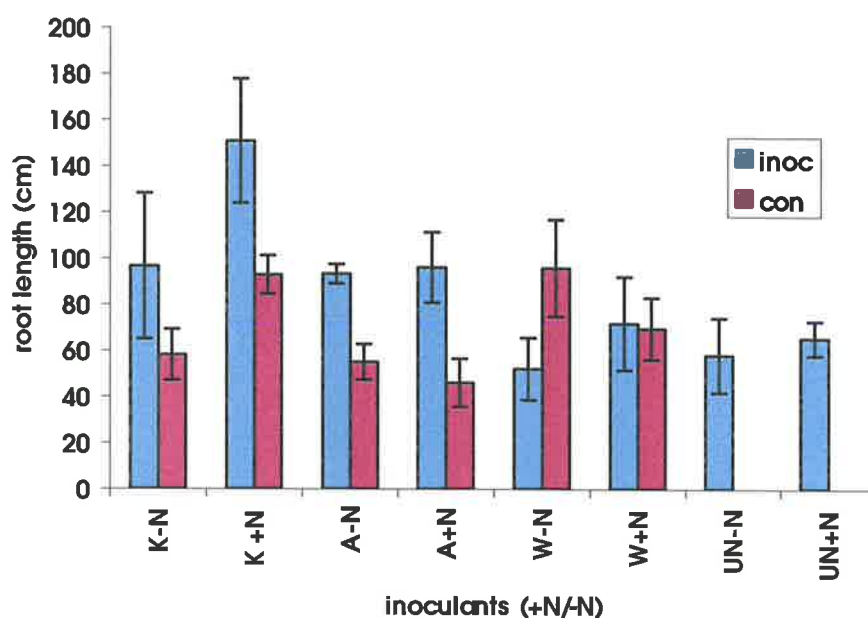
Fig.4.6 Total root length of plants of inoculated plants with or without added nitrate.

Length of inoculated roots was measured after growth in the spermosphere tubes for 28 days. Treatments included added $NaNO_3$ (3.5mM, +N), or no added nitrate (-N) Tukeys HSD = 22.5.
K = Kapunda, A = Avon, W = Waite, Un = uninoculated, inoc = inoculated, Con = control, un = uninoculated.
Mean and standard errors of the mean of 3 replicates.

4.5. Petri Dish Bioassay to Detect the Effects of Inoculation on Root Growth

4.5.1 Materials and methods

4.5.1.1 Preparation for germinated seed for Petri dish assay.

Surface sterilised wheat seeds, germinated for 48 hours and inoculated for 2 hours in rhizobacterial preparations were placed in Petri dishes containing river sand and water (see below). The rhizobacterial preparations were obtained from wheat plants that had been grown for 28 days in the presence of macerate suspensions from either Kapunda, Avon or Waite soils in N-free semi solid medium in spermosphere tubes. These plants were removed from their media and the roots macerated. In these experiments, inoculants were used diluted 1:4 and 1:100 in phosphate buffer (0.05 M, pH 7.00). The seeds were placed in a consistent orientation in a glass Petri dish together with 200 μl of inoculum, which was applied to the emerging roots. The Petri dishes (9 cm x 1.3 cm), contained washed river sand (25 g) diameter (1-2 mm), and RO water (5 ml). They had been autoclaved on 3 consecutive days at 121° C for 20 min. There were 3 replicates for each treatment as described in the previous experiment (Table 4.2). The Petri dishes were angled at 45° and the lower two thirds of the dish covered with alfoil to encourage roots to grow down into the river sand matrix. The dishes were randomised and grown for 7 days in a controlled growth environment at 15°C with a 12 hour photoperiod, a relative humidity with 0-70% and irradiance 240 $\mu E\ m^{-2}\ s^{-1}$.

4.5.1.2. Root length in Petri dish bioassay

The length of lateral branches and the total length of the roots were measured with a ruler.

4.5.1.3. Seed borne bacteria

It was assumed that any bacteria present in the rhizosphere of the uninoculated or heat-killed suspension in control plants (4.4.2.1.) originated from within the plant. Therefore, to test for the presence of seed borne bacteria, 24 wheat seeds were surface-sterilised and pre-germinated on nutrient agar plates for 48 hours at 25°C to check for residual surface contamination. Six of the seedlings were then macerated in phosphate buffer

(500 µl) and the macerate pipetted (10 µl x 6 replicates) onto nutrient agar. To ensure effective surface sterilisation, a further six seedlings were surface-sterilised a second time prior to being macerated in phosphate buffer (500 µl). This macerate was then dispensed onto nutrient agar (10 µl x 6). These nutrient agar plates were incubated at 28°C and checked for bacterial growth.

4.5.2 Results

4.5.2.1 Root length

There were no differences in root length of 7 day-old plants inoculated with rhizobacterial preparation from Kapunda, Avon and Waite soils that had been diluted 1:100 (results not shown). However, root length of plants inoculated with the 1:4 diluted rhizobacterial preparation from both Kapunda and Avon soils were significantly longer than the heat-killed controls. Plants inoculated with the rhizobacteria from Kapunda soil also had significantly longer roots than plant roots from uninoculated plants (P<0.05, Fig.4.7.). The rhizobacteria derived from the Waite soil had no effect on root growth, whether heat-killed or not.
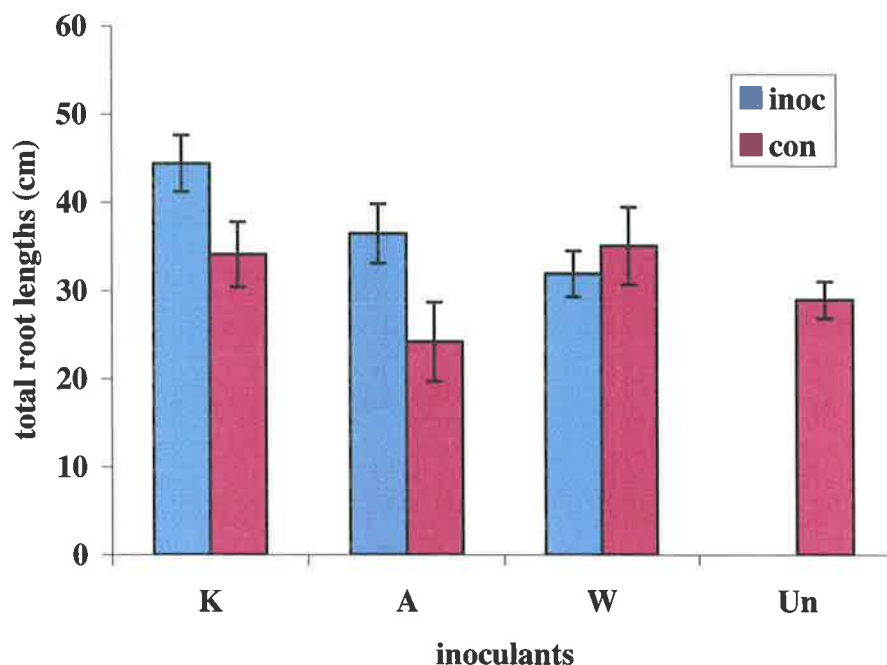
Fig. 4.7 Total lengths of wheat roots inoculated with rhizobacterial preparations grown for 7 days in Petri dishes

Inoculants for the Petri dish bioassay were obtained from wheat roots grown in spermosphere tubes for 28 days (4.4.1.2) and diluted 1:4. Plants were grown in sterile river sand and the root lengths measured after 7 days.

K = Kapunda, A = Avon W = Waite, Un = Uninoculated, inoc = inoculated, con = heat-killed control. Error bars represent standard errors of 3 replicates.

4.5.2.2 Seed borne bacteria

Following surface sterilisation of the seeds, no surface contamination was observed after 48 hours on agar plates. When macerates from 6 seeds were grown on NA, the numbers of cfu/seed grown on each NA plate were 75, 0, 0, 0, 250 and 58. Following a second surface-sterilisation, macerates from another 6 seeds were grown on NA. Numbers of cfu/seed were 58, 0, 0, 0, 58 and 0 on each of the NA plates. Up to 50% seeds released bacteria from inside the seed.

4.6. Discussion

The focus of experiments in this chapter was the selection of a soil source from one of three field sites in order to select N_2-fixing bacteria as potential inoculants for wheat growth. The data indicate that the Kapunda soil is the most promising source, based on evidence for an input of N from atmospheric N_2-fixation in the plant rhizosphere (^{15}N) and stimulation of root growth by bacterial consortia from this soil. As discussed previously, (2.2.3) generation of $^{15}N_2$ is an expensive procedure and due to the exploratory nature of the experiment it was not repeated. The addition of a nitrate supplement to the inoculated plants, up to 28 days after germination, was associated with a positive inoculation effect. However, as the plants did not appear to require nitrate in addition to seed reserves for up to 28 days after germination in the spermosphere system, it is likely that other factors in addition to N_2 fixation are responsible for beneficial plant responses.

4.6.1 N_2 fixation

Evidence for the occurrence of N_2-fixation in association with plant roots following exposure of the plant to $^{15}N_2$ was confirmed by the detection of raised levels of ^{15}N in plant tissue by mass spectrometry. However, only a small amount of N was transferred into the plant from N_2 fixation in the rhizosphere. Data from this $^{15}N_2$ experiment have been supported by subsequent work showing evidence for N_2 fixation by bacteria grown in association with wheat, but with no significant transfer of fixed N into the plant by 26 days (Merbach et al, 1998).

It was anticipated that the contribution from free-living N_2-fixing bacteria to the N uptake by the plants would be small, given the high energy levels required by bacteria to enable them to fix N_2 (Sprent, 1979). As bacterial biomass increased 100 fold over the duration of the experiment, conditions were sufficient to sustain bacterial growth and N_2 fixation. To detect significant differences in total plant N attributed to N_2 fixation, it would be necessary to extend the growth of the plants beyond 28 days. This factor has been followed up in the longer-term experiments described in Chapter 6.

4.6.2 Stimulation of root growth due to microbial inoculation

The addition of nitrate to the growth medium enabled differentiation to be made between N_2-fixing bacteria from the 3 soil sources used to inoculate wheat. Root elongation was greatest in the plants inoculated with the bacteria derived from the Kapunda soil and suggests that an inoculation effect other than, or in addition to, N_2 fixation contributed to the plant growth response. Indeed, the presence of available nitrate may or may not repress nitrogenase activity, depending on concentration and bacterial species. For example, nitrate (0.5 mM) did not affect nitrogenase activity in *Azospirillum* NR- and NR+ (El-Komy et al, 2003) but nitrogenase activity was repressed in *Azospirillum* 10SW when grown with added nitrate, but the repression was reduced when the bacterium was grown in association with wheat (Bhattarai and Hess, 1997). Optimal growth in *Azospirillum lipoferum* has been observed with oxygen (5%), nitrate (2 g/l) and molybdenum (0.5 g/l) (Furina et al, 1999). Inhibition of nitrogenase activity was noted in *Acetobacter diazotrophicus* when grown with ammonium chloride and ammonium nitrate (25 mM) but low levels of N (1/10 MS liquid medium) did not repress nitrogenase acitivity (Muthukumarasamy et al, 2002). The suggestion that a mechanism other than N_2 fixation is responsible for stimulating root growth is borne out by data obtained in the Petri dish bioassay. Enrichment of dominant bacteria after 28 days of growth in the spermosphere model system with subsequent re-inoculation onto wheat grown in river sand for 7 days in Petri dishes, resulted in stimulation of roots by bacteria from the Kapunda soil source. The presence of bacteria in the inoculum transferred from uninoculated and heat-killed inoculated plant roots to the seedlings in the Petri dishes, may have originated within the seeds providing controls against which inoculation effects could be measured. The differences in bacterial numbers between heat-killed controls from Kapunda, Avon and Waite soils (Fig 4.5b) may be attributed to compounds produced by heat sterilisation as it was assumed that any seed-borne bacteria would be similar. No nutrients were added to the river sand and with N available from seed reserves, stimulation of root growth 7 days after inoculation is therefore not likely to be attributable to N_2 fixation.

Inoculated plants grown in N-free semi-solid medium showed significant increases in seminal, crown and lateral root lengths when compared with plants inoculated with heat-killed inoculum. In this study the macerate suspensions used to inoculate wheat were also prepared as controls after being heat-killed by autoclaving. Methods for the

sterilisation of soils include pasteurisation, gamma irradiation and fumigation as well as by autoclaving. Increased nutrient levels may occur in response to heat or irradiation with associated increases in availability of manganese (Cawse, 1975). In a comparative study of the effects of sterilization by autoclaving or irradiation the effects of respiration, nitrogen levels and direct counts of soil bacteria were used to demonstrate that in autoclaved soil, bacterial cells lysed rapidly, CO_2 levels were recorded at almost zero and after an initial NH_4^+ flush the level remained steady. However, in irradiated soils cell lysis was less and CO_2 and NH_4^+ levels increased over time (Ramsay and Bawden, 1983). Other studies compared the growth of a fungus *(Laccaria bicolor)* added to either autoclaved or irradiated soil and results from that study also indicated that there were differences between the two methods of sterilisation. There was no fungal growth following autoclaving but rapid growth followed the irradiation of the soil (Brulé et al, 2001). Preliminary studies to compare the dry weights of plants, either inoculated with heat-killed macerate suspension (autoclaved) or uninoculated, demonstrated that after 4 weeks of growth there were no significant differences. The decision to use the autoclaving to kill bacterial cells in preference to irradiation was based on the above studies and the ease with which it could be performed. Results of the inoculation experiment (4.3.1) showed it was possible to detect effects of inoculation but not possible to determine which of the bacterial consortia from the three soil sources under investigation stimulated the best root growth. When inoculation density was reduced from 10^{10} cfu/ml to approximately 10^6 cfu/ml in the presence of added N, it was possible to discriminate between the effects on root growth by the bacterial consortia from the three soil sources.

Increased length of lateral roots in inoculated plants grown for 28 to 30 days may have been partly due to improved N availability, attributable to N_2 fixation by root-associated bacteria. An alternative reason for increased root length in inoculated roots may be a plant-induced response to depleted mineral supply, due to competition in the rhizosphere from bacteria. Indeed, after 28 to 30 days of growth in the spermosphere tubes in association with wheat, bacterial counts on three selective N-free media, showed that a range of N_2-fixing bacteria from the Kapunda, Avon and Waite soil sources were present in the rhizosphere, ranging in density between 10^7 to 10^8 cfu/ml. All bacteria including non N_2-fixing bacteria were enumerated by plating onto Nutrient

Agar (NA) but the data was not included as it did not represent significantly more bacteria than were accounted for after enumeration on N-free media.

The N_2-fixing community from the Kapunda soil source was selected for further study and characterisation. This is described in Chapter 5.

4.7 Conclusion

In conclusion, this chapter has raised the notion that inoculants of macerate suspension from rhizospheres derived from wheat grown in soils from Kapunda, Avon and Waite might contain microbiota that will enhance the early growth characteristics of wheat. In consequence, exploratory experiments to this end have shown that inoculation of wheat by bacteria capable of growing on N-free media from Kapunda resulted in stimulation of wheat roots and transfer of fixed N into the wheat tissue more effectively than inoculation by bacteria from Avon and Waite soil sources. Chapters 5 and 6 provide more concrete evidence about the mediators that support these notions.

Chapter 5: Characterisation of a Community of Bacteria from Kapunda using Fatty Acid Methyl Ester Analysis (GC-FAME) and Molecular Techniques

5.1 Introduction

A growth response in wheat plants, elicited through the plant rhizosphere by bacterial communities from Kapunda, Avon and Waite soils was described in Chapter 4. Conditions for plant growth were in N-free medium, and it was concluded that in spite of the predicted likelihood of enhancement from Avon and Waite soils, Kapunda yielded the better potential. Therefore, since the Kapunda bacterial community contained significant numbers of N_2-fixing bacteria the experiments discussed in this chapter were designed to characterise the Kapunda bacterial community. This approach was made in order to determine whether the observed plant stimulation occurred in association with dominant species within the community or whether species diversity was the significant characteristic of the community. The characterisation studies also sought to establish whether dominant bacteria in the community carry N_2-fixing genes.

Fatty acid methyl ester (GC-FAME) analysis has been variously used to characterise the structure of microbial communities (Kaneda, 1991; Frostegård et al, 1993; Olsson and Wallander, 1998). Comparisons have also been made between endophytic and rhizosphere microbial communities in association with different cultivars of canola and wheat (Siciliano et al, 1998). Techniques can be extended to compare N_2-fixing communities from different sources.

A focus of interest was to confirm the presumption that the effect of Kapunda soil bacteria on wheat growth was better than that of Avon and Waite. The detection of differences between the fatty acid profile of the N_2-fixing bacterial community from the Kapunda soil source and from Avon and Waite soil sources was so determined. It was proposed that variations in the fatty acid profiles could provide a key to identify groups or species of bacteria within the microbial communities, which, in turn, may differ between soil sources and which of more importance may be responsible for plant growth-promotion.

To complement information obtained in the GC-FAME analysis, molecular techniques were also employed to classify individual bacteria isolated from the Kapunda soil source using rRNA-directed oligonucleotide probes (Woese, 1987). The use of comparative 16S and 23S rRNA sequence analysis of various bacterial genera has facilitated the compilation of phylogenetic databases (Olsen et al, 1994). Information on both highly conserved and variable regions within the rRNA molecular structure, identical in phylogenetically related taxa, has resulted in the development of both species and group specific rRNA directed probes, to detect target sites of aligned sequences. Within the cell, the large numbers of copies of rRNA have also enabled whole cell identification using rRNA-directed probes (Kirchhof et al, 1997). The oligonucleotide probes used in this chapter differentiated the alpha, beta and gamma sub-groups of the Proteobacteria through hybridisation studies.

Characterisation studies also included amplification of *nif*D (encodes for MoFe protein) and *nif*H DNA (product is NifH, Fe protein) by PCR (polymerase chain reaction), which was performed on bacterial isolates from the N_2-fixing community from the Kapunda soil source to detect structural genes for nitrogenase.

Identification of the isolates using GC-FAME analysis and comparison of GC-FAME profiles with the Sherlock database (MIDI Inc[R]. Newark, Delaware) and data obtained through molecular techniques was to provide information to highlight similarities and differences between individual bacteria isolated from the Kapunda soil. Using selected isolates as inoculants for wheat, fatty acid profiles from the rhizosphere were compared with those obtained from the bacterial community from the Kapunda soil source. The fatty acids present in the uninoculated plant rhizosphere were assumed to be derived from both plant and seed borne bacterial sources so it was necessary to discount them from the analysis of inoculated plants. The GC-FAME profiles from the individual rhizobacteria of interest could then be compared with the bacterial community profiles to evaluate contributions to the community profile by individual bacteria.

The source of a consistently dominant fatty acid (18:1w9c) within the bacterial community from Kapunda soil was also investigated as part of the overall project to select potential inoculants to promote the growth of wheat.

5.1.1 Aims

The aims of experiments in this chapter were as follows:

- to compare the GC-FAME profiles of three bacterial communities from Kapunda, Avon and Waite soils, after 6 weeks of growth in wheat rhizospheres, in order to confirm relevant differences between the fatty acid profiles of the Kapunda community and those of Avon and Waite (section 5.2.),

- to classify bacteria isolated from the microbial community from Kapunda soil into sub-groups of the Proteobacteria using rRNA-directed hybridisation probes and relate species within the subgroups to the fatty acid profiles obtained with GC-FAME (section 5.3),

- to screen bacterial isolates from the Kapunda soil source for the presence of the structural nitrogenase gene *nif*D and *nif*H using PCR to select isolates carrying the nitrogenase gene for GC-FAME studies (section 5.4),

- to detect similarities between isolates using a repetitive sequence based PCR (InterLINE) to reduce duplication of selected isolates,

- to compare GC-FAME profiles from dominant bacterial strains isolated from the Kapunda soil microbial community with GC-FAME profiles of the community after inoculation of wheat, to facilitate interpretation of fatty acid peaks,

- to investigate the origin of a dominant fatty acid within the microbial community from the Kapunda soil source (section 5.5).

5.2 Comparison of GC-Fame Profiles of Three Bacterial Communities

5.2.1 Introduction

Bacterial communities isolated from Kapunda, Avon and Waite soil sources were grown in association with wheat for 6 weeks under N-free conditions, after which the GC-FAME profiles from the rhizosphere bacteria were analysed to detect any underlying differences between their fatty acid profiles. GC-FAME analysis was carried out directly on rhizosphere bacterial communities without the process of subculture onto enriched media to include unculturable microorgansms in the analysis.

5.2.2 Materials and methods

5.2.2.1 Preparation of inoculated wheat plants

Wheat was inoculated with macerate suspensions from wheat plants (*Triticum aestivum* var Stiletto) grown in soil from Kapunda, Avon and Waite as described in section 3.2.1.6. The plants were grown in N-free washed sand, semi-solid or porous agar media in pots (7.5 cm x 6.6 cm) for 6 weeks prior to GC-FAME analysis in the rhizosphere bacterial community. These three growth substrates were selected to provide the opportunity to identify an optimal condition for root colonisation by N_2-fixing bacteria in the experiment and following this, provide sufficient material on which fatty acid profiles of the bacterial community could be determined. Initial experiments determined that inoculated wheat grown in N-free medium in glass tubes provided insufficient bacteria for the GC-FAME analysis and larger containers were therefore selected. Preparation of the three substrates (N-free) with the addition of nutrient supplements for the growth of wheat is described in section 3.2.4.2. Nutrient supplements were similar to experiments described in Chapter 4. The substrates were autoclaved in pots and inoculated with bacterial suspensions from Kapunda, Avon and Waite soil sources as described in 3.2.1.8. Two wheat seeds, immersed for 2 hours in each suspension (1 ml) were sown into each pot. The roots from the first plant were used for GC-FAME analysis and roots from the second plant were macerated and used to estimate bacterial numbers at harvest. There were 3 replicate pots per treatment.

Transparent sterile sun bags (Sigma), manufactured with a patch to allow the exchange of gases, were used to cover the plants to prevent contamination. A perforated grid

(Fig. 5.1.) gave support to plants grown in semi-solid medium. The plants were randomised and grown at 15°C in a controlled environment cabinet with a photoperiod of 12 hours, temperature 15°C, at a relative humidity 60-70% and with irradiance 240 μE m^{-2} s^{-1}. The pots were watered to weight with sterile RO water every 3 days for 42 days.

transparent Sunbag fits over
autoclavable pot

pore for air transfer

ties
grid
medium
roots
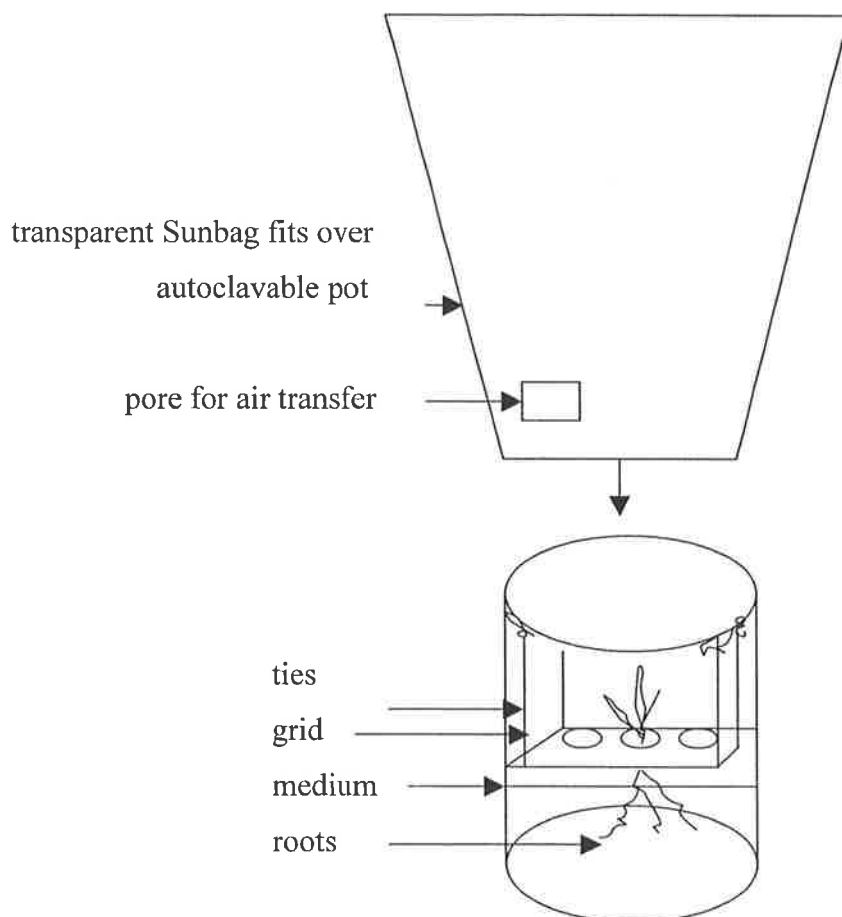
Fig. 5.1 Apparatus for growth of inoculated wheat supported by a perforated grid and grown in semi-solid medium

5.2.2.2 Plant dry weights

Root and shoot dry weights were obtained after drying the plant material at 60°C for 3 days. Results were analysed statistically using ANOVA (3.2.10).

5.2.2.3 Bacterial numbers

The numbers of N_2-fixing bacteria in the soil bacterial suspensions and from the root rhizosphere following harvest of the plants were determined by 10 fold serial dilution and growth onto 4CS, PCAT and Nfb medium selective for N_2-fixing bacteria (Appendix 4). To compare the numbers of bacteria introduced at the commencement of the experiment (1 ml) with the numbers of bacteria counted on the roots at the end of the experiment (whole root system macerated in 1 ml) total numbers were expressed as cfu/ml.

5.2.2.4 Preparation of the bacterial communities from the wheat rhizosphere for GC-FAME

Inoculated plants were harvested under sterile conditions. The roots from one of the two plants from each pot were rinsed in PBS to eliminate sand and agar and then placed in heat-resistant glass screw-top tubes (13 mm x 100 mm) containing PBS (10 ml). The tubes were consistently shaken 20 times, roots removed from the tubes and rhizosphere suspension centrifuged at maximum speed of 4,000 rpm for 5 min (Econospin, Sorvall Instruments). Centrifugation would have been more efficient at a higher speed to ensure no bacterial loss but to prevent loss of sample by transfer from smaller tubes able to withstand greater centrifugal force to heat resistant tubes required for GC-FAME analysis, the glass tubes were used for the entire sample preparation. The resulting pellet was resuspended in fresh PBS (2 ml), and centrifuged once more for 5 min. The supernatant was aspirated from the pellet, which was prepared and used directly for GC-FAME analysis (3.2.4). Profiles of fatty acids were generated for each sample to identify differences between the communities derived from different soil sources and types of growth substrates. The contribution of individual fatty acids is expressed as a percentage of the total.

5.2.3 Results

5.2.3.1 Dry weight of plants

The mean dry weight of the shoots was significantly greater when plants were grown in sand than in the semi-solid or porous medium ($P<0.05$). Conversely, the mean dry weight of roots was significantly heavier when plants were grown in semi-solid medium than in porous agar ($P<0.05$) as shown in Table 5.1. However, there were no significant differences in weights of plants inoculated with bacteria from different soil sources (data not shown).

Table. 5.1. Weight (mg) of shoots and roots of wheat grown in sand, porous medium and semi-solid medium for 6 weeks

	Sand	Porous agar	Semi-solid
Shoots	780 ± 51 a	640 ± 56 b	590 ± 34 b
Roots	490 ± 37 ab	360 ± 36 b	510 ± 50 a

Means and standard errors of means of 3 replicates. Means (shoots or roots) followed by the same letter are not significantly different ($P< 0.05$).

5.2.3.2. Estimation of bacterial numbers in inoculum and rhizospheres

At the beginning of the experiment wheat seeds were inoculated with 1 ml of a suspension containing 10^7 cfu/ml bacteria. After 6 weeks of growth in the rhizosphere, the rhizobacteria from the Kapunda soil grown in the sand treatment had increased in numbers 10-fold to *ca* 10^8 cfu/ml (Fig. 5.2). It was possible to estimate bacterial numbers from the sand samples as serial dilutions for determining numbers on agar plates were taken from a suspension. However it was not possible to eliminate sand from the pellet prepared for GC-FAME analysis and therefore it did not reflect an accurate assessment of the bacterial community. When grown either on PCAT or Nfb media, numbers of bacteria from Kapunda, Avon and Waite soils were significantly higher in sand than when grown on semi-solid or porous media.

a)

(b)

(c)
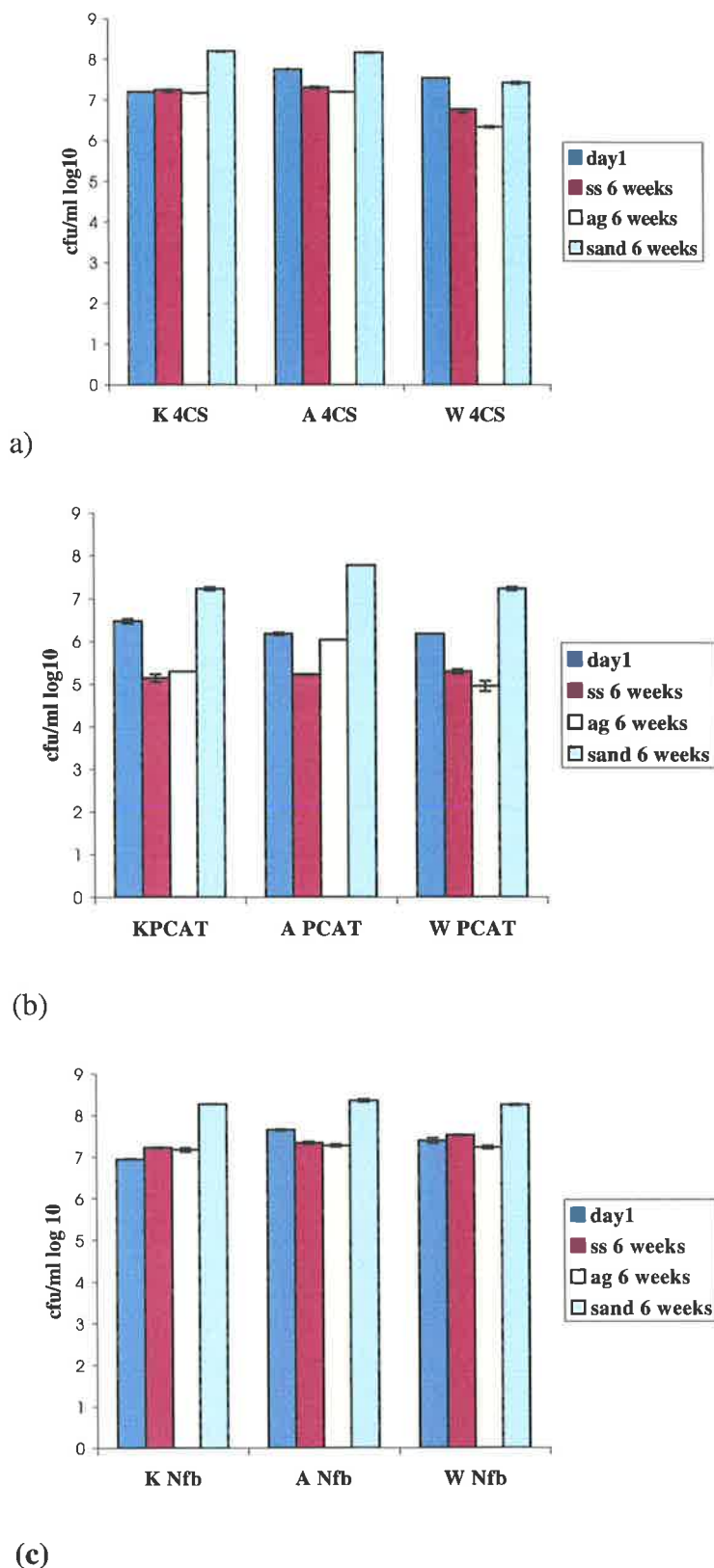
Fig. 5.2 Estimation of numbers of bacteria used to inoculate wheat and recovered after 6 weeks in the wheat rhizosphere.

(a) Bacteria grown on 4CS medium, Tukeys HSD=0.4951 (b) PCAT medium, Tukeys HSD= 0.3633 (c) Nfb medium, Tukeys HSD=0.1700. Means and standard errors of means of 3 replicates.

5.2.3.3 GC-FAME

FAME analysis revealed that the microbial community from the Kapunda soil source, recovered from the wheat rhizosphere after 6 weeks of growth in semi-solid medium, had a more diverse fatty acid profile than the rhizosphere communities of plants from any source grown on the porous agar medium (Table 5.2). Similar fatty acids were present in both porous and semi-solid media. However additional fatty acids were present in the semi-solid medium. The major fatty acids within the profile from the porous and semi-solid medium were also similar. Difficulty in eliminating sand from the sample in preparation for GC-FAME analysis resulted in a loss of bacteria from the sample. The concentration of fatty acids in the rhizosphere of plants grown in sand was therefore below the limits of detection.

Twenty one fatty acids (contributing 0.2% or more of the total) were detected in the wheat rhizosphere communities. The highest percentage of fatty acids detected in the analysis were from 14:0, 16:0, 16:1w9c, 18:0, 18:1w9c, Sum feature 4 (16:1w7 c/15iso2OH or 15iso2OH/16:1w7c), Sum feature 6 (18:2w6,9c/18:0ante or 18:0ante/18:2w6,9c). Fatty acids 16:1 and 18:1w9c) were common to all samples and contributed approximately 20% and 50% respectively. The composite Sum features were also detected in all samples. Significance of Sum features is discussed in Appendix 2. Total number of fatty acids detected was higher in rhizospheres of wheat inoculated with Kapunda soil suspension grown on semi-solid medium (21) compared with all other samples (6 to 9, Table 5.2).

Seven of the fatty acids from the Kapunda GC-FAME profile from semi-solid medium were identified as bacterial fatty acids commonly detected in Gram negative bacteria. Five of these fatty acids did not appear in the profiles of bacterial communities from either Avon or Waite soils.

TABLE 5.2 Mean percentage contribution of individual fatty acids in the GC-FAME profiles of bacterial communities from Kapunda, Avon and Waite recovered from the rhizosphere of wheat plants grown in semi-solid medium and porous medium.

Fatty acids	SEMI-SOLID MEDIUM			POROUS MEDIUM		
	Avon	Waite	Kapunda	Avon	Waite	Kapunda
10:03OH			0.15(±.01)			
12:00			0.37(±.06)	0.92 (±.03)	0.42 (±.18)	
140iso					0.44 (±.11)	
14:00	1.2 (±.05)		1.20 (±.1)	1.38 (±.06)	0.87 (±.21)	1.3 (±.25)
15:0iso *			0.21(±.02)		0.52 (±.2)	
15:0ante-iso*			0.28(±.05)		0.76 (±.27)	1.2 (±.48)
15:00 *			0.27(±.02)			
16:0ante-iso	0.82 (±.11)	1.57 (±.3)	0.96(±.006			
16:0	**21.2 (±.83)**	**19.9 (±.9)**	**20.6(±0.3)**	**19.1 (±.7)**	**18.4 (±2.2)**	**22 (±1.34)**
16:1w9c	3.26 (±.19)	4.73 (±.7)	3.1 (±.03)			3.2 (±.14)
17:0 cyclo *			0.27(±.02)			
18:0	2.11 (±.2)	2.3 (±.005)	2.3 (±.03)			2.7 (±.51)
18:0iso			0.26(±.01)			
18:1w9c	**52.1 (±2.1)**	**49.55 (±4)**	**47 (±1.2)**	**59.7 (±4.5)**	**47.0 (±6.3)**	**45.9 (±3.8)**
18:3w6c (6,9,12)			0.4 (±.03)			
19:0iso *			0.3 (±.03)			
19:0 cyclow8c *			0.24(±.02)			
20:00 *			0.31(±.04)			
20:1w9c			0.49(±.04)			
20:4w6, 9,12,15c			0.40(±.03)			
Sum 3	3.5 (±.06)	11.5 (±.88)	6.88 (±.2)	3.41 (±.6)	3.7 (±1.48)	4 (±1.03)
Sum 5	9.85 (±.42)	7.51 (±.7)	10.5 (±.2)	7.76 (±.2)	7.55 (±.55)	10 (±1.53)
Total number of fatty acids in FAME analysis	8	7	21	6	9	8

Fatty acids detected in the rhizospheres of plants inoculated with bacterial communities from Kapunda, Avon and Waite soils grown in pots in porous or semi-solid medium (not detected in the rhizosphere of plants grown in sand). Fatty acids with highest percentage of the total profile are shown in bold.

Fatty acids commonly detected in Gram negative bacteria (*). Sum feature 3 (16:1w7c/15iso2OH or 15iso2OH/16:1w7c), Sum feature 5 (18:2w6,9c/18:0ante or 18:0ante/18:2w6,9c). Means and standard errors of means of 3 replicates.

5.2.4 Conclusion

Trace amounts of diverse fatty acids were detected in the GC-FAME analysis of the microbial community from Kapunda. The presence of fatty acids 16:0 and 18:1w9c, comprising 70% of the total fatty acids within the GC-FAME profile, prompted further investigation to identify common bacterial groups from the Kapunda soil.

5.3 Characterisation of the Kapunda Community of N_2-fixing Bacteria using rRNA Group-specific Probes

5.3.1 Introduction

Group-specific oligonucleotide probes were used to classify bacteria isolated from the community from the Kapunda soil source into sub-groups of the Proteobacteria. Identification of isolates from the Kapunda soil source (4.3.1.2) using GC-FAME analysis was carried out to validate the molecular experimentation.

5.3.2 Materials and Methods

5.3.2.1 Hybridisation of bacteria using rRNA-directed group-specific probes
5.3.2.1.1 Nucleic acid extraction

Bulk nucleic acid extractions were prepared to contain substantial ribosomal DNA content (Stahl and Flesher, 1987). Eighty bacteria isolates from the Kapunda soil were taken from frozen stocks, grown on agar plates containing DYGS medium (Appendix 4.) and streaked to form single colonies. *Rhizobium leguminosarum* and *Azospirillum brasilense*, classified within the alpha sub-group of the Proteobacteria (Woese, 1987) were included as reference strains. DNA extractions were carried out as described in Chapter 3.2.8.1. MOPS buffer (30 µl, Appendix 3.) and DNA (10 µl) were then gently mixed and applied to a Hybond-N+ filter (9 x 12 cm, Amersham Pharmacia Biotech) using a dot blot apparatus. The samples were applied according to the instructions of the manufacturer (Bio Rad). The membrane was then air dried and fixed for 5 min by UV cross linkage.

5.3.2.1.2 Hybridisation of DNA to group-specific oligonucleotide probes

Hybridisation was carried out according to Kirchhof and Hartmann, (1992). The fixed membranes were placed in hybridisation bottles and pre-washed in 0.1 x SSC, 0.5% SDS for 1 hour at 37°C. The membranes were pre-hybridised for 1 hour at 60°C in buffer containing 5 x SSC, 5 x modified Denhardt's solution and 1% sarkosyl (15 ml). Denhardt's solution was diluted from concentrated stock (100 x modified Denhardt's solution: 2% polyvinylpyrrolidone, 2% Ficoll 400, 2% polyethylene glycol 8000). The membranes were prepared in sufficient numbers for hybridisation to each of the probes.

The sequences for group-specific rRNA-directed oligonucleotide probes, ALF 1b (alpha sub class), BET 42a (beta sub class), GAM 42a (gamma sub class, Manz et al, 1992), AZO (*Azospirillum*) are tabulated in Table 5.3 and referred to (3.2.8.2). The probes were labelled with [^{32}P] using a 3'end labelling kit (Amersham) and the labelling reaction carried out at 37°C according to the manufacturer's recommendations. After incubation, the labelling reaction was added to the pre-hybridisation buffer in the bottles and incubated overnight with the membranes in a rotating oven, set at the melting temperatures of the oligonucleotide probes.

TABLE 5.3 Sequences and target sites for oligonucleotide probes ALF 1b, BET 42a, GAM 42a, AZO

Probe	Sequence	Target site rRNA position	Melting temperatures (T_m)
ALF1b	5'-CGTTCG(C/T)TCTGAGCCAG-3'	16s, 19-35	50°C
BET42a	5'-GCCTTCCCACTTCGTTT-3'	23s, 1027-1043	47°C
GAM42a	5'-GCCTTCCCACATCGTTT-3'	23S, 1027-1043	47°C (Manz et al,1992)
AZO	5'-GGGGCT(A/G)TTTCC(C/T)GG-3'	23s	48°C (Kirchhof and Hartmann, 1992)

Probes were labelled with [^{32}P] using a 3' end labelling kit (Amersham). Overnight hybridisation of membranes was carried out at the melting temperature of individual oligonucleotide probes as shown.

Membranes were washed in 2 x SSC/0.1% SDS at room temperature for 20 min followed by a further wash at hybridisation temperature for 5 min. The membranes were then air-dried and exposed to a phosphor-imaging screen overnight. Visualisation of the images was carried out following exposure to a phosphor imager and the data quantified

using Storm Phosphor Image software (Molecular Dynamics). The intensity, background value and area of the image were analysed and an average value (intensity-background/area) was calculated. Hybridisation was recorded as positive within the range of average values of 800-1999 and strongly positive with average values >2000. The ranges were determined using the reference samples as indicators of strongly positive average values.

5.3.2.2 Identification of bacterial isolates using GC-FAME analysis

GC-FAME analysis and identification of isolates was carried out as described (3.2.4.1). The GC-FAME profiles were compared with the Sherlock TSBA aerobe library version 3.8 (MIDI Inc[R]. Newark, Delaware) and a computer generated similarity index was constructed to compare the GC-FAME profile from the sample with the GC-FAME database. The likely identity of samples was suggested by reference to the similarity between the GC-FAME profile in the database and sample GC-FAME profile.

5.3.3 Results

5.3.3.1 Hybridisation of bacteria to group-specific probes

Results are presented in Table 5.4. Of the 80 isolates (excluding 2 reference strains) hybridised to ALF1b, BET42a, Gam42a and AZO probes, 21 hybridised to the GAM42a probe and 19 to the ALF1b probe. The remaining 26 isolates did not hybridise to the group-specific probes.

Three isolates hybridised to both GAM42a and ALF1b and one isolate hybridised to BET42a and weakly to AZO. The reference strains *Rhizobium leguminosarum* and *Azospirillum brasilense* hybridised to both ALF1b and AZO probes as expected, with *Azospirillum brasilense* hybridising strongly to the AZO probe. The majority of isolates identified by GC-FAME analysis correlated well with classification of the isolates into sub-groups of the Proteobacteria following hybridisation to oligonucleotide probes (Table 5.4). For example, the isolate identified by GC-FAME as *Agrobacterium tumefaciens*, which is classified within the alpha sub-group of the Proteobacteria (Woese, 1987), hybridised positively to the ALF1b probe. Apart from one exception, the Enterics (eg *Pantoea agglomerans* and *Enterobacter taylorae*) hybridised to the GAM42a probe as expected. The bacteria identified as *Pantoea agglomerans* and

Enterobacter taylorae each comprised 24% of the total number of isolates identified by GC-FAME.

TABLE 5.4 Numbers of bacteria hybridising to rRNA-directed group-specific probes ALF1b (α), BET42a (β),GAM42a (γ) and AZO

Probe	Number of bacteria	FAME Identification	Hybridisation (+)	(++)
ALF1b	19	*Agrobacterium tumefaciens* (2) *Enterobacter taylorae* (1) *Xanthomonas sp.*(1)	8	13
BET42a	8	*Burkholderia cepacia* (1) *Cytophaga* (1)	6	2
GAM42a	21	*Enterobacter taylorae* (2) *Pantoea agglomerans* (3) *Pseudomonas putida* (1)	15	6
Azo	2	Identification unknown	3	1
Total No. of isolates	**54**			

Positive hybridisation of calculated areas between the range of 800 to1999 (+), strongly positive >2,000(++), n = 80. 26 isolates did not hybridise to the probes. The number of isolates which hybridised to probes and were identified by FAME are in parentheses. The remainder of isolates were unidentified.

5.3.4 Conclusion

The majority of bacteria fell into the alpha and gamma sub-groups of the Proteobacteria while identification of the isolates by GC-FAME analysis indicated that they were represented by a range of genera, many of which are known to contain N_2-fixing bacteria. The next step was to investigate the presence of the nitrogenase gene in the isolates and assess genetic similarities between isolates.

5.4 Molecular Characterisation of Bacteria

5.4.1 Introduction

Isolates from the Kapunda soil source were screened for the presence of the structural nitrogenase gene *nif*D and *nif*H using specific PCR amplification as well as hybridisation studies. A small study was also carried out to determine whether *nif* genes were located on bacterial chromosomes or plasmid. InterLINE PCR used as a

fingerprinting method was used to assess similarities between the isolates and reduce duplication. The results from the characterisation studies were then used to select isolates carrying the nitrogenase gene for further study.

5.4.2 Materials and methods

5.4.2.1 Purification of bacteria on selective media

To screen bacteria from the Kapunda soil source for the presence of the nitrogenase gene, bacteria from the rhizosphere and within the roots and stems of inoculated wheat grown in semi-solid N-free medium in the spermosphere system (4.3.1.2, 3.2.15) were taken from frozen stocks. Additional bacteria isolated from the rhizosphere of wheat grown directly in Kapunda soil were also included (3.2.1.4). The bacteria were selected from N-free media (PCAT, 4CS and Nfb) and the selection of the isolates was based on dominance and growth on selective media.

5.4.2.2 Polymerase chain reaction (PCR)

Cell lysates were prepared from purified isolates for use in PCR as described in section 3.2.8.5. Samples were placed in PCR tubes on a block in a thermal cycler (MJ Research.). A negative control of RNAase-free water was included with every PCR to ensure that the products were free from contamination.

5.4.2.2.1 PCR using *nif* genes

A 370 base pair fragment of *nif* H gene, which corresponded to positions 111 and 483 of the *Bradyrhizobium japonicum nif*H gene was amplified with primers shown in Table 5.5 (modified from Zehr et al, 1995). *Bradyrhizobium japonicum* and *Azospirillum brasilense* were used as reference strains.

TABLE 5.5 Oligonucleotide *nif* H primers used for PCR

Primer 1	5-GGAATTCTGTGATCCTAAAGCTGA-3
Primer 2	5-AGCATACATTGCCATCATTTCACC-3

The amplification reaction mix (25µl) is shown in Table 5.6.

TABLE 5.6 Reagents for amplification of *nif*H genes in bacterial cell lysates.

Reagents	final conc	Quantity per reaction	Supplier *
10 X buffer	(1X)	2.5 µl	Promega
MgCl$_2$ 25 mM	(2mM)	2 µl	Promega
d NTP 10 mM	(2mM)	0.5 µl	
Primer 1 20 µM	(0.1µM)	1.25 µl	Gene Works
Primer 2 20 µM	(0.1µM)	1.25 µl	Gene Works
RNAase-free sterile water		16 µl	
Taq Polymerase 5U/µl (0.1U)		0.5 µl	Promega
Cell lysates		1 µl	
Total volume		**25 µl**	

* Suppliers: Promega , Castle Hill NSW; GeneWorks, Adelaide, South Aust.

The initial denaturation step was performed at 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 45°C for 2 min and 72°C for 30 sec. A final extension step was then carried out at 72°C for 3 min. The products were electrophoresed on a 1% agarose gel in 1x TAE buffer and stained with ethidium bromide and visualised under UV light.

Sequences for the *nif*D primers (260 and 261) are shown in Table 5.7.

TABLE 5.7 Oligonucleotide primers used for *nif*D PCR.

Primer 260	5-TC(A/G)TTI(C/T)CIAT(A/G)TG(A/G)TG(A/G/C/T/)CC-3
Primer 261	5-TG GGI CCI (A/G)TI AA(A/G)GA(C/T)ATG-3

The reaction mix and thermal programme for the *nif* D PCR was as described by Rademaker and de Bruijn, (1997) (Table 5.8*). Bradyrhizobium japonicum* and *Azospirillum brasilense* were used as control strains.

TABLE 5.8 Reagents for amplification of *nif*D genes in bacterial cell lysates

Reagents	final conc	Quantity per reaction	Supplier *
5 X Gitschier buffer (1x)		5 µl	(see 3.2.6.3).
BSA 20mg/ml (16mg/ml)		0.2 µl	Promega
DMSO 100% (10%)		2.5 µl	Sigma Aldrich,
d NTP 100mM mixed 1:1:1:1 to give 25 mM each (0.5mM)		0.5 µl	Boehringer Mannheim,
RNAase-free sterile water		12.65 µl	
Primer 260 10 µM (0.4 µM)		1 µl	Gene Works Adelaide
Primer 261 10µM (0.4µM)		1 µl	Gene Works Adelaide
Taq Polymerase 5U/µl (0.08U)		0.4 µl	Promega
Cell lysates		1 µl	
Total volume		**25 µl**	

* Suppliers: Promega, Annandale, NSW Aust.; Sigma Aldrich, Castle Hill, NSW Aust.; GeneWorks, Adelaide, S.Aust.;Boehringer Mannheim, Geneva, Switzerland.

The PCR cycling conditions were as follows: denaturation at 95°C for 7 min with 35 cycles of 94°C for 1 min, 52°C for 1 min and 65°C for 8 min. A final extension was carried out at 65°C for 16 min. The products were electrophoresed on a 1% agarose gel in 6 x loading buffer, stained with ethidium bromide and visualised under UV light.

5.4.2.2.2 InterLINE PCR

A repetitive sequence-based PCR (InterLINE) was carried out to generate species-specific PCR product patterns. The long interspersed DNA elements (LINES) occur in close proximity to each other and one LINE primer is used in the PCR for amplification of products (Smida et al, 1996). The sequence for the GRK primer is shown in Table 5.9. Similarity of bands was compared by eye.

TABLE 5.9 Sequence for the GRK InterLINE oligonucleotide primer used in the

GRK primer	5-GAGTTTGGCAAAGACCC-3

The PCR master mix for the InterLINE PCR was prepared in a final volume of 50 µl with the following reagents as shown in Table 5.10.

TABLE 5.10 Reagents for the repetitive sequence based InterLINE PCR.

Reagents final conc	Quantity per reaction	Supplier *
5 X buffer (Appendix 3) (0.5x)	5 µl	
MgCl₂ 25mM (1.5mM)	3 µl	Promega
d NTP 10 mM (0.2mM)	1 µl	Boehringer Mannheim
RNAase-free sterile water	34.6 µl	
GRK primer 50 pmole (1p/mole)	1 µl	Gene Works
Cell lysates	5 µl	
Taq Polymerase 5U/µl added after a hot start (0.04U)	0.4 µl	Promega
Total volume	50 µl	

* Suppliers: Promega, Annadale, NSW, Aust.; Boehringer Mannheim, Geneva, Switzerland; GeneWorks, Adelaide, S.Aust.

The samples were placed in a thermal cycler and the block temperature was held at 84°C for 10 min then reduced to 80°C while Taq Polymerase 5U/µl (0.4 µl) was added to the PCR mixture. Denaturation of the samples was carried out at 93 °C for 90 sec, which was followed by five cycles of 93°C for 90 sec min, 37°C for 90 sec, 72°C for 2 min. This was then followed by 25 cycles of 93°C for 1 min, 52°C for 1 min and 72°C for 2 min with a final extension at 72°C for 10 min.

5.4.2.3 Preparation and hybridisation of *nif* probes

5.4.2.3.1 Generation of probes

DNA from *Bradyrhizobium japonicum* and *Azospirillum brasilense* was amplified by *nif*D and *nif*H PCR. The amplification products were electrophoresed on 1% agarose gel and the bands corresponding to the *nif*D genes (400bp) and *nif*H genes (370 bp) were excised. The DNA from the excised bands was purified using the QIAquick gel extraction kit protocol (QIAGEN, Santa Clarita, USA) according to the manufacturer's instructions and presence of product size confirmed by gel electrophoresis. The purified DNA was then used as a hybridisation probe. The sequence for the oligonucleotide *nif*D probe (kindly provided by Dr. G.Kirchhof, unpublished) is shown in Table 5.11.

Table 5.11 Sequence of *nif*D oligonucleotide probe

*nif*D	5'-GACATCGTNTTCGGCGGCGA-3"

5.4.2.3.2 Hybridisation of *nif* amplification products

The products from *nif* D and *nif* H PCR (25 µl) were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and then visualised under UV light. The gel was depurinated, denatured and neutralised (section 3.2.8.3.) and the DNA transferred to Hybond N+ membranes using a Southern blot (Sambrook et al, 1989). Hybridisation to *nif* probes was performed as described in section 3.2.8.4. Following washing, the membranes were then air-dried and exposed to a phosphor screen for 24 hours after which time a phosphor-imager was used to visualise the bands.

Plasmid miniprep extractions from selected bacterial isolates were carried out to determine whether *nif* genes detected by PCR were located on the plasmid. In the preparation for extracting plasmid DNA, bacteria were grown in Luria broth (5 ml) at 28°C in a shaking incubator overnight and the plasmids extracted according to the method of Birnboim and Doly (1979). *Agrobacterium radiobacter* K84 was used as a positive control. Plasmid DNA was resuspended in TE buffer with RNase A (20 µg / ml) and stored at -20°C until used. The plasmid DNA (25 µl) with 6 x loading buffer (4 µl) was applied to a 0.8% agarose gel and electrophoresed in 1 x TAE at 4°C for 36 hours. Southern blots and subsequent hybridisation to *nif*D DNA probe and *nif* D oligonucleotide probe were then carried out as described above.

5.4.3 Results

5.4.3.1 *nif*H, *nif*D and InterLINE PCR

A product of 370 bp was consistently amplified from *Bradyrhizobium japonicum,* the positive control following *nif*H PCR. Of the 29 isolates tested, 11 also gave a similar band. Examples are shown in Fig.5.3.

Following amplification of *nif* D, positive control *Bradyrhizobium japonicum* showed a band of 400bp. Of the 86 isolates tested, 21 consistently gave a band of 300bp. Examples are shown in Fig 5.4.

Five groups of three or four isolates were identified with similar banding patterns following InterLINE PCR. (6% Polyacrylamide gels were electrophoresed by Dr. G. Kirchhof, results not shown).

a   b   c   d   e   f   g   h   i   j   k
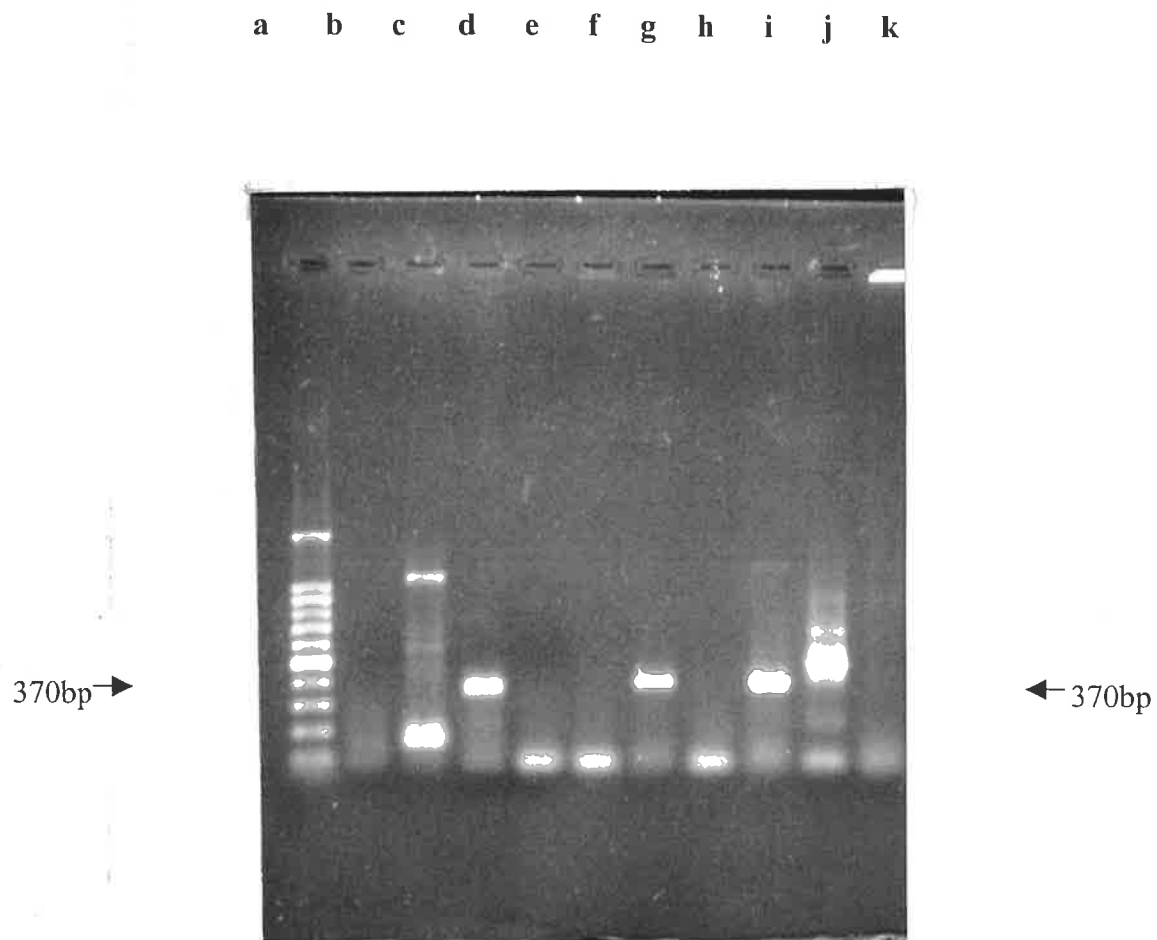
370bp→

←370bp

Fig.5.3. *nif*H **amplification of DNA from bacterial strains isolated from the rhizosphere of wheat grown under N-free conditions in the spermosphere model.** Lane (a) 100bp DNA ladder (Promega),(b) isolate 1 (c) isolate 2 (d) *Bradyrhizobium japonicum* (370bp) (e) isolate 3 (f) isolate 4 (g) KL1.1 (h) isolate 5 (i) P5.2 (j) *Azospirillum brasilense*, (k) isolate 6. KL1.1, P5.2 = 370bp.
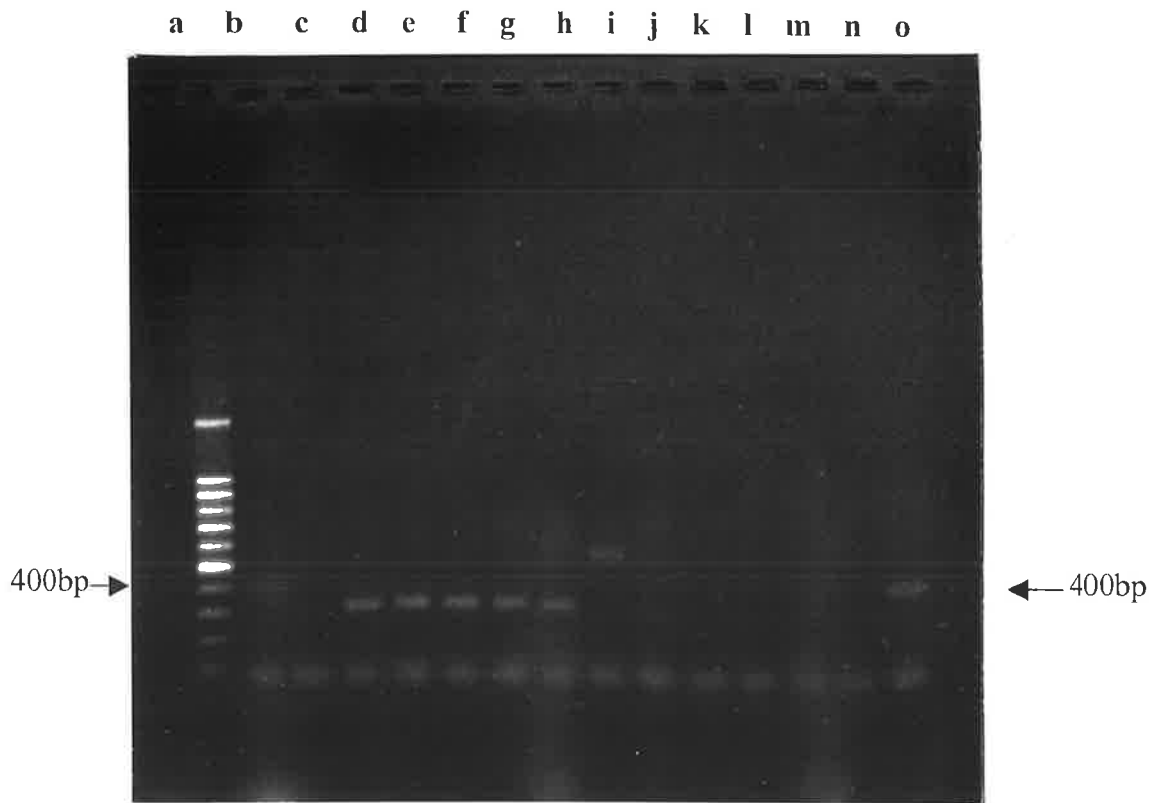
Fig.5.4. *nif*D amplification of DNA from bacteria isolated from the rhizosphere of wheat grown under N-free conditions in the spermosphere model.

Lane (a) 100bp DNA ladder (Promega), (b) *B.japonicum*, (c) H_2O, (d) KL2.3, (e) KL1.1, (f) KL1.3, (g) KL5.4, (h) KL2, (i) KL6.4, (j) KL4, (k) KL5, (l) KL6, (m) KL7, (n) KL8, (o) *Bradyrhizobium japonicum* (400bp), KL2.3, 1.1, 1.3, 5.4,2 = 350bp.

Information obtained from amplification of *nif* genes and from species-specific PCR product band patterns produced by InterLINE PCR, was used to construct a table into which 5 groups of isolates were placed (Table 5.12). Included in the table is information obtained from hybridisation to group-specific probes (5.3.2.1). and identification of isolates following GC-FAME by comparison with the Sherlock TSBA aerobe library version 3.8 (3.2.4).

With one exception Group 1 isolates were selected on Nfb medium (requiring malic acid as a carbon source), all hybridised to GAM42a probe and showed a 300bp band following amplification by *nif*D PCR. Identification by the Sherlock database indicated that three of the four isolates tested were *Pantoea agglomerans*. Two of these isolates also showed bands of 370 bp after amplification by *nif*H PCR. Group 2 isolates were also isolated using malic acid as the carbon source in the medium (Nfb medium), but none was positive using *nif*D PCR. All Group 2 isolates hybridised to BET42a probe. The three isolates from Group 3 all hybridised to GAM42a probe and two of the three isolates produced a 300 bp band after amplification with after *nif*D PCR. Group 4 isolates were all isolated on Nfb medium, two hybridised to GAM42a, and all showed a 300 bp band after *nif*D PCR amplification. Both of these isolates also produced a 400 bp band after *nif*H PCR. Identification of 2 of the 3 isolates by the Sherlock database indicated that they were *Pantoea agglomerans*. Group 5 isolates were all selected on PCAT medium (utilising azelaic acid as a carbon source). Two hybridised to both ALF1b and GAM42a probes and showed no bands after *nif*D PCR. However, one of the three isolates showed a 400 bp band following amplification of DNA with *nif*H primers.

TABLE 5.12 Bacterial isolates grouped by InterLINE PCR, *nif*D and *nif*H PCR, rRNA group-specific probes and identification by Sherlock database after GC-FAME analysis.

	Name of isolate	*nif*D	*nif*H	Hybridisation to rRNA-directed probes	Identification Sherlock database after FAME (Similarity index 0-1)
1	P 5.2	+	+	GAM42a	*Pantoea agglomerans* (0.9)
1	KL 1.1	+	+	GAM42a	*P.agglomerans* (0.9)
1	KL 1.3	+		GAM42a	*P.agglomerans* (0.7)
1	KL 4.2	+		GAM42a	unidentified
2	KL 2.1	-		BET42a	unidentified
2	KL 5.2	-		BET42a	unidentified
2	KL 6.1	-		BET42a	unidentified
2	KL 2.2	-		No hybridisation	*P.agglomerans* (0.6)
3	4CS 1.4	+		ALF1b	*Salmonella typhimurium* (0.88)
3	P 5.1	+		GAM42a	unidentified
3	KS 2.2	-		GAM42a	*Enterobacter taylorae* (0.92)
4	P 3.1	?	+	ALF1b/GAM42a	unidentified
4	P 3.2	-		No hybridisation	unidentified
4	P 4.3	?	+	ALF1B/GAM42a	unidentified
5	KL 2.3	+		GAM42a	*P.agglomerans*
5	KL 3.1	+	+	GAM42a	*P agglomerans* (0.84)
5	KL 4.3	+	+	No hybridisation	*P agglomerans* (0.90)

Similarity of band pattern after InterLINE PCR and grouped 1-5. Banding patterns were compared by eye.

nif D and *nif*H bands are indicated as present or not by (+) and (-) respectively.

Bacterial strains were isolated on P (PCAT), KL (Nfb) and 4CS(4CS) medium. KS was isolated from within the stem of wheat.

5.4.3.2 Hybridisation to *nif* probes

Hybridisation to amplification products from positive control bacteria (*Bradyrhizobium japonicum* and *Azospirillum brasilense*) occurred when either *nif*H or *nif*D probes were used. When the stringency of hybridisation conditions for the *nif*H probe was reduced by lowering the hybridisation temperature from 65°C to 60°C, bands from amplification products from three isolates hybridised to the *nif*H probe. This confirmed that *nif*H genes were present in the isolates tested but a reduction in hybridisation stringency implied a lower homology to the *nif*H genes of the control strains.

When amplification products from the same isolates were hybridised to a *nif*D oligonucleotide probe, there was a weak band from only one isolate and no bands when hybridised to the *nif*D *Azospirillum brasilense* probe.

5.4.4 Conclusion

There was evidence for the presence of the nitrogenase gene within bacteria isolated from Kapunda soils. Hybridisation of amplification products from *nif*H PCR to *nif*H probes confirmed homology to the *nif*H genes present in the controls and although a consistent band was shown for *nif*D amplification products, homology to the controls was poor. This indicates that *nif*D genes had low homology to the control strains. This information was used to reinforce selection of isolates containing the nitrogenase gene for further study.

5.5 Comparison of the GC-FAME Profiles from Inoculated and Uninoculated Wheat

5.5.1 Introduction

Plant macerate suspension (3.2.1.6) or individual bacteria isolated from Kapunda soil were used to inoculate wheat grown in N-free semi-solid medium to compare fatty acid profiles of the bacterial community after 6 weeks of growth in the rhizosphere. Individual bacteria isolated from the rhizosphere of wheat grown for 6 weeks in semi-solid medium in the spermosphere model system. The experiment aimed to investigate the identity of fatty acids comprising the highest percentage of the GC-FAME profile of the bacterial community.

5.5.2 Materials and Methods

5.5.2.1 Comparison of bacterial fatty acid profiles from the plant rhizosphere inoculated with either individual isolates or mixed rhizobacteria from Kapunda soil

Wheat was inoculated with each of 8 isolates and grown for 6 weeks in N-free semi-solid medium in the spermosphere model system. Mixed rhizobacteria from Kapunda soil (3.2.1.7) were also used to inoculate wheat and grown as above. The fatty acid profiles were compared. Selection of the isolates was based on positive results from *nif* D and *nif*H PCR, hybridisation to group-specific probes and identification on GC-FAME (Table 5.13). Two of the bacterial strains (4KR1.2 and 4KS6.1) had been previously isolated from the wheat root and stem respectively. A heat-killed control was included in the experiment in addition to reference strains *Azospirillum brasilense* and *Burkholderia cepacia*.

TABLE 5.13 Bacteria selected for GC-FAME to compare individual fatty acid profiles with the community profile after 6 weeks growth in the wheat rhizosphere.

Isolate	Identification on GC-FAME	*nif* D	*nif* H	Hybridisation to probe
KL 1.1	*Pantoea agglomerans*	+	+	GAM42a
KL 2.1	*unidentified*	+		BET42a
KL 4.4	*P.agglomerans*	+		GAM42a
KL 5.4	*P.agglomerans*	+	+	-
KL 6.4	*Enterobacter taylorae*	-	+	ALF1b
P 5.2	unidentified	+	+	GAM42a
4KR 1.2	*E.taylorae*	+	-	-
4KS 6.1	*E.taylorae*	+	?	GAM42a
Azospirillum brasilense	*Azospirillum brasilense*	+	+	ALF1b
Burkholderia cepacia	*B.cepacia*			
Kapunda				
Kapunda control				
Uninoculated				

GC-FAME identification of 8 isolates from the bacterial community from Kapunda. Selection of bacteria was based on positive *nif*D and *nif*H PCR and hybridisation to a variety of group specific probes.

Bacterial isolates were grown overnight as broth cultures (5 ml) in TSB on a shaking incubator at 28° C. The cultures were adjusted to 10^8 cfu/ml, washed and resuspended in

phosphate buffer (pH 7.00). Surface-sterilised seeds were immersed for 2 hours in the bacterial suspension and uninoculated control seeds were placed in phosphate buffer. Autoclavable pots (7.5cm x 6.6cm) containing C- and N-free semi-solid agar (4CS, 160 ml) with 1.75 g agar/l were used to grow the wheat seedlings as described in section 3.2.4.2. Four seedlings were planted per pot and there were 3 replicate pots for each of 13 treatments. Plants were grown under the same conditions as described previously (5.2.2.1).

5.5.2.2 Harvest of inoculated plants in preparation for GC-FAME analysis

Fatty acid profiles from the eight isolates selected as inoculants and mixed rhizobacteria were generated by GC-FAME analysis. After 6 weeks of growth the roots from the four plants in each pot were pooled and placed in sterile centrifuge tubes (50 ml) containing PBS (10 ml). The tubes were placed on an orbital shaker for 20 min, the roots removed and the tubes centrifuged at 10,000 g for 10 min. The resulting pellet was then transferred to glass Pyrex tubes for GC-FAME analysis (3.2.4.1.) Fatty acid profiles were compared. The fatty acids from uninoculated plants were subtracted from the profiles of inoculated plants to eliminate all plant related fatty acids from the analysis.

5.5.2.3 Investigation into the identity of dominant fatty acids in the FAME profile from the bacterial community from the Kapunda soil

Consultation of the GC-FAME Gram negative database (Sherlock) showed that the bacterium *Stenotrophomonas maltophilia* could be partly identified by the presence of fatty acid 18:1w9c. *Bradyrhizobium japonicum* could be partly identified by fatty acids 19:0cyclow8c and Sum features 3 (16:1 w7c / 15iso2OH, 15iso2OH / 16:1w7c), all of which corresponded with fatty acids present in the profile of the mixed rhizobacterial community from the Kapunda soil. Yeast mannitol medium (YM) was used to isolate additional bacteria from the wheat rhizosphere of plants inoculated with bacterial community from the Kapunda soil harvested in section 5.5.2.1. Twenty isolates were prepared for GC-FAME analysis as described in section 3.2.4.1.

5.5.3 Results

5.5.3.1 GC-FAME analysis of bacteria before and after inoculation of wheat

Fatty acid profiles from bacteria recovered from the rhizosphere of wheat 6 weeks after inoculation indicated that 60-80 % of the fatty acids recorded at the time of inoculation were retained. An example of data for 4 isolates is shown in Table 5.14.

Table 5.14 Fatty acids in the GC-FAME profile from bacteria before and after recovery from the rhizosphere

Fatty acid	Percentage contribution of fatty acids to total profile							
	4KR1.2 before	4KR1.2 after	KL4.4 before	KL4.4 after	P5.2 before	P5.2 after	4KS6.1 before	4KS6.1 after
9:0	.09					4.2		
10:03OH		1.2	2.49	.84	2.8	1.23		.84
12:0	3.57	2.01	1.58	2.3	1.69	2.3	3.5	1.76
12:02OH			4.31	.97	4.58			.76
12:03OH		1.62	3.75	1.38	3.92	1.21		.93
13:0	.26						.2	
14:0	6.19	2.43	.56	2.57	.49	2.86	6.17	2.4
Un 14.5	.77						.75	
15:0iso		2.3		2.7		2.75		1.8
15:0anteiso		3.49		3.4		3.55		5.2
15:0	1.74						1.32	
16:0	27	12.43	31.44	18.2	30.86	14.9	27.07	15.088
16:1w5c	.19	1.05					.19	
17:0	1.05						.81	
17:0cyclo	11.42	6.004	5.07	4.7	4.2	5.8	9.93	6.14
18:0	.25	.93	1.29	1.83	1.22	1.45	.25	1.19
18:1w7c	21.1	10.5	16.52	13.8	16.81	13.7	22.4	5
19:0cyclo8c	.45	6.5	.57	5.16	.48	5.59	.33	4.07
sum2	7.59	2.45	.2	2.77		2.88	7.64	2.47
sum3	17.73	8.4	31.82	7.85	32.29	9.14	19.01	7.73
sum4	.41	30.04	.43	17.75	.65	23.77	.39	23.46
sum5		5.2		4.75		4.2		3.75
Total fatty acids	17	16	13	16	13	16	15	16

Isolates 4KR1.2, KL4.4, P5.2 and 4KS6.1 before and after recovery from rhizosphere. Fatty acids retained after recovery from rhizosphere are highlighted in bold.
Sum2 (12:0alde? unknown 10.928, 16:1 isoI / 14:03OH or 14:03OH / 16:1 isoI)
Sum3 (16:1 w7c / 15iso2OH, 15iso2OH / 16:1w7c); Sum4 (17:1isoI / anteiB or 17:1anteiso B /iI); Sum5 (18:2w6,9c / 18:0ante or 18:0ante / 18:2w6,9c) Replicates=3.

Fatty acid 18.1w9c represented 47.4% of the total fatty acids from the bacterial community profile (Table 5.15). The fatty acid profile from the inoculated plants showed greater numbers of fatty acids than the controls.

TABLE 5.15 GC-FAME analysis of fatty acids from the wheat rhizosphere, 6 weeks after inoculation with bacteria from the Kapunda bacterial community (Kapunda), the heat-killed Kapunda control (Kapunda control) and uninoculated plants.

Fatty acid	% fatty acids Kapunda	% fatty acids Kapunda control	% fatty acids Uninoculated
9:0	0.49 ± 0.16	3.9 ± 0.62	
10:03OH	0.22 ± 0.01	0.78 ± 0.08	1.05 ± 0.04
12:0	0.42 ± 0.08	4.06 ± 0.45	1.96 ± 0.08
12:02OH		1.9 ± 0.41	1.47 ± 0.10
12:03OH		2.69 ± 0.82	1.375 ± 0.104
14:0	0.56 ± 0.06	1.87 ± 0.19	3.04 ± 0.17
14:0iso	0.2 ± 0.03		
15:0iso	0.39 ± 0.04		2.47 ± 0.4
15:0anteiso	0.5 ± 0.05	4.67 ± 0.38	2.86 ± 0.41
16:0	**17.4 ± 0.32**	**18.72 ± 1.29**	**17.6 ± 1.79**
16:0anteiso	0.63 ± 0.19		
16:1w9c	1.54 ± 0.34		
17:0anteiso	0.16 ± 0.017		
17:0cyclo	0.82 ± 0.14	6.67 ± 0.58	8.15 ± 0.9
18:0	2.25 ± 0.15	1.9 ± 0.244	2.28 ± 0.4
18:0iso	0.4 ± 0.08		
18:1w7c	4.1 ± 0.5	10.75 ± 1.5	7.5 ± 1.5
11Me18:1w7	0.43 ± 0.05		
18:1w9c	**47.4 ± 2.19**		
19:0cyclo8c	0.65 ± 0.12		2.8 ± 0.9
20:1w9c	0.53 ± 0.08		
20:46,9,12,15c	.29 ± .05		
sum2	0.2 ± .04	1.35 ± 0.26	2.05 ± 0.11
sum3	3.28 ± .19	10.58 ± 1.2	8.8 ± 1.2
sum4	3.28 ± 0.19	23 ± 2.7	26.08 ± 1.3
sum5	**13.7 ± 1.4**	4.9 ± 0.9	7.3 ± 1.2
Total fatty acids	24	15	16

Values in bold print record a high percentage of individual fatty acids from the profile.
Sum2 (12:0alde? unknown 10.928, 16:1 isoI / 14:03OH or 14:03OH / 16:1 isoI)
Sum3 (16:1 w7c / 15iso2OH, 15iso2OH / 16:1w7c)
Sum4 (17:1isoI / anteiB or 17:1anteiso B /iI)
Sum5 (18:2w6,9c / 18:0ante or 18:0ante / 18:2w6,9c). Means and standard errors of means of 3 replicates.

Fatty acids from the rhizosphere of uninoculated plants were omitted from the profiles of all inoculated plants to identify fatty acids specifically associated with the bacterial populations and exclude plant fatty acids. Nine fatty acids were identified as only

occurring in the Kapunda community fatty acid profile (Table 5.16). The dominant fatty acid 18:1w9c was not present in the uninoculated rhizosphere. The reference strains *Burkholderia cepacia* and *Azospirillum brasilense* and isolates P 5.2, KL 5.4 also contained fatty acids not present in the rhizosphere of uninoculated plants.

TABLE 5.16 Fatty acids from the rhizosphere of inoculated plants (Kapunda) retained after omitting the fatty acids from the rhizosphere of uninoculated plants from the Kapunda fatty acid profile.

	Percentage of fatty acids				
Fatty acid	**Kapunda**	***B.cepacia***	***A.brasilense***	**P 5.2**	**KL 6.4**
9:0	0.49 ± 0.16		3.8 ± 0.28	4.21 ± 1.6	
10:0		0.63 ± 0.15	0.81 ± 0.13		
11:0iso					1.01 ± 0.39
14:0iso	0.2 ± 0.03				
15:0		1.00 ± 0.2			0.85 ± 0.01
16:0anteiso	0.63 ± 0.19				
16:1w9c	1.54 ± 0.34				
16:03OH					
16:0Nalcohol		0.79 ± 0.1	0.93 ± 0.15		
17:0anteiso	0.16 ± 0.01				
18:0iso	0.4 ± 0.08				
11Me 18:1w7c	0.43 ± 0.05				
18:1w9c	**47.4 ± 2.19**				
20:1w9c	0.53 ± 0.08				
20:4w6,9,12,15c	0.29 ± 0.05				
Number of fatty acids retained after omitting fatty acids similar to those in uninoculated rhizosphere	**10**	**3**	**3**	**3**	**2**

Fatty acid 18:1 w9c with highest percentage of the total profile is in bold print. Means and standard errors of means of 3 replicates.

5.5.3.2 GC-FAME analysis of *Stenotrophomonas maltophilia* isolated from the Kapunda microbial community

Sixty percent of the bacteria isolated on YM medium from the bacterial community from the Kapunda soil source were identified, through GC-FAME analysis, as likely to be *Stenotrophomonas maltophilia*. Two fatty acids 16:1w9c and 18:1w9c, which contributed to the bacterial community fatty acid profile from the Kapunda soil were identified only in the fatty acid profile of *Stenotrophomonas maltophilia* (see Table 5.17).

TABLE 5.17 Fatty acid content from GC-FAME analysis of rhizosphere bacteria from the Kapunda microbial community and *Stenotrophomonas maltophilia*.

FATTY ACID	PERCENTAGE OF FATTY ACID IN GC-FAME ANALYSIS	
	Kapunda	*Stenotrophomonas maltophilia*
9:0	0.49 ± 0.16	
10:0		0.72 ± 0.05
10:03OH	0.22 ± 0.01	
11:0iso		2.5 ± 0.03
11:0iso3OH		1.08 ± 0.01
Unknown 11.79		1.17 ± .01
12:0	0.42 ± 0.08	
12:0iso3OH		1.0 ± .37
12:03OH		2.54 ± 0.27
13:0iso		.511 ± 0
13:0 2OH		.71 ± 0.008
13:0iso 3OH		1.74 ± 0.02
14:0	0.56 ± 0.06	5.3 ± 0.05
14:0iso	0.2 ± 0.03	1.87 ± 0.06
15:0iso	**0.39 ± 0.04**	**26.5 ± 0.3**
15:0anteiso	0.5 ± 0.05	16.5 ± 3
15:0		1.3 ± 0.01
16:0	**17.4 ± 0.32**	**8.19 ± 0.02**
16:0iso		3.32 ± 1.4
16:0anteiso	0.63 ± 0.19	
16:1w9c	**1.54 ± 0 .34**	**3.8 ± 0.07**
17:0iso		1.7 ± 0.04
Iso17:1w9c		2.48 ± 0.04
17:1w7c		0.41 ± 0.01
17:0anteiso	0.16 ± 0.017	
17:0cyclo	0.82 ± 0.14	
18:0	2.25 ± 0 .15	
18:0iso	0.4 ± 0.08	
18:1w7c	**4.1 ± 0 .5**	**1.05 ± 0.17**
11Me18:1w7	0.43 ± 0.05	
18:1w9c	**47.4 ± 2.19**	**1.21 ± 0.08**
19:0cyclo8c	0.65 ± 0.12	
20:1w9c	0.53 ± 0 .08	
20:46,9,12,15c	.29 ± .05	
sum2.	0.2 ± .04	
sum3	**3.28 ± .19**	**14.06 ± 0.32**
sum4	**3.28 ± 0.19**	**0.85 ± 0.06**
sum5	**13.7 ± 1.4**	

Fatty acids 14:0iso, 16:1w9c and 18:1w9c (highlighted) were common to *Stenotrophomonas maltophilia* and the Kapunda rhizosphere community after omission of fatty acids present in the rhizosphere of uninoculated plants
Sum2 (12:0alde? unknown 10.928, 16:1 isoI / 14:03OH or 14:03OH / 16:1 isoI),
Sum3 (16:1 w7c / 15iso2OH, 15iso2OH / 16:1w7c), Sum4 (17:1isoI / anteiB or 17:1anteiso B /iI), Sum5 (18:2w6,9c / 18:0ante or 18:0ante / 18:2w6,9c) n=3.

5.5.4 Conclusion

The majority of fatty acids contributing to the fatty acid profile of isolates used to inoculate wheat were recovered from the rhizosphere were similar before and after inoculation. As expected however there were variations in the percentage of the fatty acids recovered. Two of the isolates tested had fatty acids in common with the community of bacteria, but these represented only a small percentage of the whole profile. *Stenotrophomonas maltophilia* also isolated from the community of bacteria, but unlike other isolates tested, had similar fatty acids in common with the bacterial community. These were 14:0iso, 16:1w9c and 18:1w9c.

5.6 Discussion

GC-FAME analysis provided the basis for comparing differences between the microbial communities from Kapunda, Avon and Waite soils. The bacteria were washed from the wheat rhizosphere after growth in C-and N-free semi-solid medium for 6 weeks and used directly for GC-FAME analysis without the disadvantage of further culture and potential loss of unculturable bacteria (Cavigelli et al, 1995; Richie et al, 2000). The fatty acid profile from the bacterial community from Kapunda soil showed a greater diversity of fatty acids within the profile, compared with the communities from the Avon and Waite soils. Comparison between the fatty acid profiles of the bacterial community from Kapunda soil, in either porous or semi-solid media, enabled the detection of similar fatty acids within the profiles from both media but a greater diversity of fatty acids was present in the semi-solid medium, albeit in trace amounts. Therefore it was assumed that similar species were present in both media but with a greater opportunity for the proliferation of additional species provided in the semi-solid medium. Statistical analyses carried out in other GC-FAME studies have included trace amounts of fatty acids (<0.3%) in the analysis of the total profile from sediment and humus samples (Rajendran et al, 1992; Bååth et al, 1994). However, only fatty acids that contributed to greater than 1% of the total in at least one of three replicates was included in another analysis (Lawler et al, 2000). The nutrient supplements for sand, porous and semi-solid media were similar to those used previously in Chapter 4 and it was anticipated that growth in the semi-solid medium would encourage the presence of the same species grown in larger growth pots as grown in the smaller tubes used

previously. Differences between the physical structures of the different media may have contributed to small changes noted in fatty acid content between porous and semi-solid media particularly from bacterial communities from the Waite soil, although the major fatty acids in the profile were retained. The study was designed to address differences between bacterial communities from the 3 soil sources to account for the positive growth-promotion in wheat inoculated with bacteria from the Kapunda soil (Chapter 4). GC-FAME analysis showed that the range of bacterial species from the Kapunda soil was likely to be greater than from within the bacterial communities from Avon or Waite soils.

Although the levels of some of these fatty acids were present in trace amounts, they are known to occur in Gram negative bacteria (GC-FAME Sherlock Aerobe Library vs 3.8). As part of the broader investigation to identify bacteria within the Kapunda soil profile contributing to plant growth, it was therefore considered worth including in the study. Plant growth-promoting bacteria such as *Stentrophomonas maltophilia* are known to contain fatty acids 15:0 ante-iso, 15:00 and 15:iso (in trace amounts) in addition to 16:1w9c, 16:0 and 18:1w9c present as 3.1, 20.6 and 47% of the profile respectively (GC-FAME Sherlock Aerobe Library vs 3.8. see 3.2.4). Free-living N_2-fixing bacteria *Enterobacter taylorae* and *Pantoea agglomerans* contain fatty acids 12:0 and 17:0cyclo, *Pseudomonas* sp. 12:0, 10:03OH and 17:0cyclo and *Rahnella aquatilis* 17:0cyclo all of which correspond to the fatty acids detected in the GC-FAME profile from rhizosphere bacteria derived from the Kapunda soil. It is also known *Corynebacterium* sp. used as a biodegradation agent (Rahman et al, 2002) contains 18:1w9c (Haack et al, 1994). N_2-fixing bacteria such as *Klebsiella* sp. contain the major fatty acids found in Sum feature 3, with 15:00 also present in trace amounts. The major fatty acids 16:0, 18:1w9c and Sum feature 3 are common to many bacteria for example *Arthrobacter* spp., *Azotobacter* spp., *Xanthomonas* spp., *Paenibacillus* spp., *Pseudomonas* spp., and *Azospirillum brasilense* (Sherlock Aerobe Library). Sum feature 3 is present in *Klebsiella* spp., *Burkholderia cepacia, Enterobacter* spp., *Rahnella aquatalus, Salmonella* spp. and *Azospirillum brasilense*. It was therefore likely that the bacterial community from Kapunda, grown in N-free conditions in the wheat rhizosphere, contained a more diverse range of bacteria compared with the two other soil sources. The assumption was made that growth in N-free medium would advantage N_2-fixing bacteria in the rhizosphere and encourage their growth.

The study also highlighted the presence of several dominant fatty acids, providing a focus for investigating the identity of bacteria likely to play a major role within the community from the Kapunda soil source. Following inoculation of wheat by bacteria isolated from the bacterial community of Kapunda soil, GC-FAME analysis was carried out both prior to and following the recovery from the rhizosphere after 6 weeks of growth, and results indicated that the majority of dominant fatty acids were retained by individual isolates. The proportions of fatty acids within the profile were frequently altered but the observation supported other work indicating that environmental conditions, temperature and conditions for growth may often alter the proportions of dominant fatty acids but that the fatty acids were generally retained and identifiable (Haack et al, 1994). Differences observed in the fatty acid profile from individual isolates before and after growth in the wheat rhizosphere show some differences in fatty acids after harvest. The fatty acids detected by GC-FAME after growth in the rhizosphere could be a contribution by seed-borne bacteria and further studies would be needed for clarification.

The subtraction of fatty acids, which were present in the rhizosphere of uninoculated plants from the fatty acid profile of inoculated plants, highlighted the presence of fatty acids of microbial origin. The dominant fatty acids from the inoculated Kapunda rhizosphere, 18:1w9c and 16:1w9c, were not present in the rhizosphere of uninoculated plants or plants inoculated with individual strains (isolated from the potentially N_2-fixing bacterial community). However, subsequent isolation of *Stenotrophomonas maltophilia* from the rhizosphere bacteria from Kapunda and GC-FAME analysis provided evidence for the likely contribution of fatty acids 14:0iso, 18:1w9c and 16:1w9c by *Stenotrophomonas maltophilia* to the community FAME profile. The bacterium was sub-cultured successfully at a later date on N-free medium (data not shown) indicating that *Stenotrophomonas maltophilia* grows in N-free conditions in the rhizosphere. Recent studies have cited *Stenotrophomonas maltophilia* strain SB-K88 as a biocontrol agent which produces antifungal xanthobaccins one of which (A) suppressed damping-off disease (Nakayama et al, 1999) and mutagenesis of strain W81 improved biocontrol of *Pythium* spp. (Dunne et al, 2000). *Stenotrophomonas maltophilia* strain C3 prevented the growth of *Rhizoctonia solani* Kuhn on grass (Giesler and Yuen, 1998). The bacterium also has potential plant growth-promoting properties (Koch et al, 1998). The interest in the bacterium as biocontrol agent and plant

growth promoter supported the decision to test it as a potential inoculant in the current work.

The combination of GC-FAME and molecular techniques provided information for the compilation of a set of data of isolates classified within subgroups of the Proteobacteria, and identification of isolates carrying the nitrogenase (*nif*) gene, thus reducing the number of isolates targeted for further study. Indeed the recovery of rhizobacteria, with subsequent culture on N-free selective medium, indicated that N_2-fixing bacteria had proliferated in the wheat rhizosphere after 6 weeks of growth in N-free conditions. Screening of isolates using amplification of both *nif*D and *nif*H PCR products with subsequent comparison of data, supported evidence for the presence of the nitrogenase gene within isolates. There was, however, a consistently different band size between the amplification of DNA from the *nif*D PCR products of reference strain *Azospirillum brasilense* and the DNA of isolates tested. The poor results obtained from hybridisation of isolates to the *nif*D probes may indicate a poorly conserved *nif*D region within the isolates under investigation and may also account for the difference in band size of *nif*D amplification products. Sequencing of PCR products from several isolates following *nif*D PCR was initiated but not completed due to time constraints and would no doubt clarify poor results.

Subsequent to this study, approaches in addition to or instead of GC-FAME analysis have been published to improve the identification of microbial communities. As GC-FAME analysis does not provide positive identification of microorganisms within the soil community, additional complementary methods can be used. For example GC-FAME or phospholipid fatty acid analysis (PLFA) has been used in association with specific polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to examine changes in microbial structure over time (Thirup et al, 2003; Kozdrój and van Elsas, 2001). The effectiveness of PCR-DGGE however depends on the design of primers based on sequences of already identified bacteria and DNA from unknown bacteria may not be amplified due to differences in sequences (Marschner et al, 2001). New methods for culturing previously unculturable bacteria (Sait et al, 2002; Janssen et al, 2002), will contribute to the genetic database of known organisms. GC-FAME length heterogeneity PCR (LH-PCR or terminal restriction fragment polymorphism (T-RFLP) to provide information on nucleic acid diversity of soil

bacteria (Richie et al, 2000) uses fluorescently-labelled primers for amplification of DNA derived from microorganisms (Dierkson et al, 2002). Comparative 16s rRNA sequence analysis and fluorescent *in situ* hybridisation (FISH) has also been used successfully to study microbial diversity in environmental samples (Amman et al, 1995; Amman et al, 1996) The success of these approaches again depends on the matching of sequences with those published within databases and the use of multiple group-specific probes (Daims et al, 1999).

In Chapter 6, selection of potential inoculants for screening wheat growth-promotion was based on information derived from characterisation studies of Chapter 5. The inclusion of *Strenotrophomonas maltophilia* for further testing was also based on assessment of GC-FAME profiles.

Chapter 6: Co-Inoculation of Wheat to Improve Early Growth

6.1 Introduction

The experiments described in this chapter were carried out to assess the potential for improving the growth of wheat using bacterial inoculants isolated and selected from the Kapunda soil source. This chapter is divided into two sections. The first section contains the selection and screening of isolates to exclude antagonistic responses between them. The second section describes experiments designed to assess plant dry weights and concentration of total plant N in co-inoculated wheat, grown in N-sufficient and N-limited plants.

The GC-FAME and molecular studies described in Chapter 5 was considered to provide sufficient information to support the selection of isolates from Kapunda for these further studies. Criteria for selecting such isolates included their ability to grow on N-free medium, a presence of the nitrogenase gene and a range of genera represented within the alpha, beta and gamma sub-groups of the Proteobacteria.

Stenotrophomonas maltophilia, a possible plant growth-promoting bacterium (Koch et al, 1998) present in the Kapunda soil source was selected as a potential inoculant through comparative fatty acid studies. The isolates were identified by GC-FAME analysis.

Hybridisation studies, using rRNA probes, classified isolates within sub-groups of the Proteobacteria and subsequent DNA fingerprinting reduced the chance of the duplicating isolates selected for further study.

The combination of more than one microorganism as co-inoculants can result in a synergistic effect. *Azospirillum lipoferum* and *Phialophora radicola*, an avirulent fungus found in grasses have been used together to inoculate wheat and *Azospirillum*

brasilense and *Glomus* sp. strain have been also used as co-inoculants for wheat with significantly improved growth of plants (Flouri et al, 1995; Gori and Favilli, 1995). As discussed previously (2.6.3), a mix of bacteria as inoculants can stimulate significant plant growth promotion. In the first section of this chapter, preliminary testing was carried out using single isolates, later a mix of two. Three bacterial isolates were finally selected as co-inoculants on wheat.

The second section of this chapter returns to the issue of N-fixation. Measurement of the N in bacteria recovered from the roots of inoculated plants was carried out to determine whether the N bacterial biomass could affect the estimation of plant N if bacteria were retained on the plant roots despite vigorous washing.

6.1.1 Aims

The aims of the work can be categorised as follows as follows:

Screening and selection of isolates

- to screen isolates for possible antagonism to each other (6.2.1.1),
- to select potential isolates as co-inoculants for wheat (6.2.1.2) and
- to determine whether selected bacterial isolates persisted in the wheat rhizosphere following inoculation (6.2.1.3).

Co-inoculation of wheat to enhance growth

- to compare dry weights and total plant N following co-inoculation of wheat with 3 bacteria with or without added ammonium nitrate or with increasing concentrations of ammonium nitrate (6.3.1.1, 6.3.1.2 and 6.3.1.3),
- to verify that the N content of the bacterial biomass that remain on the root do not contribute significantly to total plant N following inoculation of wheat (6.3.1.4).

6.2 Section 1: Screening and Selection of Bacterial Isolates as Potential Co-Inoculants for Wheat

6.2.1 Materials and methods

6.2.1.1 Antagonism studies

In order to select co-inoculants, by their ability to not exhibit antagonism to each other, 12 bacterial isolates were studied. Purified isolates (KL1.1, KL2.1, KL2.4, KL3.1, KL4.2, KL4.4, KL5.4, KL6.4, KS2.1, P5.2, 4KS6.1, 4KR1.2) were separately cultured overnight in DYGS liquid broth (5 mls) (Appendix 4) in a shaking incubator (28°C). Aliquots (10 µl) of each these bacterial cultures to be tested for antagonistic effects were applied to the centre of a Petri dish of modified Stonier's agar (Appendix 4) and incubated for 2-3 days. The bacteria were then killed by chloroform. Each bacterium to be tested was grown overnight in DYGS broth as described above and added (0.5 ml) to the overlay medium (5 ml) (3.2.5.13). The overlay medium was poured over the Stonier's agar (i.e. killed bacteria) and the diameters of zones of inhibition in the overlay culture were measured after 3 days of incubation at 28°C. One Petri dish was prepared for testing each bacterium and the experiment was repeated 3 times. Data was pooled and expressed as the mean of the measured zones of inhibition.

6.2.1.2 Selection of isolates as potential inoculants

To make a final selection of potential inoculants, purified isolates selected from preliminary studies (results not shown) were first identified by GC-FAME or Biolog® (Biolog® identification by Dr. G. Kirchhof). Also included, as controls were *Azospirillum lipoferum* 596 and *Rhizobium meliloti*. Bacteria were grown overnight in TSB (5 ml, Appendix 4) in a shaking incubator at 28°C (Table 6.1).

TABLE 6.1 Identification of isolates by GC-FAME or Biolog®

Isolate	Identification by FAME (F) or Biolog® (B)	FAME Similarity Index 0-1
KL1.3	*Pantoea agglomerans* (F)	0.709
KL2.2	*Pantoea agglomerans* (F)	0.606
KL3.1	*Pantoea agglomerans* (F)	0.849
KL5.4	*Pantoea agglomerans* (F)	0.823
KS2.1	*Enterobacter taylorae* (F)	0.915
P 5.2	*Pantoea agglomerans* (F)	0.905
KL2.4	*Stenotrophomonas maltophilia* (B)	

GC-FAME (F) identification, Biolog® (B, Dr. G. Kirchhof)
KL and KS isolates were selected on Nfb medium and the P isolate
was selected on PCAT medium.

The cultures were washed twice in PBS and the concentration of cells adjusted to 10^7 cfu/ml in phosphate buffer (0.05 M, pH 7.0). Isolates KL5.4 and KL2.4 were combined as a dual inoculant as were the two reference strains *Azospirillum lipoferum* 596 and *Rhizobium meliloti*. Pre-germinated wheat seeds were surface sterilised, immersed in the bacterial suspensions for 2 hours and placed in Petri dishes on sterile washed river sand with sterile water (5 ml). There were 3 replicates (5 seeds per replicate) for each of 9 treatments, including an uninoculated control. The Petri dishes were angled at 45° and the lower two-thirds of the dish covered with aluminium foil to encourage roots to grow down into the river sand. Dishes were randomised and plants grown for 7 days in a controlled growth environment at 15°C with a 12-hour photoperiod, relative humidity 60-70% and an irradiance 240 µE/m/s. Root lengths were then measured with a ruler.

6.2.2 Results

6.2.2.1 Antagonism studies

Isolate KL2.1 (unidentified) was inhibited by isolates KL3.1, KL4.4, KL5.4, P5.2, (all identified as *Pantoea agglomerans*). The data for each isolate tested was pooled and expressed as a mean size zone of inhibitions (Table 6.2). No other inhibitory effects were found (results not shown).

TABLE 6.2 Diameter of zones of inhibition of isolate KL2.1 tested against 11 bacterial isolates isolated from Kapunda soil

Isolate	Identification	Inhibition of KL2.1 Size of zone (mm)
KL1.1	*P.agglomerans*	0
KL2.4	*S. maltophilia*	0
KL3.1	*P. agglomerans*	29 ± 0.6
KL4.2	unidentified	0
KL4.4	*P.agglomerans*	33 ± 0.3
KL5.4	*P.agglomerans*	21 ± 0.6
KL6.4	*E.taylorae*	0
KS2.1	*E.taylorae*	0
P5.2	*P.agglomerans*	29 ± 0.6
4KS6.1	*E. taylorae*	0
4KR1.2	*E.taylorae*	0

Isolates tested for antagonistic responses to each other included *Pantoea agglomerans*, *Stenotrophomonas maltophilia*, *Enterobacter taylorae* and an unidentified strain.
Means and standard errors of means of the size of zone of inhibition from each of the 3 experiments.

6.2.2.2 Selection of isolates as potential inoculants

There were no significant differences were between the root length of wheat grown in association with different test isolates or uninoculated. However wheat roots inoculated with dual inoculants KL54/KL2.4 and with isolate KS2.1 were the longest of those tested (Table 6.3).

TABLE 6.3 Mean root length per plant of wheat grown in association with bacterial isolates for 7 days

Isolate	Mean root length (cm)	se
A. lip	50.1	1.65
uninoculated	48.0	1.82
KM	44.9	1.98
KL5.4/KL2.4	52.8	2.38
KL3.1	50.0	2.30
KS2.1	52.2	2.89
A.lip /R.mel	50.8	2.53
P5.2	44.1	2.77
KL2.2	47.7	2.22

A.lip=*Azospirillum lipoferum*, R.mel= *Rhizobium meliloti*.
Means and standard errors of means of 3 replicates.

6.2.3 Summary

Three isolates (KL5.4, KS 2.1 and KL2.4) were selected as potential inoculants for further testing on wheat for the following reasons. (a) Inoculation with these isolates was associated with increased length of wheat roots in a Petri dish bioassay. Isolate KL5.4 (*Pantoea agglomerans*) was *nif* D and *nif*H positive (Table 5.14) and was antagonistic to another isolate. (b) Isolate KS2.1 (*Enterobacter taylorae*), isolated from within the stem of the plant. (c) The third isolate KL2.4 (identified on Biolog as *Stenotrophomonas maltophilia)*, grew well in association with KL5.4. The selection of *Stenotrophomonas maltophilia* as a potential inoculant for wheat is supported by evidence from the GC-FAME profile of Kapunda soil, suggesting the presence of this bacterium (Chpt. 5, section 5.8).

6.3 Section 2: Co-Inoculation of Wheat to Enhance Growth

6.3.1 Materials and methods

6.3.1.1 Effect of ammonium nitrate on wheat growth

A preliminary experiment was conducted (prior to co-inoculation studies) to determine whether the difference in dry weights of wheat grown with or without added ammonium nitrate could be measured after 6 weeks of growth. Wet sand (pH 6.4) was autoclaved and dried. Nutrients were added to the dry sand and mixed well (3.2.5). Two treatments were set up one with and one without added NH_4NO_3 (350 mg/kg sand). PVC cylindrical pots (26.6 cm x 6.2 cm), lined with plastic bags, and containing sand (1 kg) were planted with 2 surface-sterilised wheat seeds. There were 10 replicate pots for each treatment.

The water content of the pots was maintained at 12%. Polyalkathane beads were placed on the surface of the sand and the treatments were randomised and grown at 15°C in a controlled environment cabinet as previously described (6.2.1.3.). After 6 weeks of growth, the plants were washed to remove sand from the roots, dried at 60°C for 48 hours. Dry weights of roots and shoots were recorded. Statistical analyses were carried out using Analysis of variance (ANOVA), Statistix vs. 7 and Tukey's honestly significant difference (HSD).

6.3.1.2 Effect of bacterial inoculation on wheat grown in the presence of added ammonium nitrate

The effect of bacterial inoculation on wheat grown with added ammonium nitrate was studied. Wheat was grown in sand with added nutrients as described (6.3.1.1). Two plants per pot were grown with or without added NH_4NO_3 (350 mg/kg). They were either inoculated with a mixture of bacterial isolates (KL5.4, KS2.1, KL2.4), or inoculated with the reference *Azospirillum lipoferum* 596, or with mixed rhizobacteria bacteria from Kapunda soil source as described (3.2.1.7), or uninoculated (ie 8 treatments). There were 5 replicate pots per treatment. The bacteria were cultured overnight in N-free 4CS broth, washed in phosphate buffer and the concentration of bacteria adjusted to 3×10^8 cfu/ml before immersing the seeds in the bacterial suspension for 2 hours prior to planting. After harvest at 6 weeks, dry weights of wheat were measured. Total plant N was determined by total combustion gas chromatography (3.2.6.2).

6.3.1.3 Effect of bacterial inoculation on wheat growth at different levels of added ammonium nitrate

The aim was to test the effect on inoculated wheat grown in sand after adding different levels of NH_4NO_3. The sand was supplemented with nutrients (6.3.1.1). The concentration of NH_4NO_3/kg added to sand was 0 mg, 117 mg, 223 mg or 350 mg. Wheat seeds were inoculated or not with a mixture of 3 bacteria (KL5.4, KS2.1, KL2.4) as described above. The concentration of inoculum was adjusted to 5×10^8 cfu/ml. There were 5 replicates of each treatment.

The dry weights, total plant N were measured. The number of tillers and leaves on plants were also counted and recorded after harvest as described by Zadoks Decimal Code (Zadoks et al, 1974). Plants were harvested after 6 weeks of growth.

6.3.1.4 Contribution of bacterial biomass N to measure total plant N

This experiment was conducted to check whether the N from the biomass of any bacteria retained in the rhizoplane after washing, contained sufficient N to create false measurements of total plant N. The measurements of bacterial N were made on the total root bacteria in the wheat rhizosphere.

Isolate KL5.4 was cultured overnight in N-free 4CS broth (5 ml) in a shaking incubator at 28°C. The culture was centrifuged 10,000 g (2 min), cells washed with PBS and concentration adjusted to 10^8 cfu/ml. An aliquot of cell suspension (1 ml) was seeded into flasks containing 4CS broth (100 ml) supplemented with either 0 mg/l, 15 mg/l, 50 mg/l, 200 mg/l, 350 mg/l or 700 mg/l of NH_4NO_3. The broth cultures were shaken (190 rpm) at 28°C for 24 hours, centrifuged at 10,000 g (5 min) and pellets washed twice with sterile water. The bacterial pellets were dried at 60°C and weighed. Serial dilutions on NA plates were carried out from an aliquot of bacterial culture (1 ml) from each flask to estimate the number of cfu/ml. Total bacterial N was then measured (3.2.6.1). The concentration of bacterial N was calculated per cfu/ml.

Surface-sterilised wheat seeds were inoculated with isolate KL5.4 (10^8 cfu/ml) after resuspending the overnight culture (grown in N-free 4CS medium) in phosphate buffer (pH 7.0). The wheat was then grown for 6 weeks in tubes in semi-solid, N-free medium with either 15 mg/l, 50 mg/l, 200 mg/l, 350 mg/l or 700 mg/l of NH_4NO_3 added as a supplement. There were 3 replicates for each inoculated and uninoculated treatment. At harvest, the wheat roots were macerated in PBS (1 ml). Estimations of bacterial numbers were made from serial dilutions of macerate on NA plates. Data obtained from calculating bacterial biomass N per colony forming unit of bacteria from broth culture were used to calculate total bacteria biomass N associated with roots grown in increasing concentrations of added N.

The macerated wheat roots were then dried and weighed and total plant N measured (3.2.6.1).

6.3.2 Results

6.3.2.1 Effect of ammonium nitrate on wheat growth

The dry weights of roots and shoots at 6 weeks were significantly greater in plants grown with added NH_4NO_3 compared with plants grown without NH_4NO_3 (Fig 6.2).
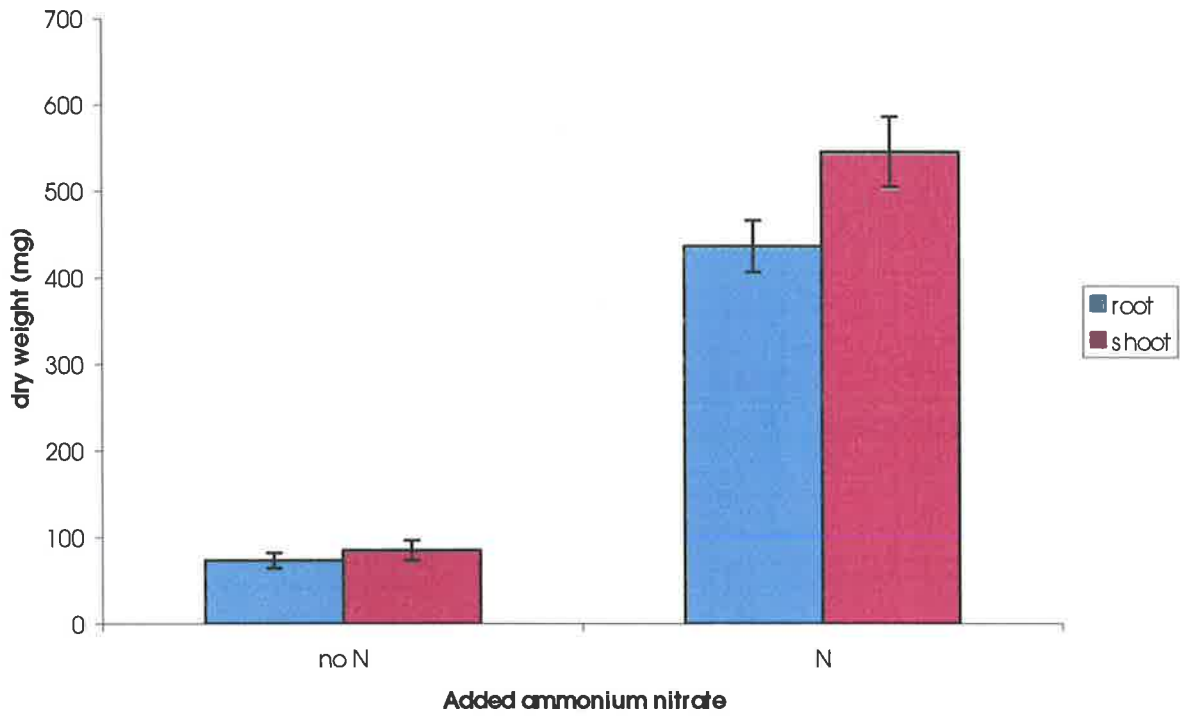
Fig. 6.2 Dry weight of wheat roots and shoots grown with or without added NH_4NO_3, (350mg / kg)

Means and standard errors of means of 10 replicates.

6.3.2.2 Effect of bacterial inoculation on wheat grown in the presence of added ammonium nitrate

In treatments where nitrate was added to the sand, the dry weights of roots and shoots were all significantly greater than treatments without added NH_4NO_3. There were no significant differences in the dry weights of shoots or roots for any treatments without added nitrate (Fig. 6.3). However, when NH_4NO_3 was added, the root dry weights in plants inoculated with the reference strain were significantly greater than the co-inoculated plant roots, (Tukeys HSD: root= 315.19).
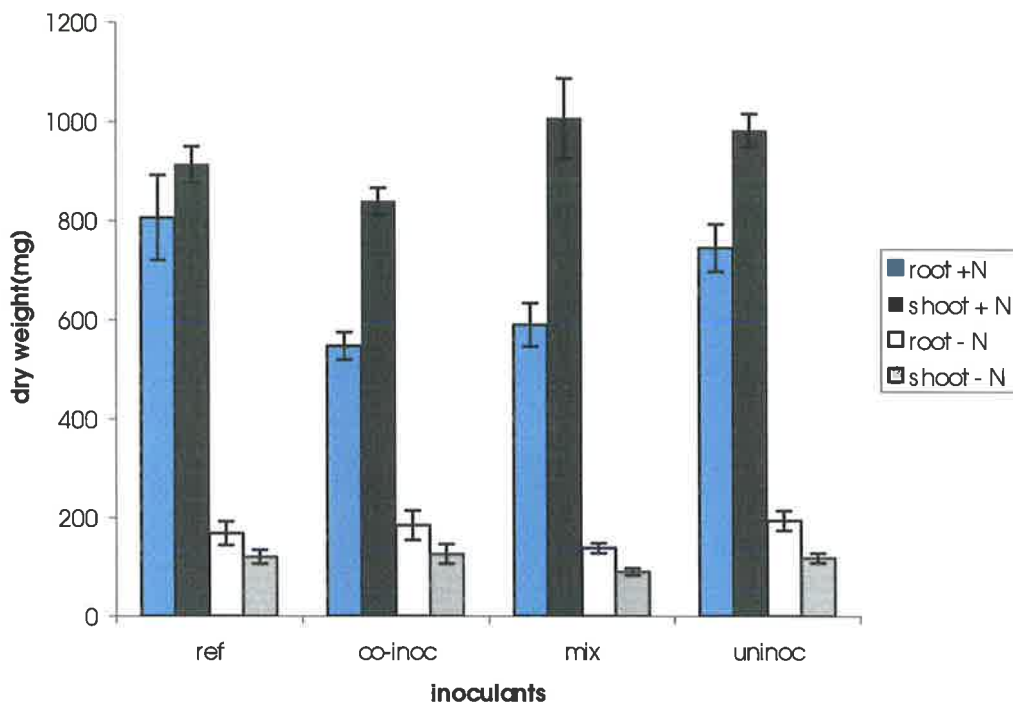
Fig. 6.3 Dry weight of roots and shoots of wheat grown either with or without added NH_4NO_3, (350mg / kg) and with or without inoculum

Ref = *Azospirillum lipoferum*, co-inoc = KL5.4/KL2.4/KS2.1, mix = mixed rhizobacteria from Kapunda, uninoc = uninoculated. (+N/-N = NH_4NO_3)
Tukeys HSD: root +N = 315.19, Tukeys HSD: shoot+N = NS,
Tukeys HSD: root-N = NS, Tukeys HSD: shoot-N = NS
Means and standard errors of means of 5 replicates.

When plants were grown without N supplement, the concentration of N/g dry weight was greater in the roots than in the shoots, but there were no significant differences in N content between inoculation treatments for either roots or shoots (Fig. 6.4).

When NH_4NO_3 was added as a supplement, tissue concentrations of N were greatly increased and shoot N (mg N/g DW) was greater than root N. Shoot N was not significantly different between inoculation treatments. However, the concentration of N in roots of plants co-inoculated with KL5.4, KL2.4 and KS2.1, was significantly higher than in uninoculated roots or those plants inoculated with *Azospirillum lipoferum* (Tukeys HSD = 9.1)
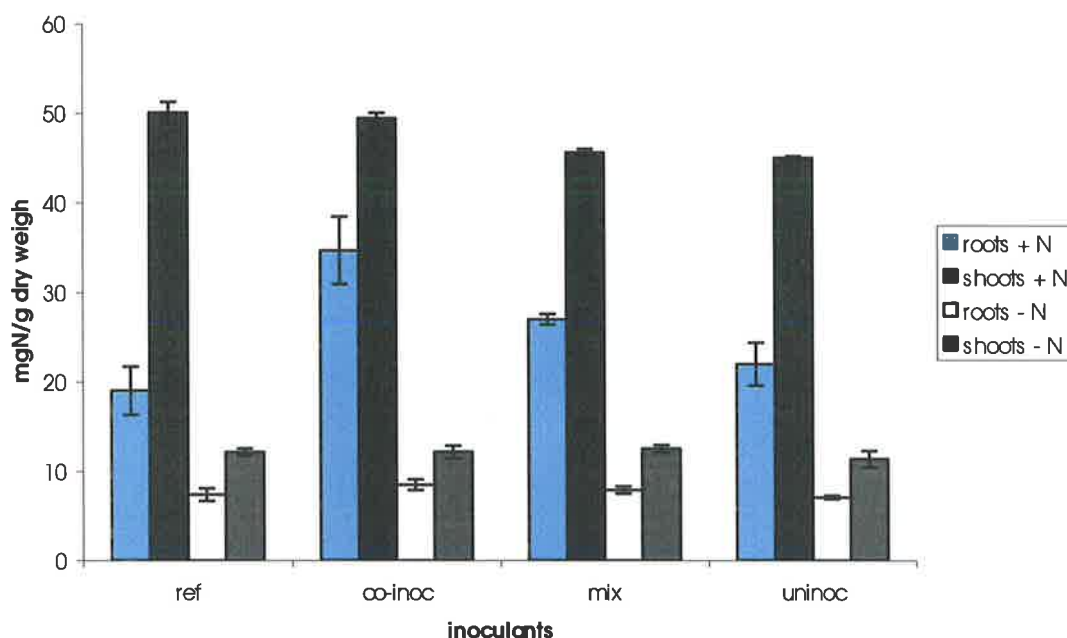
Fig. 6.4 N concentration of roots and shoots of wheat grown either with or without NH_4NO_3, (350mg / kg) and with or without inoculum

Ref = *Azospirillum lipoferum*, co-inoc = KL5.4/KL2.4/KS2.1, mix = mixed bacteria from Kapunda, uninoc = uninoculated In the legend (+M/-N) = NH_4NO_3
Means and standard errors of means of 5 replicates.
Tukeys HSD: root+N = 9.1 Tukeys HSD: shoot+N = NS,
Tukeys HSD: root-N = NS, Tukeys HSD: shoot-N = NS.

6.3.2.3 Effect of bacterial inoculation on wheat growth at different levels of added ammonium nitrate

Dry weights of inoculated wheat roots grown in 350mg NH_4NO_3/kg were significantly greater than those of the uninoculated plants grown with all other levels of NH_4NO_3 supplement (Fig. 6.5). Similarly, the shoot dry weights of inoculated wheat plants grown in concentrations of NH_4NO_3 (350 mg/kg) were significantly greater than the dry weights of plants grown with lower concentrations of N supplement.
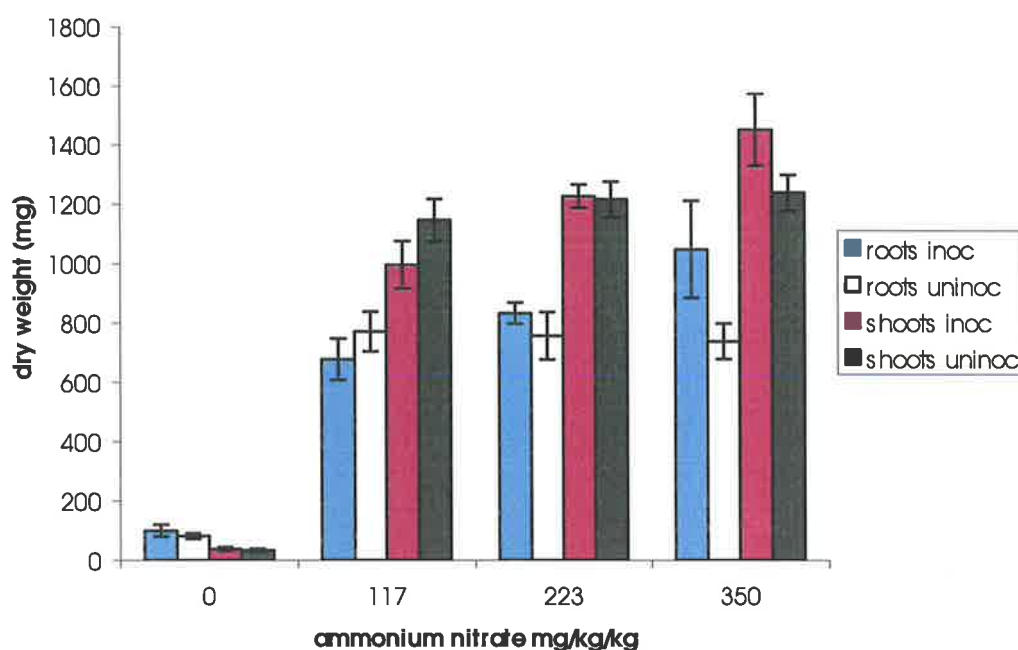
Fig. 6.5 Dry weight of root and shoots of wheat grown either with or without increasing concentrations of NH_4NO_3 and with or without inoculum

Inoc = KL5.4/KL2.4/KS2.1, uninoc = uninoculated, NH_4NO3 = (0/mg/kg, 117 mg/kg, 223 mg/kg, 350 mg/kg). Means and standard errors of means of 5 replicates.

The number of leaves and tillers on plants grown with added NH_4NO_3 were significantly greater than on plants grown without added NH_4NO_3 (P <0.001) but there was no inoculation effect.

Total plant N (mg/plant) increased with increasing NH_4NO_3 addition for both inoculated and uninoculated treatments (Fig. 6.6). There was no effect of inoculation between 0 and 223 mg/kg (NH_4NO_3) sand on total plant N. However, at 350 mg of NH_4NO_3/kg the N content of the inoculated plants was significantly greater than uninoculated plants.
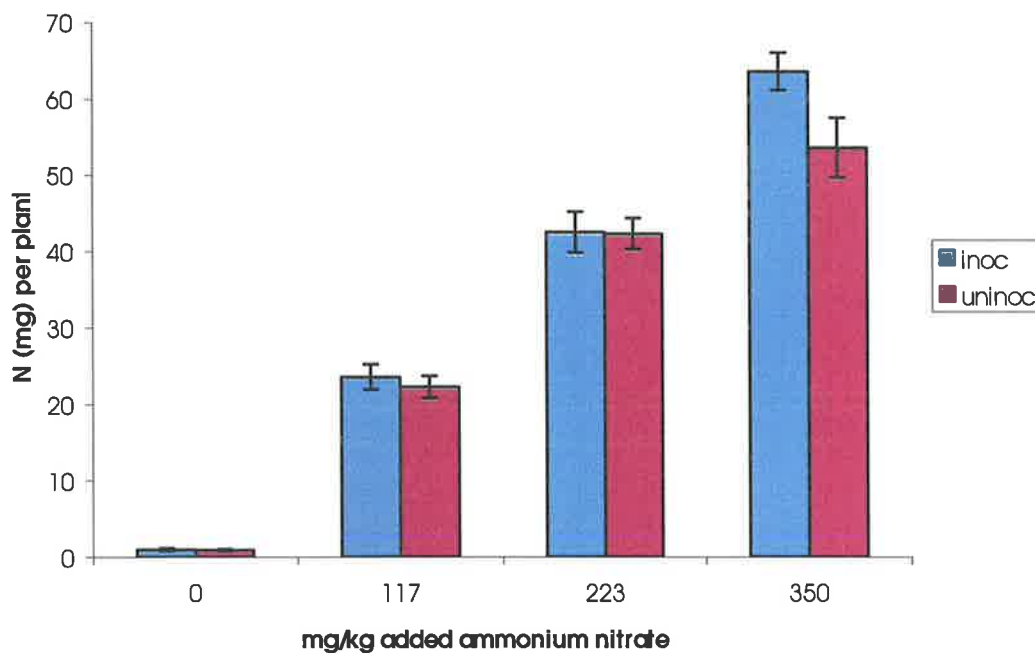
Fig.6.6. Total plant N in inoculated and uninoculated wheat grown in increasing concentrations of NH₄NO₃

Inoc = KL5.4/KL2.4/KS2.1, ininoc = uninoculated. NH_4NO_3 = (0/mg/kg, 117mg/kg, 223mg/kg, 350mg/kg).
Means and standard errors of means of 5 replicates.

6.3.2.4 Contribution of bacterial biomass N to measured total plant N

After 24 hours, growth in numbers of bacteria (measured in cfu/ml of strain KL5.4) was higher with increasing N concentration in the culture broth (Fig. 6.7). The concentration of bacterial N in broth increased from 6 µg/ml to 132 µg/ml over the range of 0 to 700 mg/l (NH_4NO_3). The total numbers of bacteria in the plant roots ranged from 4 x 10^7 cfu/ml without added N, to 4 x 10^8 cfu/ml when grown with 350mg NH_4NO_3 (Fig. 6.8). These data were used to calculate the potential contribution of bacterial N to the N concentration and content of roots (Table 6.4).
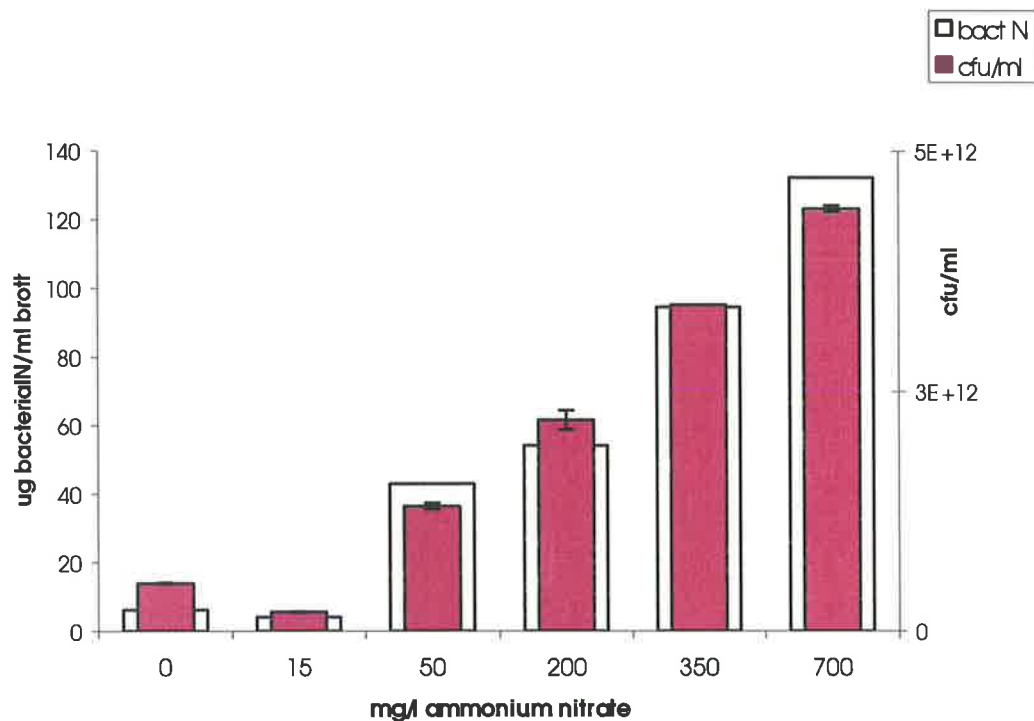
Fig. 6.7 Numbers of bacteria (KL 5.4) and concentration of bacterial N associated with increasing concentrations of NH_4NO_3 in culture broth

Addition of NH_4NO_3 to the broth culture ranged from 0 to 700mg/l.
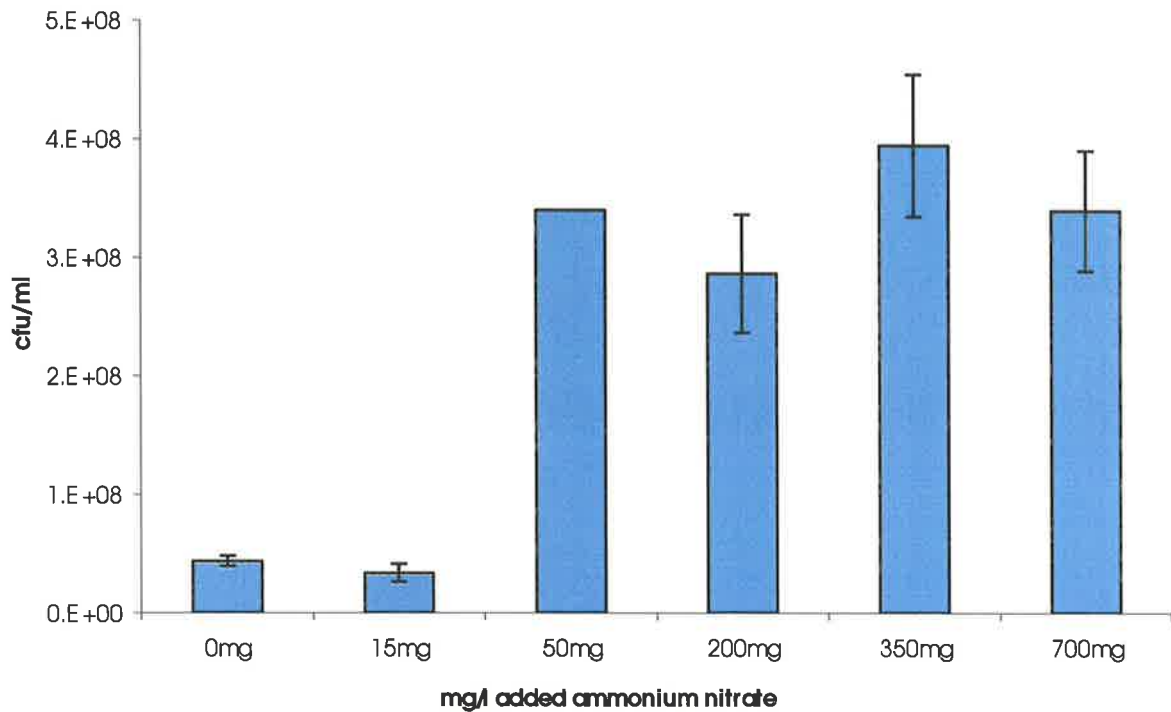
Fig 6.8 Numbers of bacteria in plant roots at increasing concentrations of NH₄NO₃ in plant growth medium

Means and standard errors of means of 3 replicates.

The N content of roots was 77.19, 91.22 and 92.98 μg in the 0, 350 and 700 mg/l (NH₄NO₃) treatments respectively. Bacterial biomass could have only contributed between 0.0005 μg N (0.00004%) and 0.0109 μg N (0.009%) of the total root N (Table 6.4).

TABLE 6.4 Concentration of N (μg) in plant roots and estimation of the contribution to root N of bacteria adhering to the roots

NH₄NO₃ mg/l broth	mean μg N/root		mean μg N/rhizosphere bacteria	
0	77.19	(± 6.0)	0.0005	(± 0.00005)
15	71.9	(± 11.5)	0.0006	(± 0.0001)
50	56.14	(± 1.7)	data not available	
200	45.6	(± 3.5)	0.007	(± 0.002)
350	91.22	(± 7.6)	0.0109	(± 0.004)
700	92.98	(± 6.3)	0.0102	(± 0.002)

Means and standard errors of the means of 3 replicates.

6.3.3 Summary

Root N in co-inoculated plants grown with an N supplement of 350mg/kg was significantly higher than that in uninoculated or plants inoculated with the reference bacterium *Azospirillum lipoferum*. Bacterial biomass N was very low and did not appear to contribute significantly to root N content.

6.4 Discussion

One of the aims of the experiments described in this chapter was to compare the growth of inoculated and uninoculated wheat grown with or without an added source of N. Co-inoculation of plants with a mixture of 3 bacterial strains (KL5.4 *Pantoea agglomerans*, KL2.4 *Stentrophomonas maltophilia* and KS2.1, *Enterobacter taylorae*) in the presence of NH_4NO_3 (350mg/kg) resulted in a significant increase in concentration of N in plant roots (24%) compared with uninoculated plants. In contrast, the concentration of wheat root N grown in association with *Azospirillum lipoferum* was 36% lower than uninoculated plants. The increase in plant N content in co-inoculated plants may be attributed to N_2 fixation, as N from bacterial biomass in the rhizosphere populations did not make a significant contribution to the total plant N. If N_2 fixation was responsible for increased plant N content, added nitrate in the growth medium is likely to be necessary to significantly enhance N_2 fixation by the bacterial inoculants.

The source of N and conditions for growth appear to greatly affect nitrogenase activity. There are few reports in the literature on the role ammonium nitrate plays on the ability of N_2-fixing bacteria to fix dinitrogen. Generally low N supply in the soil does not affect N_2 fixation (El-Komy et al, 2003), but high levels of ammonium nitrate (25 mM) are reported to reduce nitrogenase activity (Muthukumarasamy et al, 2002). The results outlined in this chapter agree with an inoculation study of hydroponically grown wheat which showed a doubling of the rate of N_2 fixation by *Pantoea agglomerans* and a lack of response by *Azospirillum* spp. following the addition of KNO_3 (100 mg/l) to the medium (Ruppel and Merbach, 1997). The addition of NH_4Cl (100 mg/l) did not significantly alter nitrogenase activity compared with no additional N. However, in pure culture the addition of NH_4Cl significantly repressed the nitrogenase activity of *Azospirillum* sp. and to a lesser extent that of *Pantoea agglomerans* (Merbach et al,

1998). When no N supplement was provided to inoculated wheat plants, *Azospirillum* sp. fixed more atmospheric N_2 than *Pantoea agglomerans* (Ruppel and Merbach, 1997). In the work described here, inoculated wheat grown in sand (not hydroponically as above) required a concentration of 350mg NH_4NO_3/kg before an inoculation effect occurred. At 350mg NH_4NO_3/kg of added N, the total plant N for each plant averaged 64 mg, with 0.8 mg attributed to residual seed N (4.2.2.1). Therefore, the total content of plant N measured in the 2 plants per pot accounted for 126.4 mg of available N in the sand. With only a single application of N at the commencement of the experiment, over the 6 weeks of wheat growth, nitrate ions could be washed to the bottom of the pot, limiting their availability in the soil solution but providing conditions conducive for nitrogen fixation. By extending the length of the growing period, N supply would become growth limiting. If plant N were assessed at intervals during that period, further information on the relationship between added N, N_2 fixation and improved N uptake by the plant may be revealed. Further studies (acetylene reduction), to assess the effect of varying concentrations of ammonium nitrate on nitrogenase activity of the 3 bacteria used as co-inoculants would also contribute to the understanding of the plant/bacteria interaction. The inclusion of non-N_2-fixing bacteria as inoculants may also have been useful in plant N-uptake experiments for comparative studies.

Roots and shoots in uninoculated plants grown with or without added NH_4NO_3, (Fig. 6.2) weighed less than the subsequent experiment (Fig. 6.3) In the second experiment, plants were grown for only 3 days longer but all other conditions including growth room temperature and humidity were similar and differences cannot be explained. Root and shoot dry weights of co-inoculated wheat supplemented with NH_4NO_3 (350 mg/kg, Fig. 6.3), were significantly less than those inoculated with *Azospirillum lipoferum* and uninoculated plants, but were significantly greater than uninoculated plants in the follow-up experiment (Fig. 6.5). Growth conditions in the two experiments were very similar and the reasons for differences in dry weights are unclear. Despite the differences in dry weights, inoculation in both experiments increased plant N.

The aim of the project was to select potential inoculants to improve the early growth of wheat. The screening of isolates for antagonism to each other eliminated the selection of one isolate that was inhibited by the four others identified as *Pantoea agglomerans*. Strains *of Pantoea agglomerans* have been reported as biological control agents. In an

antagonism study, *Pantoea agglomerans* (strain EII318) inhibited the growth of *Erwinia amylovora*, the bacteria responsible for causing fire blight (Wright et al, 2001). Antibiotics pantocin A and B are also produced by this strain of *Pantoea agglomerans*. Strain (CPA-Z) has been used as an anti-fungal biological agent in post harvest disease in apples (Nunes et al, 2002). The selection of *Pantoea agglomerans* (KL5.4) as a potential inoculant for wheat may provide a competitive advantage in the rhizosphere. The growth of at least one bacterium was inhibited by the presence of isolate KL5.4 but further antibiosis studies were not undertaken.

Use of GC-FAME fatty acid profiles to highlight likely candidate bacterial isolates (Chpt. 5) contributed to the selection of 3 locally adapted bacteria KL5.4 (*Pantoea agglomerans)*, KL2.1 (*Stenotrophomonas maltophilia)* and KS2.1 (*Enterobacter taylorae)* which have demonstrated a potential benefit when used in association with wheat

Chapter 7: General Discussion, Summary of Findings and Future Research

7.1 General discussion

The aim of the current study was to develop an effective screening system to select free-living N_2-fixing bacteria from South Australian soils for their use as potential inoculants to improve the early growth of wheat. The screening system was designed to identify a source most likely to contain plant growth-promoting bacteria and to select bacteria from that soil for use as co-inoculants to increase the N content of wheat.

The selection and isolation of soil bacteria as inoculants has reinforced general interest in the potential for both improving agricultural crop yields and for reducing detrimental effects from excess nitrogenous fertilisers (Merbach et al, 1998; Bloemberg and Lugtenberg 2001). One of the dilemmas associated with screening of N_2-fixing bacteria as inoculants has been to distinguish between the notions of testing large number of soil isolates from a range of genera or of focussing on specific genera, described as plant growth-promoting bacteria (Bashan and Holguin, 1997). An example of such bacteria is *Azospirillum*. The approach taken in this study was to first identify a community of N_2-fixing bacteria, which stimulated root growth and total plant N content before studying specific bacteria from the soil microbial community.

N_2 fixation in three test soils

Early work confirmed that it was possible to differentiate between growth responses of plants grown in association with N_2-fixing communities from three soils (Chpt. 4). The three representative soils were collected from long-term wheat trials at Kapunda, Avon and Waite in South Australia. All of the sites had all been sown to continuous wheat for more than 20 years and none of the soils had been supplemented with nitrogenous fertilisers. Increased concentrations of soil N were observed at two of the sites (Waite and Avon), which implicated the input of N from free-living N_2-fixing bacteria. Each of the three soils was assessed for evidence of N_2 fixation by free-living bacteria. $^{15}N_2$ was

used to directly measure active N_2 fixation. This technology demonstrated transfer of fixed N from the Kapunda soil to wheat plants by bacteria. The $^{15}N_2$ method was used in preference to the acetylene reduction assay (ARA), which provides indirect evidence for N_2 fixation (Turner and Gibson, 1980). If ARA had been used in parallel with mass spectrographic measurement study of $^{15}N_2$ incorporation by plant tissue, the evidence for the presence of nitrogenase activity within the bacterial communities may have been more robustly confirmed. However, as mentioned previously, the use of $^{15}N_2$ is unfortunately expensive. Consequently this limited more extensive experimentation by this method. The application of ARA, particularly for the determination of nitrogenase activity in individual isolates, is likely to support further molecular studies to determine the absence or presence of an N_2-fixing gene. However, this study provides evidence to show that bacteria from the Kapunda soil, which grow on N-free media, could significantly increase the length of wheat roots when compared with the effect of bacteria from Avon and Waite soils or uninoculated controls. The two assessment criteria, the measurement of $^{15}N_2$ and the determination of root length, indicated that bacteria in the Kapunda soil source were more effective at fixing N_2 and stimulating root growth than bacteria from either Avon or Waite soils (Chpt. 4). Hence the Kapunda soil was used for isolation of potential inoculants.

The status of Azospirillum in this study

Azospirillum has been used successfully as an inoculant to improve yield for crops such as rice (Omar et al, 1992). Dry weight and root development were significantly enhanced in maize (Fulchieri and Frioni, 1994) and specifically the application of *Azospirillum lipoferum* CRT1 improved the N content of maize (Berge et al, 1990). The strain *Azospirillum lipoferum* CRT1 has been marketed as Azogreen® and its application has achieved consistent improvement of plant N content in the field in 13 locations over 4 years (Fages, 1992). Azogreen® (Lipha, Meyzieu, France) has increased early growth and yield in maize as measured by increases in plant height, root length and root fresh weight (Jacoud et al, 1998). It was anticipated that the soils from Kapunda, Avon and Waite would contain *Azospirillum* spp., which could be isolated for testing as potential inoculants. Indeed, numerous *Azospirillum* spp. had been previously isolated from wheat grown in Kapunda soil (Dr. J. Balandreau, 1995 pers. com.). Bulk soils collected at the end of the growing season from Kapunda, Avon and Waite were used to grow wheat as trap plants for N_2-fixing bacteria. Ironically they yielded few *Azospirillum* spp.

The most common bacteria isolated from the Kapunda soil were *Pantoea agglomerans*, *Enterobacter* spp. and *Stenotrophomonas maltophilia*. Nevertheless since microaerophilic bacteria such as *Azospirillum* spp. are known to grow well in N-free semi solid medium (Krieg and Dobereiner, 1984) and because they have been well studied (Steenhoudt and Vanderleyden, 2000) and successfully used as inoculants (Vázquez et al, 2000), *Azospirillum* spp. were chosen as a model for comparative wheat growth-promotion studies in this project. Specifically, isolates of *Azospirillum lipoferum* 596, isolated from eastern Australia (New and Kennedy, 1989) and *Azospirillum brasilense* Sp7 were used as reference bacteria. Indeed these reference bacteria were not as effective as the locally isolated bacteria either at inducing increased root length (Chpt. 4) or improving total N content of the wheat (Chpt. 6) which were grown in pots under laboratory conditions. This observation supports the view, put forward in the Introduction (1.1) that local bacteria that were adapted to climatic conditions and competitive pressures, may prove to be more effective as inoculants than those introduced from elsewhere.

Bacterial isolates from local sites

After the selection of the Kapunda soil as a potential source of growth-promoting bacterial strains, the next step was to select bacteria from that soil for further testing as potential inoculants. Bacteria were classified into the sub-groups of the Proteobacteria and an initial selection was made from a range of genera within these sub-groups. Many known bacteria belong to sub-groups of the Protebacteria (Woese, 1987). The classification of isolates into such sub-groups provided the supporting evidence for the valid identification by GC-FAME analysis. Bacteria carrying the nitrogenase gene were identified by *nif*D and *nif*H hybridisation as well as by PCR studies (Chpt. 5). While identification of the strains was carried out by GC-FAME analysis, it could not provide sufficiently precise identification. However fingerprinting PCR, using InterLINE PCR, enabled a better identification and grouping of similar species and the selection of isolates for testing as plant growth promoters could be made without unnecessary duplication of the bacteria (Chpt. 5).

The use of GC-FAME analysis

Identification of a dominant bacterial species within the N_2-fixing community in the Kapunda soil (*Stenotrophomonas maltophilia*) was achieved by comparing the fatty

acid profiles of a known culture with field isolates (Chpt. 5). The use of GC-FAME analysis was adopted in preference to phospholipid fatty acid analysis (PLFA). Although similar results were obtained using both approaches, a smaller sample of the rhizosphere bacterial community was required for GC-FAME and analysis is more easily performed than PLFA.

A more diverse fatty acid profile was observed in the bacterial community isolated from the Kapunda soil source, after growth in the wheat rhizosphere, than from either Waite or Avon soils. The diversity of fatty acids reflects the diversity of bacterial types from within the Kapunda soil that are able to colonise the wheat rhizosphere. The data from GC-FAME analysis in the screening process supported initial observation indicating that the bacteria present in the community from the Kapunda soil source differed from those from Avon and Waite soils.

GC-FAME analysis has been used in other studies to compare the fatty acid composition of microbial communities found within roots and in the soil. Fatty acid profiles of microbial communities from within the roots of different cultivars of wheat and canola were compared with microbial communities from the rhizosphere soils (Siciliano et al, 1998). In one canola cultivar, different fatty acid profiles were observed in the microorganisms in roots and in the rhizosphere soil. In a phytoremediation study to monitor the degradation of 2- chlorobenzoic acid (2CBA) in the soil, *Pseudomonas aeruginosa* strain R75 and *Pseudomonas savastanoi* strain CB35 were used as inoculants for meadow brome (*Bromus biebersteinii*) (Siciliano and Germida, 1998). GC-FAME was used to characterise the microbial communities. After inoculation of meadow brome with the pseudomonad strains, fatty acid profiles of the microbial community on the root surface altered. Associated with the changes in fatty acid profiles was an increased potential for the degradation of 2CBA.

GC-FAME and other analytical techniques
The investigation and monitoring of complex soil microbial populations have resulted in the use of a combination of a variety of methods. The fatty acid profiles generated by GC-FAME are representative of a microbial population or community and the profile can be used as a fingerprint to monitor change (Cavigelli, 1995: Griffiths, 1999). To provide better resolution of the change, length heterogeneity PCR (LH-PCR) was used

in association with GC-FAME (Dierksen et al, 2002). In that study, a limited number of marker fatty acids and LH-PCR fragments were used as variables to monitor changes in the microbial community following different tillage and ground cover practices in an agricultural soil from Oregon. However, the combination of methods provided information about different aspects of the microbial community and did not improve the information derived from GC-FAME analysis. It is difficult to select appropriate fatty acid and LH-PCR fragments as markers. Another study compared two DNA techniques (terminal restriction fragment length polymorphisms (T-RFLP) and LH-PCR to monitor microbial community dynamics. Inconsistencies associated with restriction digests in T-RFLP were found and the investigators achieved a better resolution of the microbial community structure using LH-PCR (Mills et al, 2003).

The fatty acid profiles generated by GC-FAME analysis may indicate changes in microbial communities over time, but GC-FAME analysis is limited in its ability to identify distinct groups of microorganisms within a community. Substrate utilisation by Biolog® has been widely used in association with GC-FAME (Bååth and Arnebrånd, 1994; Pennanen et al, 1996; Lawler et al, 2000) but determination of background reading in the substrate utilisation assay may require a preliminary count of bacteria on TSBA (Lawler et al, 2000) and unculturable bacteria are not included.

In the present study, cloning and sequencing of 16S rDNA was not attempted, as GC-FAME was perceived to be a fast and simple method to achieve a similar outcome. The combination of GC-FAME and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) or LH-PCR would have provided a more comprehensive understanding of the structure of microbial communities. As LH-PCR profile analyses are based on variation in the sequence lengths of specific regions of DNA phylogenetically dissimilar organisms may appear closely related if the same length fragment is detected (Mills et al, 2003). Patterns of microbial community change could, however, be monitored. Some GC-FAME studies are reported to use samples directly extracted from the soil (Buyer and Drinkwater, 1997) and fatty acid profiles may be complex and difficult to interpret due to the contamination of plant and animal fatty acids amongst those from microorganisms. In the current work, the bacteria of interest were cultured under N-free growth conditions for 4 to 6 weeks. The diversity of the soil bacterial communities had been altered to favour N_2-fixing bacteria. Hence, the fatty

acid profiles generated by GC-FAME are likely to provide more focussed information about the N_2-fixing bacteria than on the other organisms that are less likely to grow in these N-free conditions. Following from these observations that there were differences between the diversity of fatty acids from the bacterial community from Kapunda soil and those from Avon and Waite soils (present in trace amounts) the identity of the group or groups of bacteria responsible for the differences can now be addressed.

Fatty acids for bacterial identification

Examination of the fatty acid profile from *Stenotrophomonas maltophilia* (isolated from Kapunda soil), showed that the dominant fatty acids were 14:0iso, 16:1w9c and 18:1w9c, (Chpt. 5). These are similar to those in the fatty acid profile derived from the rhizosphere community from Kapunda. This finding is therefore consistent with the observation that *Stenotrophomomas maltophilia* is dominant in that soil. This bacterial species, a known plant growth-promoting bacterium (Koch et al, 1998) was selected for further testing as a potential inoculant as described previously (5.6).

GC-FAME analysis was used to validate molecular characterisation and identification of individual isolates. Within the Proteobacteria, γ-purple bacteria contain sub-groups including fluorescent pseudomonads and enterics (Woese, 1987). Identification of bacteria such as *Pantoea agglomerans* and *Enterobacter taylorae* by GC-FAME was consistent with the attribution of these bacteria to the γ sub-group of the Proteobacteria by hybridisation studies using rRNA-directed probes. Classification of individual bacteria into sub-groups of the Proteobacteria provided the basis for selecting a range of taxa from within the bacterial sub-groups for preliminary testing of bacteria as growth promoting microorganisms for wheat.

Further selection of isolates was based on information obtained from characterisation studies for specific traits. Characterisation studies of bacteria within the sub-groups, was aimed to identify whether the structural nitrogenase *nif* gene was present and whether the bacteria were antagonistic to each other. Studies that focussed on the nitrogenase gene, demonstrated amplification of *nif* PCR products after using *nif*D and *nif*H PCR primers. However, hybridisation of these PCR products to the *nif*H probe only occurred after reducing the hybridisation stringency conditions (Chpt. 5). There was little

hybridisation of amplified *nif* D product to the *nif*D probe (based on gene sequences from *Klebsiella pneumoniae*), indicating that these sequences are apparently not well conserved in these organisms. This is contrary to evidence that structural genes for nitrogenase *nif*HDK are strongly conserved, with the *nif* genes being homologous to *Klebsiella pneumoniae* found in many N_2-fixing bacteria (Kennedy, 1993, de Zamaroczy, 1995). Sequencing of the *nif* PCR products should confirm whether the bacteria do or do not contain *nif*HDK and would be the next step in future investigations. Comparison of bands following PCR fingerprinting enabled commonality between strains to be identified. This reduced unnecessary duplication of the bacteria selected for further testing in the wheat rhizosphere (Chpt. 5).

The process for selecting potential inoculants included the testing strains for their ability to inhibit growth of other bacteria (Chpt. 6). Of the 12 bacteria tested, 4 of the strains, identified as *Pantoea agglomerans*, were antagonistic to an unidentified isolate (KL2.1). As discussed previously (6.1) *Pantoea agglomerans* is known to produce the antibiotics pantocin A and B (Nunes et al, 2002) and this characteristic, as well as the presence of *nif* genes, influenced the final selection of isolate KL5.4 as one of the co-inoculants for wheat. Further studies are necessary to establish whether the strain could be an effective biocontrol agent.

Bacterial inoculants and plant growth

One of the reported benefits of applying free-living diazotrophic bacteria as inoculants in the field is an improved N nutrition of plants. The magnitude of the effect depends upon plant cultivar, soil type and level of nitrogenous fertiliser. Benefits of bacterial inoculation to the cultivation in rice occur in N deficient crops (Omar et al, 1992), and in wheat supplied with NH_3NO_3 at 0, 40 and 80 kg/ha (Kapulnik et al, 1983) and in maize with NH_4NO_3 at 160 kg/ha (Berge et al, 1990). The authors of the second study acknowledged that the high level of 160 NH_4NO_3 kg/ha was necessary to produce a grain yield of 14.4 t/ha. This could only be obtained with uninoculated plants fertilised with 240 kg N/ha. Inoculation with *Azospirillum lipoferum* therefore achieved a benefit to the plant equivalent to that achieved with 80 kg N/ha. The high levels of N fertiliser necessary to significantly increase grain yield suggest that the larger root system which was observed in the plants led to increased plant nutrition associated with more fertile tillers per unit area. N_2 fixation was unlikely to have contributed to the observed benefit.

This thesis describes root length increase in inoculated plants supplemented with nitrate (4.4.2.2) and concentrations of plant N also increasing in inoculated wheat when ammonium nitrate was supplied at 350 mg/kg (6.3.2.2). These data also suggest that N_2 fixation is not the responsible mechanism for eliciting a beneficial plant response. Fixed N, (^{15}N) measured in 28-day-old wheat plants exposed to no added N was low (4.2.2.2) This was to be expected as the seed reserves had maintained the young plants in the early stages of growth. After the seed reserve was utilised, plant N content was only improved in inoculated plants that had been supplemented with nitrate. It is unclear whether improved plant N content and concentration occurred in response to N_2 fixation or stimulation of plant N uptake by bacteria.

Estimation of the proportion of non-fixing to N_2-fixing bacteria after growth in semi-solid, N-free medium in the wheat rhizosphere was carried out early in the project by plating bacterial communities onto nutrient agar plates (not reported) as well as selective media described in Chapter 4 of the thesis. The differences between bacterial numbers growing on nutrient agar medium and on N-free media were not significant and data were not reported. A mathematical model devised to assess benefits from N_2 fixation under optimal conditions, predicted that the rates of N_2 fixation were dependent on the proportion of N_2-fixing bacteria within the microbial community and could reach rates up to 20 kg N/ha per year (Jones et al, 2003). The monitoring of proportions of N_2-fixing bacteria from the soils throughout the project may have clarified the argument for plant N levels being higher in inoculated than uninoculated plants particularly following ammonium nitrate supplementation (Chpt. 6). However, this approach has limitations. Bacterial counts obtained on selective media would not have reflected true bacterial numbers, because unknown, unculturable species would not have been assessed. Improvements in growth media with associated identification of new bacteria are beginning to recognise the problem of unculturable bacteria (Janssen et al, 2002; Sait et al, 2002) and a resolution of this may lead to a better understanding of the dynamics of the bacteria within the community. Further studies will be required to clarify the mechanism responsible for the increase of N in inoculated plants.

Roots from plants grown in association with N_2-fixing bacteria isolated from Kapunda, Avon and Waite soils were up to 50% longer than those from plants inoculated with heat-killed bacteria. The addition of a nitrate supplement to the medium enriched with

soil bacteria enabled significant increases in root length to be detected between plants grown with bacteria from the Kapunda soil source when compared with plants grown with bacteria Avon and Waite soils. The mechanism or mechanisms responsible for stimulating the growth plant roots by bacteria was not determined. However the notion can be put that root elongation results from a combination of factors. Predominantly, bacterial production of phytohormones will stimulate growth or through improved N nutrition via N_2 fixation. Data from the Petri dish bioassay provided evidence that bacteria stimulated the growth of 7 day-old plant roots that only had access to nutrients from the seed source. Therefore it can be argued that improved growth is due to bacterial phytohormone production. Some rhizosphere bacteria such as *Azospirillum brasilense*, produce indoleactetic acid, which could be responsible for enhanced plant growth. It has been reported that growth characteristics such as root exudates, and high levels of bacterial inoculation (10^9 cfu/seedling) may cause either positive or negative effects on root elongation (Harari et al, 1989; Omay et al, 1992; Bothe et al, 1992). *Pantoea agglomerans* and other free-living Enterobacteriaceae also produce phytohormones (Hofflich and Ruppel, 1994: Zimmer et al, 1994). The work described in this thesis pre-empts future studies in the interaction of phytohormones between plants and bacteria.

Endophytes

The application of *Pantoea agglomerans, Stenotrophomonas maltophilia* and *Enterobacter taylorae* as co-inoculants for wheat under controlled growth conditions demonstrated an increase in concentration and content of plant N in the early stages of growth of the wheat plants (Chpt. 6). In this study, *Enterobacter taylorae* was isolated from within the stem of wheat. Studies elsewhere have shown that *Enterobacter taylorae* could be isolated from within the wheat stem and that *Pantoea agglomerans* was in the intercellular spaces of the root cortex respectively (Ruppel et al, 1992). The status of these species as possible endophytes has not been addressed in the thesis but it is recognised that advantages for endophytes include protection from desiccation and a reduction in competitive nutritional pressures from the rhizosphere microbes. Colonisation in the intercellular spaces of the plant and access to plant metabolites by non-pathogenic bacteria may, in turn provide an advantage to the plant by enhancing host defence against pathogens such as *Fusarium oxysporum* f.sp.*vasinfectum* (Chen et

al, 1994). Endophytes within the plant may also be retained within the seed and passed from one plant generation to the other without the necessity for annual re-inoculation.

Host specificity

One of the proposals made at the commencement of the study was that bacteria, locally adapted to the environment, would enhance the growth of wheat. Since only one variety of wheat (*Triticum aestivum* var Stiletto) was used in the current study, other varieties can now be tested for growth responses to the selected co-inoculants. In addition to the adaptation of bacteria to local conditions, host specificity may be a factor to be considered when selecting bacteria as potential inoculants for wheat. The natural progression from this study would be to address adaptation of bacteria to specific varietal hosts.

7.2. Summary of findings

Differential selection of the Kapunda soil from two other soil sources Waite and Avon, as a source for potential inoculants was based on:

- detection by mass spectrometry of ^{15}N in plant tissue of wheat that had been grown in association with a macerate suspension (Chpt. 4)
- significant increase in the lengths of lateral roots when wheat was inoculated with the macerate suspension from the Kapunda soil that was supplemented with nitrate as compared with that from Waite or Avon soils (Chpt. 4)
- greater diversity of fatty acids in the GC-FAME profile of the bacterial community from the Kapunda soil source than the diversity found in Waite or Avon soils, ie a wider diversity of bacteria within the community (Chpt. 5)

Selection of locally adapted bacterial isolates from the Kapunda soil source as potential inoculants was based on:

- similar fatty acids within the GC-FAME profile shared by the isolate *Stenotrophomonas maltophilia* and the predominately N_2-fixing community from Kapunda (Chpt. 5)
- identification of the nitrogenase gene, as demonstrated by PCR and hybridisation studies (Chpt. 5)
- resistance to antagonism from other selected isolates (Chpt. 6)

Final selection of co-inoculants *Pantoea agglomerans, Stenotrophomonas maltophilia* and *Enterobacter taylorae* improved the content and concentration of plant N when grown in sand in pots.

7.3 Future directions

The assessment of plant growth-promotion mediated by an association with bacteria under controlled laboratory conditions (agar or sand culture) was the first step towards selecting potential inoculants for use in the field. Now a more reductionist approach will focus on mechanisms responsible for the growth features identified in this thesis. In future prior to testing co-inoculants in the field, replicated pot experiments with field soil, using temperature, soil moisture and light conditions that equate to the estimates of realistic field conditions.

Only one variety of wheat was used throughout the current study. Genetically dissimilar varieties may now be tested with identical inoculants to determine the status of host specificity. As new cereal varieties become available, the ability of the inoculants to thrive in the rhizospheres of the different varieties must be established. Although some bacteria are ubiquitous to the rhizosphere of diverse plants, bacteria used as inoculants need to be validated on a broad range of genetic plant material.

Monitoring the success of inoculants in the rhizosphere was initiated using *gfp* labelling and two of the potential inoculants (*Pantoea agglomerans* and *Enterobacter taylorae*) were labelled (results not shown). Further study using differential combinations of all three bacteria of interest (*Pantoea agglomerans*, *Enterobacter taylorae* and *Stenotrophomonas maltophilia*) can provide data about the competitive ability between them and native rhizosphere microbial populations. Limited *in situ* hybridisation experiments using fluorescently labelled rRNA-targeted oligonucleotides and confocal laser-scanning microscopy to visualise bacteria have been carried out (data not shown). Extension of *in situ* hybridisation to monitor the colonisation patterns and persistence of the inoculants particularly in non-sterile soil would provide useful data for the development of the beneficial inoculants. Other techniques have been used in another study to monitor colonisation of bacteria in the rhizosphere. The study identified *Azospirillum brasilense* in wheat rhizosphere using a specific AB oligonucleotide probe complementary to 23s rRNA, confirming colonisation of the bacteria within root hairs (Assmus et al, 1995). *In situ* hybridisation work has been carried out by other groups using group-specific probes based on sub-class of the Proteobacteria to monitor bacteria

in inactivated sludge (Amman et al, 1996; Snaidr et al, 1997). The development of specific fluorescently labelled probes to track the co-inoculants will assist monitoring rhizosphere colonisation in future.

In other studies two of the bacteria selected as potential inoculants *Pantoea agglomerans* and *Enterobacter taylorae* have been isolated from within the cortex of the plant. In future, a determination of whether the two bacterial species occur within the rhizosphere or within the plant will be pivotal to the development of technologies directed to the application of inoculant to either the seed or seedlings as a foliar spray. Foliar sprays have been used to deliver endophytic inoculants into cotton (Musson et al, 1995), and diazotrophic bacterium *Klebsiella* sp. (KUPOS) onto rice to increase grain yield under water stress conditions (Razi and Sen, 1996). Bacterial biocontrol agents have also been applied successfully to pearl millet and rice as foliar sprays (Umesha et al, 1998; Vidhyasekaran et al, 1997).

The experimentation to determine the presence or absence of N_2 fixation should be confirmed by including acetylene reduction assays in addition to $^{15}N_2$ fixation described (Chpt. 4). It would provide a cheap and rapid method for routine determination of N_2-fixing potential. Verification of the presence of N_2-fixing genes within individual isolates may also be carried out with greater emphasis on sequencing *nif*PCR products. Molecular techniques such as PCR-DGGE, LH-PCR, may provide more detailed information on the bacterial changes within the community and if so, might complement GC-FAME analysis.

The focus of work in this thesis is the isolation and selection of the co-inoculants but in future the work might be expanded to include a more detailed examination of endophytes isolated from the local soil environment. Survival of endophytes as inoculants may be more successful than bacteria colonising solely the outside the plant and this direction for future work is strongly recommended

Appendix 1: Quantification of Exchangeable Nitrate Levels in the Soil

The concentration of exchangeable nitrate in the soils was significantly higher in the Waite soil compared with the other soils from Kapunda and Avon P< 0.001 (Fig 1.1). The nitrate concentration ranged from 10.7 and 9.2 µg N/g soil DW in Kapunda and Avon soils respectively with the highest concentration of 23.9 µgN/g soil nitrate present in Waite soil.
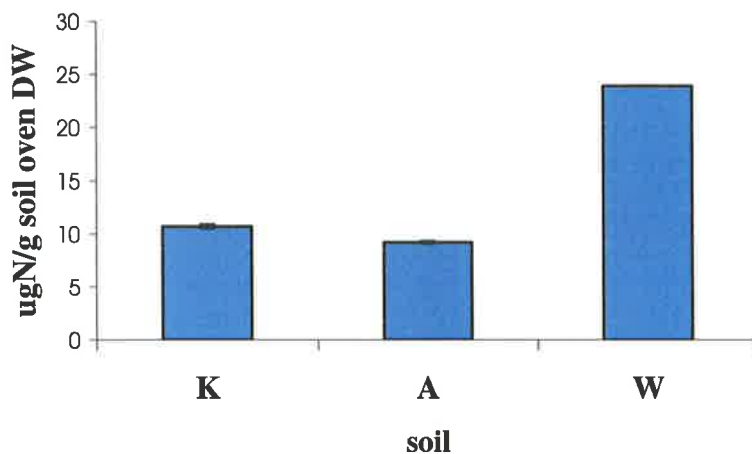
Fig.1.1 Exchangeable nitrate (µg N/g soil) in Kapunda (K), Avon (A) and Waite (W) soils, LSD < .001.

Kjeldahl method

Total N was determined in a sulfuric acid digest of the plant material by a micro Kjeldahl method (Bremner and Mulvany, 1982). The total N in the diluted digest solution was measured colorimetrically in an autoanalyser by the nitroprusside/dichloro-S-triazine modification (Blakemore et al, 1987) of the Berthelot indophenol reaction (Searle, 1984). The autoanalyser employed a Technicon peristaltic pump and a Chem Lab Spectrophotometer. The standards (ammonium sulfate) ranged from 0-50 mg/l.

Total combustion gas chromatography

Total combustion gas chromatography for the analysis of total N in plant material was carried out in a Carlo Erba Nitrogen Analyser. Samples are oxidised by flash combustion and resulting gases are separated in a chromatographic column and detected by a thermal conductivity detector. The analyser is calibrated with the following standards: corn bran standards C25 and C23 (National Institute of Standards and Technology, USA Department of Commerce), 2-[N-Morpholino] propane sulfonic acid (MOP, BDH), Acetanilide (Alpha Resources, USA). Sucrose is used after the standards to return the analyser to baseline.

Appendix 2: Description of Fatty Acids

The convention for describing fatty acids is the ratio of the total number of carbon atoms to the number of double bonds. The position closest to the aliphatic end (w) is also included in the description as well as cis (c) and trans (t). The following prefixes are also applied to describe the fatty acid: iso (i), anteiso (a), cyclopropyl fatty acids (cyclo). For example a monounsaturated fatty acid common to Gram negative bacteria is 18:1w7c, having 18 carbon atoms and one double bond located at the seventh carbon atom from the aliphatic end of the molecule. In most analyses, several specific fatty acids overlap the same peak in the gas chromatogram. Discrimination of the proportion of the fatty acids within the peak is therefore difficult and the two are added together as sum features. The percentage of Sum features of the GC-FAME analysis may therefore include two fatty acids one of which may be the dominant or indeed the only fatty acid present.

Gas Chromatography of Fatty Acid Methyl Esters (GC-FAME) for identification of soil bacteria

The procedure for the Microbial Identification System version 3.8 (MIS, Midi, Inc[R]. Newark, Delaware, 1994) was carried out as recommended by the manufacturer.

Cell Harvest

Bacterial isolates were checked for purity on trypticase soy broth agar (TSBA). The cultures were then streaked onto four quadrants of agar plates and incubated at 28°C for 24 h. As the most stable composition of fatty acids come from the late log phase, cells (40mg ± 2) were gently scraped from the third quadrant of the plate with a 4 mm teflon inoculating loop and transferred into the base of a glass pyrex screw-top tube 13 mm x 100 mm in preparation for saponification of fatty acids. Direct GC-FAME analysis on microbial communities did not include the growth on TSBA.

Saponification of fatty acids

The cells in the glass tubes were lysed by the addition of methanolic base (1 ml). The methanolic base comprised: NaOH, 45 g; methanol, reagent grade 150 ml; distilled water 150 ml.

The tubes were vortexed for 5-10 s, heated at 100°C for 5 min, vortexed a second time for 5-10 s and heated for a further 25 min at 100°C. The tubes were then brought to room temperature.

Fatty acid Methylation

To increase the volatility of the fatty acids for the GC analysis, fatty acids were converted to fatty acid methyl esters by the addition of 2 ml of methylation reagent (6N hydrochloric acid 325 ml; methanol reagent grade, 275 ml). The tubes were vortexed for 5-10 s and heated at 80°C for 10 min then rapidly cooled to room temperature by plunging them into cold water.

Extraction of fatty acid methyl esters

Hexane and methyl-tert-butyl ether 1.25 ml (hexane, HPLC grade 200 ml; methyl-tert-butyl ether, HPLC grade, 200 ml) was added to each of the tubes, which were mixed by rotation for 10 min. The bottom aqueous phase was then discarded using a glass Pasteur pipette.

Base wash to remove residual reagents from organic extract

Base wash 3 ml (sodium hydroxide, 10.8 g; distilled water 900 ml) was then added to each tube and mixed by rotation for 5 min. Centrifugation at 2000 rpm for 3 min was then carried out to delineate and compact the interphase between the upper hexane layer and the lower aqueous phase. The upper phase was then removed and transferred into glass GC vials (2 ml). Care was taken to avoid transferring any of the interphase and the lower phase layers.

The samples in the vials were analysed by gas chromatography (Hewlett-Packard model 5890) to obtain fatty acid methyl ester profiles. An Ultra 2 column (25 m x 0.2 mm, cross-linked 5%) was used with H_2 as the carrier gas (30 ml / min) in association with a flame ionisation detector.

Fatty acid methyl ester profiles were compared with the Sherlock TSBA aerobe library version 3.8 (MIDI Inc[R]. Newark, Delaware) and a computer generated similarity index was constructed to compare the GC-FAME profile from the sample with the GC-FAME database. The likely identity of the sample was suggested based on the similarity of FAME profile in the database and sample GC-FAME profile.

Appendix 3: Buffers

Gitschier buffer

Gitschier buffer was used for *nif*D PCR. The reagents were prepared as follows: $(NH_4)_2(SO)_4$ 1 M, (16.6 ml); Tris-HCl 1 M (67 ml); $MgCl_2$ 1 M (6.7 ml); EDTA 0.5 M 1:100 dilution, pH 8.8, (1.3 ml); β-mercapto-ethanol 14.4 M (2.08 ml) made up to 200 ml with sterile water.

InterLINE 5x buffer

10 mM Tris-HCl; 50 mM KCl; 0.005% gelatine (Smida et al, 1996).

MOPS buffer

The solution contained: 3-(N-morpholino) propanesulphonic acid (0.1 M, 100 µl), formamide (500 µl), and formaldehyde (162 µl).

Phosphate buffered saline (PBS)

PBS was prepared as follows: NaCl (8 g); KCl (0.2 g); Na_2HPO_4 (1.44 g); KH_2PO_4 (0.24 g). The pH was adjusted to 7.4 with HCl (1 N) and the volume was made up to 1 litre with RO water.

Potassium Phosphate buffer (Sambrook et al, 1989)

Potassium phosphate buffer (0.1 M) pH7.0 was prepared as follows: K_2HPO_4 1 M (61.5 ml); KH2PO4 (38.5 ml); RO water (900 ml). The 0.1 M buffer was diluted to 0.05 M by adding 500 ml buffer and 500 ml RO water.

Appendix 4: Media

Media were autoclaved at 121°C for 20 min (unless otherwise stated) and allowed to cool to 50°C prior to pouring into 9 cm sterile Petri dishes. Antibiotics and vitamins when used were filter sterilised through a Millipore 0.4 μm filter and added just prior to pouring medium.

Dextrose yeast glutamic acid medium (DYGS)

Dextrose yeast glutamic acid medium (DYGS) medium was used to grow cultures in preparation for bulk nucleic acid extractions (Rodrigues et al, 1986).

The medium contained: dextrose (2 g/l); peptone (1.5 g/l); yeast extract (2 g/l); K2HPO4 (0.5 g/l); MgSO4.7H2O (0.5 g/l); L-glutamic acid (1.5 g/l); Difco Bacto-agar (15 g/l). The pH was adjusted to 6.5 with KOH. When purity was confirmed on agar plates, bacteria were grown as overnight cultures in DYGS broth in preparation for cell lysates

Four carbon sources medium (4CS)

Four carbon sources medium (4CS) nitrogen-free medium was used to isolate a broad range of free-living nitrogen-fixing bacteria (Weaver et al, 1975) and was modified according to (Heulin et al, 1987) and the modified medium is used in this work.

Solution A: $ZnSO_4.7H_2O$ (0.43 g/l); $MnSO_4.H_2O$ (1.30 g/l); $NaMoO_4.H_2O$ (0.75 g/l); H_3BO_3 (2.8 g/l); $CuSO_4.5H_2O$ (0.025 g/l); $CoSO_4.7H_2O$ (0.07 g/l).

Supersalts: consisted of: $MgSO_4.7H_2O$ (2 g/l); $CaCl_2.7H_2O$ (2 g/l); $FeSO_4.7H_2O$ (0.44 g/l); EDTA (0.40 g/l; Solution A (10 ml/l). This stock solution was autoclaved at 121°C for 20 min.

Solution B: K_2HPO_4 (60 g/l); KH_2PO_4 (40 g/l) pH 6.8. This stock solution was autoclaved at 121°C for 20 min.

The final medium included: glucose (5 g/l); mannitol (5 g/l); soluble starch (4.5 g/l); D- malic acid (2.6 g/l); NaOH (1.7 g/l); Bacto yeast extract (0.1 g/l); supersalts (50 ml/l, as described above); solution B (15 ml/l); agar (Difco Bacto-Agar 15 g/l). The medium was adjusted to pH 6.8 using 1 N NaOH and autoclaved at 110°C for 30 min.

When prepared as a semi-solid medium for the growth of inoculated plants in the spermosphere model system, the root exudates were utilised by the bacteria as carbon sources. Glucose, mannitol, soluble starch and malic acid were therefore omitted from the medium. Difco Bacto agar was reduced to 1.75g agar/l. Bacto yeast extract (0.1 g/l) was included in this medium to facilitate initial microbial growth.

Luria Bertani medium (LB)

Luria Bertani broth (LB) was used for overnight cultures for the preparation of cell lysates and DNA extract.

The medium contained: Bacto tryptone (10 g/l); Bacto yeast extract (5 g/l); NaCl (10 g/l); pH was adjusted to 7.0 with 1 N NaOH.

Nitrogen-free malate medium (Nfb)

Nitrogen-free malate medium (Nfb) is used for the isolation of *Azospirillum* sp.

The medium was prepared with the following reagents:

Malic acid (5.0 g/l); K_2HPO_4 (0.5 g/l); $MgSO_4.7H_2O$ (0.2 g/l); NaCl (0.1 g/l); $CaCl_2$ (0.02 g/l); KOH (4.5 g/l); Fe-EDTA solution (1.64% aqueous, 4.0 ml w/v); bromothymol blue 0.5% w/v in 0.2 M KOH (2.0 ml) used as an indicator); trace element solution (2.0 ml); vitamin solution (1.0 ml) and Bacto yeast extract (20 mg). The pH was adjusted to 6.8 with KOH and agar 1.75 g/l was added. The media was made up to one litre with RO water.

FeEDTA solution was prepared as follows: $FeSO_4.7H_2O$ (708.9 mg), EDTA (948.6 mg) made up to 100 ml in RO water.

Trace element solution was prepared by adding: $NaMoO_4.2H_2O$ (0.2 g/l); $MnSO_4.H_2O$ (0.235 g/l); H_3BO_3 (0.28 g/l); $CuSO_4.5H_2O$ (0.008 g/l; $ZnSO_4.7H_2O$ (0.024 g/l).

The vitamin stock solution was prepared with biotin (Sigma 0.01 g/l) and pyridoxine (Sigma 0.02 g/l). The vitamin solution was diluted 1:50 and filter sterilised through 0.45 μm membrane filter and 1ml of the dilution added per litre.

Isolation of bacteria from the semi-solid medium was carried out by taking a loop of bacteria from the pellicle and streaking the bacteria onto solid Nfb medium containing congo red (0.25% w/v in water) and agar (1.5%). Plates were incubated at 28°C. Any red colonies were taken to be indicative of *Azospirillum* sp.

Nutrient broth (NB)

Difco nutrient broth (NB) (0.8% in RO water) was used in overnight cultures grown for the preparation of cell lysates, DNA extractions and for the growth of cultures in preparation for storage with glycerol at -80°C.

Nutrient agar (NA)

Difco nutrient agar (NA) was prepared using Difco nutrient broth (0.8% w/v) and Difco Bacto Agar (1.5% w/v) made up in RO water.

Phosphate azelaic acid trytamine medium (PCAT)

Phosphate azelaic acid tryptamine (PCAT) N-free medium is selective for *Burkholderia cepacia* (Burbage and Sasser, 1982).

The medium was prepared with the following reagents:

$MgSO_4$ (0.1 g/l); azelaic acid (2.0 g/l); tryptamine (0.2 g/l) was agitated in several drops of ethanol until dissolved; K_2HPO_4 (4 g/l); KH_2PO_4 (4 g/l); Bacto yeast extract (0.02 g/l); agar (15 g/l). The medium was adjusted to pH 5.7 with KOH (1 M).

Potato dextrose agar (PDA)

Difco potato dextrose agar (one quarter strength) contained (Difco potato dextrose broth 1%w/v) and (Difco Bacto Agar (1.5 % w/v) made up with RO water.

Tryptic Soy Broth (TSB)

Difco tryptic soy broth (TSB) broth was used in overnight cultures and for the preparation of cell lysates. The medium contained tryptic soy broth (Difco, 3% w/v) in RO water.

Tryptic Soy Agar (TSA)

Difco Tryptic soy agar (TSA) medium was used as a general growth medium and contained tryptic soy broth (Difco 3%w/v) and Difco Bacto-Agar (1.5%) made up in RO water.

Yeast mannitol medium (YM)

This medium was used to grow *Rhizobium* sp. used as reference strains for PCR and rRNA hybridisation studies.

The medium was prepared as follows: mannitol (10 g/l); Bacto yeast extract (0.4 g/l); K_2HPO_4 (0.5 g/l); $MgSO_4.7H_2O$ (0.2 g/l); NaCl (0.1g/l); Difco Bacto-agar (15g/l). The pH was adjusted to 6.8 with 1 N NaOH.

Stonier's medium for antagonism studies

Stonier's medium was prepared to test isolates for antagonistic responses to each other. The medium was prepared as an agar base and an overlay medium.

Stonier's agar (10 ml) was poured into the base of a Petri dish. The medium included the following reagents g/l. Agar (15 g), Biotin (0.002 g), $Fe(NO_3)_3.9H_2O$ (0.008 g), Potassium citrate (10.00 g), L-Glutamic acid (2 g), NH_4NO_3 (2.7 g), $NaSO_4.7H_2O$ (0.2 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (0.2 g), $MnCl_2$ (0.0009 g), $ZnCl_2$ (0.0005 g), H_2O (1 l), pH 7.0.

Overlay medium (5 ml) comprised g/l, Na_2HPO_4 (17.3 g), $Na_2H_2PO_4$ (12.2 g), Agar (Oxoid, 7 g), H_2O (1 l).

References

Alexander, D.B. and Zuberer, D.A. 1989. Impact of soil environmental factors on rates of N_2-fixation associated with roots of intact maize and sorghum plants.
In: *Nitrogen Fixation with Non-legumes*. Eds. Skinner, F.A., Boddey, R.M. and Fendrik, I. Kluwer Academic Publishers, Netherlands.

Amman, R.I., Ludwig, W. and Schleifer, K.H. 1995. Phylogentic identification and *in situ* detection of individual microbial cells without cultivation. *Microbial Review,* **59:** 143-169.

Amman, R., Snaidr, J., Wagner, M., Ludwig, W. and Scheifer, K.H. 1996. *In situ* visualisation of high genetic diveristy in a natural microbial community. *Journal of Bacteriology,* **178:** 3496-3500.

Arnold, W., Rump, A., Klipp, W., Priefer, U.B. and Puhler, A. 1988. Nucleotide sequence of a 24, 206-base-pair DNA fragment carrying the entire nitrogen fixation cluster of *Klebsiella pneumoniae. Journal of Molecular Biology,* **203:** 715-738.

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J. and Hartmann, A. 1995. *In situ* localisation of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labelled rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. *Applied and Environmental Microbiology,* **61:** 1013-1019.

Bååth, E. and Arnebrånd, K. 1994. Growth rate and response of microbial communities to pH in limed and ash treated forest soil. *Soil Biology and Biochemistry,* **29:** 995-1001.

Balandreau, J. 1986. Ecological factors and adaptive processes in N_2-fixing populations of the plant environment. *Plant and Soil,* **90:** 73-92.

Barber, D.A. and Lynch, J.M. 1977. Microbial growth in the rhizosphere. *Soil Biology and Biochemistry,* **9:** 305-308.

Barrett-Lennard, E.G. and Dracup, M. 1987. A porous medium for improving the growth of plants under sterile conditions. *Plant and Soil*, **108:** 294-298.

Bashan, Y. 1985. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biology and Biochemistry*, **18:** 297-301.

Bashan, Y., Levanony, H. and Mitiku, G. 1989. Changes in proton efflux of intact wheat roots induced by *Azospirillum brasilense* Cd. *Canadian Journal of Microbiology,* **35:** 691-697.

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

Bashan, Y., Levanony, H, and Whitmoyer, R.E. 1991. Root surface colonisation of non-cereal crops by pleomorphic *Azospirillum brasilense* Cd. *Journal of General Microbiology*, **137:** 187-196.

Bashan, Y., Puente, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin,G., Ferrara-Cerrato, R. and Pedrin, S. 1995. Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. *Applied and Environmental Microbiology*, **61:** 938-1945.

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

Bashan, Y., Hernandez, J.P., Leyva, L.A. and Bacilio, M. 2002. Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. *Biology and Fertility of Soils*, **35(5):** 359-368.

Beijerinck, M.W. 1925. Über ein Spirillum, welches freien Stickstoff binden kann? *Centralblatt für Bakerilogie*, Abt. II **63:** 353-357.

Belotte, D., Currien, J.B., Maclean, R.C. and Bell, G. 2003. An experimental test of local adaptation in soil bacteria. *Evolution,* **57:** 27-36.

Benjacob, E., Cohen, I. And Gutnick, D.L. 1998. Cooperative organisation of bacterial colonies from genotype to morphotype. *Annual Review of Microbiology,* **52:** 779-806.

Berge, O., Fages, J., Mulard, D. and Balandreau, J. 1990. Effects of inoculation with *Bacillus circulans* and *Azospirillum lipoferum* on crop-yield in field grown maize. *Symbiosis,* **9:** 259-266.

Berge, O., Heulin, T., Achouak, W., Bally, R. and Balandreau, J. 1991. *Rahnella aquatilis,* a nitrogen-fixing enteric bacterium associated with the rhizosphere of wheat and maize. *Canadian Journal of Microbiology,* **37:** 195-203.

Bevivino, A., Sarrocco, S., Dalmastri, C., Tabacchioni, S., Cantale, and Chiarini, L. 1998. Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia* – effect of seed treatment on disease suppression and growth of maize. *FEMS Microbiology Ecology,* **27(3):** 225-237.

Bhattarai, T. and Hess, D. 1997. Growth and yield response of a Nepalese spring wheat cultivar to the inoculation with Nepalese *Azospirillum* spp. at various levels of nitrogen fertilization. *Biology and Fertility of Soils,* **46:** 72-77.

Birnboim, H.C. and Doly, J.C. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research,* **7:** 1513-1522.

Blakemore, L.C, Searle, P.L. and Daly, B.K. 1987. *Methods for chemical analysis of soils.* New Zealand Soil Bureau of Science, Rep. 80.

Bloemberg, G.V. and Lugtenberg, B.J.J. 2001. Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Current Opinion in Plant Biology,* **4(4):** 343-350.

Bole, J.B. 1977. *Introductory Plant Physiology.* Second edition. Prentice-Hall Inc. New Jersey, USA.

Bothe, H., Korsgen, H., Lehmacher, T. and Hundeshagen, B. 1992. Differential effects of *Azospirillum,* auxin and combined nitrogen on the growth of the roots of wheat. *Symbiosis,* **13:** 167-179.

Bowen, G.D. and Rovira, A.D. 1969. The influence of microorganisms on growth and metabolism of plant roots. In: *Root Growth.* Ed. Whittingon, W.J. Butterworths, London. p 170-210.

Bremner, J.M. and Mulvaney, C.S. 1982. Nitrogen – total. In: *Methods of Soil Analysis Part 2, Chemical and Microbiological Properties.* Second edition. Ed. Page, A.L. Agronomy No 9, American Society of Agronomy and Soil Science Society of America, Madison, Wisconsin, USA.

Brulé, C., Frey-Klett, P., Pierrat, J.C., Courrier, S., Gérard, F., Lemoine, M.C., Rousselet, J.L., Sommer, G and Garbaye, J. 2001. Survival in the soil of the ectomycorrhizal fungus *Laccaria bicolor* and the effects of a mycorrhizal helper *Pseudomonas fluorescens. Soil Biology and Biochemistry,* **33:** 1683-1694.

Budzikiewicz, R. 1993. Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiological Reviews,* **104:** 209-228.

Burbage, D.A. and Sasser, M. 1982. A medium selective for *Pseudomonas cepacia. Phytopathology Abstracts,* **72:** 706.

Buyer, J.S. and Drinkwater, L.E. 1997. Comparison of substrate utilization assay and fatty acid analysis of soil microbial communities. *Journal of Microbial Methods,* **30:** 3-11.

Cavigelli, M.A., Robertson, G.P. and Klug, M.J. 1995. Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. *Plant and Soil,* **170:** 99-113.

Cawse, P.A. 1975. Microbiology and biochemistry of irradiated soils. *Soil Biochemistry*, **3:** 312-268.

Charlton, W.A. 1996. Lateral root initiation. In: *Plant Roots. The Hidden Half.* Second edition. Eds. Waisel, Y., Eshel, Y. and Kafkafi, U. Marcel Dekker Inc., New York.

Chen, C., Bauske, G., Musson, G. and Kloepper, J.W. 1994. Biological control potential and population dynamics of endophytic bacteria in a cotton/*Fusarium* wilt system. In: *Improving Plant Productivity with Rhizosphere Bacteria.* Eds. Ryder, M.H., Stephens, P.M. and Bowen, G.D. C.S.I.R.O. Australia.

Ciccillo, F., Fiore, A., Bevivino, A., Dalmastri, C., Tabacchioni, S. and Chiarini, L. 2002. Effects of two different application methods of *Burkholderia ambifaria* MCI 7 on plant growth and rhizosphere bacterial diversity. *Environmental Microbiology,* **4(4):** 238-245.

Chiarini, L., Bevivino, A., Tabacchioni, S. and Dalmastri, C. 1998. Inoculation of *Burkholderia cepacia, Pseudomonas fluorescens* and *Enterabacter sp.* on *Sorghum bicolor* – root colonisation and plant growth promotion of dual strain inocula. *Soil Biology and Biochemistry*, **30:** 81-87.

Chotte, J.L., Schwartzmann, A., Bally, R. and Monrozier, J.L. 2002. Changes in bacterial communities and *Azospirillum* diversity in soil fractions of a tropical soil under 3 or 19 years of natural fallow. *Soil Biology and Biochemistry*, **34(8):** 1083-1092.

Creus, C.M., Sueldo, R.J. and Barassi, C.A. 1996. *Azospirillum* inoculation in pregerminating wheat seeds. *Canadian Journal of Microbiology*, **42:** 83-86.

Daims, H., Bruhl, A., Amman, R., Schleifer, K.H. and Wagner, M. 1999. The domain specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. *Systems of Applied Microbiology*, **22:** 434-444.

Dart, P. 1986. Nitrogen fixation associated with non-legumes in agriculture. In: *Nitrogen fixation with non-legumes*. Eds. Skinner, F.A. and Uomala, P. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

de Freitas, J.R. and Germida, J.J. 1990. A root tissue culture system to study winter wheat-rhizobacteria interactions. *Applied Microbiology and Biochemistry*, **3**: 589-595.

de Freitas, J.R. 2000. Yield and N assimilation of winter wheat (*Triticum aestivum* L., var Norstar) inoculated with rhizobacteria. *Pedobiologia*, **44(2)**: 97-104.

DeLuca, T.H., Drinkwater, L.E., Wiefling, B.A. and DeNicola, D.M. 1996. Free-living nitrogen-fixing bacteria in temperate cropping systems: influence of nitrogen source. *Biology and Fertlity of Soils*, **23 (2)**: 140-144.

de Weert, S., Vemeiren, H., Mulders, I.H.M., Kuiper, I., Hendrickx, N., Bloemberg, G.V., Vanderleyden, J., De Mot, R. and Lugtenberg, B.J.J. 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonisation by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions*. **15(11)**: 1173-1180.

Delwyche, C.C. and Bryan, B.A. 1976. Denitrification. *Annual Review of Microbiology,* **30**: 241-262.

de Zamaroczy, M. 1995. Genetic control of nitrogen assimilation and nitrogen fixation in free-living *Azospirillum brasilense*: a review. In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology.* Eds Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Dierksen, K., Whittaker, G.W., Banowetz, G., Azevedo, M., Kennedy, A., Steiner, J. and Griffith, S. 2002. High resolution characterization of soil biological communities by nucleic acid and fatty acid analyses. *Soil Biology and Biochemistry,* **12**: 1853-1860.

Dobereiner, J., Baldani, V.L.D. and Reis, V.M. 1995. Endophytic occurrences of diazotrophic bacteria in non-leguminous crops. Review, In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology.* Eds Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Drew, M.C., Saker, L.R. and Ashley, T.W. 1973. Nutrient supply and the growth of the seminal rot system in barley. *Journal of Experimental. Botany,* **24:** 1198-1202.

Drew, 1975. Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytologist,* **75:** 479-490.

Dunne, C., Moenn-Loccoz, Y., de Bruijn, F.J. and O'Gara, F. 2000. Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimatum* by *Stenotrophomonas maltophilia* strain W81. *Microbiology,* **146:** 2069-2078.

Dusenberry, D.B. 1998. Fitness landscape for effects of shape on chemotaxis and other behaviours of bacteia. *Journal of Bacteriology,* **180(22):** 5978-5983.

El-Komy, H.M., Hamdia, M.A. and El-Baki, G.K.A. 2003. Nitrate reductase in wheat plants grown under water stress and inoculated with *Azospirillum* spp. *Biologia Plantarum,* **46:** 281-287.

England, L.S., Lee. H. and Trevors, J.T. 1993. Bacterial survival in soil: effect of clays and protozoa. *Soil Biology and Biochemistry,* **25:** 525-531.

Ewens, M. and Leigh, R.A. 1985. The effect of nutrient solution composition on the length of root hairs of wheat (*Triticum aestivum* L.). *Journal of Experimental Botany,* **36:** 713-724.

Fages, J. 1992. An industrial view of *Azospirillum* inoculants. Formulation and application technology. *Symbiosis*, **13**: 15-26.

Fallik, E. and Okon, Y. 1995. Inoculants of *Azospirillum brasilense*: biomass production, survival and growth promotion of *Setaria italica* and *Zea mays*. *Soil Biology and Biochemistry*, **28**: 123-126.

Fedi, S., Montaini, P. and Favilli, F. 1992. Chemotactic response of *Azospirillum* toward root exudates of C3 and C4 plants. *Symbiosis*, **13**: 101-105.

Felle, H. 1987. Proton transport and pH control in *Sinapsis alba* root hairs: a study carried out with double-barelled pH microelectrodes. *Journal of Experimental Botany*, **38**: 340-354.

Fierer, N., Schimel, J.P. and Holden, P.A. 2003. Influence of drying-re-wetting frequency on soil bacterial community structure. *Microbial Ecology*, **45**: 63-71.

Flouri, F., Sini, K. and Balis, C. 1995. Interactions between *Azospirillum* and *Phialophora radicola*. In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology*. Eds Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Frostegård, A, Tunlid, A. and Bååth, E. 1993. Phospholipid fatty acid composition, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Applied and Environmental Microbiology*, **59**: 3605-3617.

Fulchieri, M. and Frioni, L. 1994. *Azospirillum* inoculation on maize (*Zea mays*): effect on yield in a field experiment in central Argentina. *Soil Biology and Biochemistry*, **26**: 921-923.

Furina, E.K., Bonartseva, G.A. and L'vov, N.P. 1999. Effect of various concentrations of oxygen, molybdenum and nitrate, on nitrogen fixation and denitrification in *Azospirillum lipoferum*. *Applied Biochemistry and Microbiology*, **35**: 44-47.

Garcia de Salomone, I. and Dobereiner, J. 1996. Maize phenotype effects on the response to *Azospirillum* inoculation. *Biology and Fertility of Soils*, **21**: 193-196.

Gardner, W.K., Parberry, D.G. and Barber, D.A. 1982. The acquisition of phosphorus by *Lupinusalbus* L. II. The effect of varying phosphorus supply and soil type on some characteristics of soil/root interface. *Plant and Soil*, **68**: 3-41.

Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G and Van Elsas, J.D. 1999. Assessment of bacterial community structure in polymerase chain reaction and denaturing gradient gel electrophoresis. *Journal of Microbial Methods*, **38**: 1-15.

Germida, J.J. and Walley, F.L. 1996. Plant growth-promoting rhizobacteria alter rooting patterns and abuscular mycorrhizal fungi colonization of field-grown spring wheat. *Biology and Fertility of Soils*, **23(2)**: 113-120.

Giesler, L.J. and Yuen, G.Y. 1998. Evaluation of *Stenotrophomonas maltophilia* strain C3 for biocontrol of brown patch disease. *Crop Protection*, **17**: 509-513.

Giller, K.E., Day, J.M., Dart, P.J. and Wani, S.P. 1984. A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$. *Journal of Microbiological Methods*, **2**: 307-316.

Giller, K.E. and Day, J.M. 1985. Nitrogen fixation in the rhizosphere: significance in natural agricultural systems. In: *Biological interactions in soil*. Ed. Fitter, E.H. Blackwell Oxford, U.K.

Glick, B.R., Karaturovic, D.M. and Newell, P.C. 1995. A novel procedure for rapid isolation of plant growth promoting pseudomonads. *Canadian Journal of Microbiology*, **41**: 533-536.

Gori, A. and Favilli, F. 1995. First results on individual and dual inoculation with *Azospirillum – Glomus* on wheat. 1996. In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology.* Eds Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer - Verlag. Germany.

Griffiths, B.S., Ritz, K., Ebblewhite, N. and Dobson, G. 1999. Soil microbial community structure: effects of substrate loading rates. *Soil Biology and Biochemistry,* **31:** 145-153.

Haack, S.K., Garchow, H., Odelosn, D.A., Forney, L.J. and Klug, M.J. 1994. Accuracy, reproducibility and interpretation of Fatty Acid Methyl Ester profiles of model bacterial communities. *Applied and Environmental Microbiology,* **60(7):** 2483-2493.

Halsall, D.M., Turner, G.L. and Gibson, A.H. 1985. Nitrogenase activity of a range of diazotrophic bacteria on straw, straw breakdown products and related compounds. *Soil Biology and Biochemistry,* **21:** 291-298.

Halsall, D.M. 1993. Inoculation of wheat straw to enhance lignocellulose breakdown and associated nitrogenase activity. *Soil Biology and Biochemistry,* **25:** 419-429.

Han, S.O. and New P.B. 1996. The variation of nitrogen fixing ability among natural isolates of *Azospirillum.* 11th Australian Nitrogen-fixing Conference, Nedlands, W.Aust.

Hansen, A. 1994. *Symbiotic N_2 fixation of crop legumes.* Margraf verlag, Weikersheim, Germany.

Harari, A., Kigel, J. and Okon. Y. 1989. Involvement of IAA in the interaction between *Azospirillum brasilense* and *Panicum miliaceum* roots. In: *Nitrogen Fixation with Non-legumes.* p 227-234. Kluwer Academic Publishers.

Hardy, R.W.F., Burns, R.C. and Holsten, R.D. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry*, **5**: 47-81.

Harris, G.A. and Campbell, G.S. 1989. Automated quantification of roots using a simple image analyzer. *Agronomy Journal*, **81**: 935-938.

Heijnen, C.E., Hok-a Hin, C.H. and Van Veen, J.A. 1992. Improvements to the use of bentonite clay as a protective agent, increasing survival levels of bacteria introduced into soil. *Soil Biology and Biochemistry*, **24**: 533-538.

Heijnen, C.E., Hok-a Hin, C.H. and Van Veen, J.A. 1993. Root colonisation by *Pseudomonas fluorescens* introduced into soil amended by bentonite. *Soil Biology and Biochemistry*, 25: 239-246.

Hery, M., Zazaret, S., Jaffre, T., Normand, P. and Navarro, E. 2003. Adaptation to nickel spiking of bacterial communities in neocalidonian soils. *Environmental Microbiolgy*, **5**: 3-12.

Heulin, T., Guckert, A. and Balandreau, J. 1987. Stimulation of root exudation of rice seedlings by *Azospirillum* strains: carbon budget under gnotobiotic conditions. *Biological Fertility of Soils,* **4**: 9-14.

Heulin, T., Rahman, M., Omar, A.M.N., Pierrat, J.C. and Balandreau, J. 1989. Experimental and mathematical procedures for comparing N_2-fixing efficiencies of rhizosphere diazatrophs. *Journal of Microbiological Methods*, **9**: 163-173.

Hoflich, G. and Ruppel, S. 1994. Growth stimulation of pea after inoculation with associative bacteria. *Microbiological Research*, **149**: 99-104.

Hoflich, G. and Wiehe, W. 1994. Growth stimulation in graminees, legumes and crucifers by *Pseudomonas* bacteria. In: *Azospirillum VI and Related Microorganisms. Genetics-Physiology - Ecology*. Eds Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Jacoud, C., Faure, D., Wadoux, P. and Bally, R. 1998. Development of a strain-specific probe to follow inoculated *Azospirillum lipoferum* CRTI under field conditions and enhancement of maize root development by inoculation. *FEMS Microbiology Ecology*, **27:** 43-51.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. and Sait, M. 2002. Improved culturability of soil bacteria in pure culture of novel members of the divisions *Acidobacteria, Actinobacteria, Proteobacteria* and *Verrucomicrobia*. *Applied and Environmental Microbiology*, **68 (5):** 2391-2396.

Jones, D.L., Farrar, J. and Giller, K.E. 2003. Associative nitrogen fixation and root exudation – what is theoretically possible in the rhizosphere. *Symbiosis*, **35 (1-3):** 19-38.

Jones, H., Tomas, A.D., Leigh, R.A. and Jones, R.G.W. 1983. Water-relation parameters of epidermal and cortical cells in the primary root of *Triticum aestivum* L. *Planta*, **158:** 230-236.

Jungk, A.O. 1996. Dynamics of nutrient movement at the soil-root interface. In: *Plant Roots. The Hidden Half*. Second edition. Eds. Waisel, Y., Eshel, Y. and Kafkafi, U. Marcel Dekker Inc., New York.

Kaneda, T. 1991. Isi - and anteiso-fatty acids in bacteria: biosynthesis, function and taxonomic significance. *Microbiological Reviews*, **55 (2):** 288-302.

Kapulnik, Y., Sarig, S., Nur, I. and Okon, Y. 1983. Effect of *Azospirillum* inoculation on yield of field-grown wheat. *Canadian Journal of Microbiology*, **29:** 895-899.

Kapulnik, Y., Okon, Y. and Henis, Y. 1985. Changes in root morphology of wheat caused by *Azospirillum* inoculum. *Canadian Journal of Microbiology*, **31:** 881-887.

Kapulnik, Y. 1996. Chpt 32. Plant-growth promoting rhizobacteria. In: *Plant Roots. The Hidden Half.* Eds. Waisel, Y., Eshel, Y. and Kafkafi, U. Marcel Dekker Inc., New York.

Karpati, E., Kiss, P., Marini, F., Buglioni.S., Fendrik, I and Del Gallo, M. 1995. Molecular study of the interaction of *Azospirillum lipoferum* with wheat germ agglutinin. In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology.* Eds. Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Kennedy, I.R. 1988. The molecular basis of symbiotic nitrogen fixation. In: *Microbiology in Action.* Eds. Murrell, W.G. and Kennedy, I.R. pp 143-155. Research Studies Press/Wiley. Chichester, U.K.

Kennedy, I.R. 1993. *Biological Nitrogen Fixation. A manual for the study of associative systems.* Institutional Links Program Workshop, Shandong University, Jinan, China.

Kirchhof, G. 1992. Measurement of root length and thickness using a hand-held computer scanner. *Field Crops Research,* **29:** 79-88.

Kirchhof, G and Hartmann, A. 1992. Development of gene probes for *Azospirillum* based on 23S-rRNA sequences. *Symbiosis,* **13:** 27-35.

Kirchhof, G., Schloter, M., Assmus, B. and Hartmann, A. 1997. Molecular microbial ecology approaches applied to diazotrophs associated with non-legumes. *Soil Biology and Biochemistry,* **29:** 853-862.

Kloepper, J.W. and Beauchamp, C.J. 1992. A review of issues related to measuring colonisation of plant roots by bacteria. *Canadian Journal of Microbiology,* **38:** 1219-1232.

Klucas, R.V. 1991. Associative nitrogen fixation in plants. In: *Biology and Biochemistry of Nitrogen Fixation.* Ed. Dilworth, M.J. and Glenn, A.R. Elsevier, Amsterdam.

Koch, E., Kempf, H.J. and Hessenmuller, A. 1998. Characterisation of the biological activity and evaluation of potential plant growth-promoting properties of selected rhizobacteria. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz-Journal of Plant Diseases and Protection,* **105:** 567-580.

Kozdrój, J., Dirk van Elsas, J. 2001. Structrural diversity of microbial communities in arable soils of a heavily industrialised area determined by PCR-DGGE fingerprinting and FAME profiling. *Applied Soil Ecology,* **17:** 31-42.

Kozyrovska, N., Kovtunovych, G., Gromosova, E., Kuharchuk, P. and Kordyum, V. 1996. Novel inoculants for an environmentally-friendly crop protection. *Resources Conservation and Recycling,* **18:** 79-85.

Krieg, N.R. and Dobereiner, J. 1984. Genus *Azospirillum.* In: *Bergey's Manual of Systematic Bacteriology,* Vol1. Eds. Krieg, N.R. and Holt, J.G. p 94-104. Williams and Wilkins, Baltimore, London.

Kreitz, S. and Anderson, T.H. 1997. Substrate utilization patterns of extractable- non-extractable bacterial fractions in neutral and acidic beech forest soils. In: *Microbial Communities.* Eds. Insam, H. and Rangger, I. Springer Verlag, Berlin.

Lammana, A.C., Gestwicki, J.E., Strong, L.E. Bourchardt, S.L., Owen, R.M. and Kiessling, L.L. 2002. Conserved amplification of chemotactic responses through chemoreceptor interactions. *Journal of Bacteriology,* **184(18):** 4981-4987.

Lawler, K., Knight, B., Barbosa-Jefferson, V.L., Lane, P.W., Lilley, A.K., Paton, G.I., McGrath, S.P., O'Flaherty, S.M. and Hirsch, P. 2000. Comparison of methods to investigate microbial populations in soils under different agricultural management. *FEMS Microbial Ecology,* **33:** 129-137.

Lifshitz, R., Kloepper, J.W. and Kozlowski, M. 1987. Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. *Canadian Journal of Microbiology,* **33:** 390-395.

Manz, W., Amman, R., Wagner, M. and Schleifer, K.H. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Systems of Applied Microbiology,* **15:** 593-600.

Marschner, P., Yang, C.H., Liebereia, R and Crowley, D.E. 2001. Soil and plant effect on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry,* **11:** 1437-1445.

Martens, D.A. and Frankenberger Jr., W.T. 1994. Assimilation of exogenous $2/^4$C-indole-3-acetic acid and $3/^{14}$C-tryptophan exposed to the roots of three wheat varieties. *Plant and Soil,* **166:** 281-290.

Matthyse, A. 1992. Adhesion, bacterial. In: *Encyclopaedia of Microbiology Vol 1.* Academic Press.

Mehnaz, S., Mirza, M.S., Bally, R., Normand, P., Bario, A. and Malik, K.A. 2001. Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. *Canadian Journal of Microbiology,* **47:** 110-117.

Merbach, W., Ruppel, S. and Schulze, J. 1998. Dinitrogen fixation of microbe-plant associations as affected by nitrate and ammonium supply. *Isotopes in Environmental Health Stud*ies, **34:** 67-73.

Michiels, K., Vanderleyden, J. and Van Gool, A. 1989. *Azospirillum* - plant root associations : A review. *Biology and Fertility of Soils,* **8:** 356-368.

Mills, D.K., Fitzgerald, K., Litchfield. C.D. and Gillevet, P.M. 2003. A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. *Journal of Microbiological Methods,* **54:** 57-74.

Mirza, M.S., Ahmad, W., Latif, F., Haurat, J., Bally, R., Normand, P. and Malik, K.A. 2001. Isolation, partial characterisation and the effect of plant growth-promoting bacteria (PGPR) on micro-propagated sugarcane *in vitro. Plant and Soil,* **237:** 47-54.

Moulin, F., Lemanceau, P. and Alabouvette, C. 1994. Control by fluorescent pseudomonads of *Pythium aphanidermatum* root rot, responsible for yield reduction in soilless culture of cucumber. In: *Improving Plant Productivity with Rhizosphere Bacteria.* Eds. Ryder, M.H., Stephens, P.M. and Bowen, G.D. CSIRO. Australia.

Musson, G., Meinroy, J.A. and Kloepper, J.W. 1995. Development of delivery systems for introducing endophytic bacteria into cotton. *Biocontrol Science and Technology,* **5:** 407-416.

Muthukumarasamy, R., Revathi, G. and Loganathan, P. 2002. Effect of inorganic N on the population, *in vitro* colonization and morphology of *Acetobacter diazotrophicus* (syn *Gluconacetobacter diazotrophicus*). *Plant and Soil,* **243(1):** 91-102.

Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J. and Tahara, S. 1999. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar-beet damping-off disease. *Applied and Environmental Microbiology,* **65(10):** 4334-4339.

Nayudu. M., Groom, K.A.E., Fernance, J., Wong, P.T.W. and Turnbull, K. 1994. The genetic nature of biological control of the take-all fungal pathogen by *Pseudomonas.* In: *Improving plant productivity with rhizosphere bacteria.* Eds. Ryder, M.H., Stephens, P.M. and Bowen, G.D. CSIRO. Australia.

New, P. and Kennedy, I. 1989. Regional distribution and pH sensitivity of *Azospirillum* associated with wheat roots in eastern Australia. *Microbial Ecology,* **17:** 299-309.

Newbould, P. 1989. The use of nitrogen fertiliser in agriculture. Where do we go practically and ecologically? *Plant and Soil,* **115:** 297-311.

Nunes, C., Usall, J., Texido, N., Fons, E. and Vinas, I. 2002. Post harvest biological control by *Pantoea agglomerans* (CPA-Z) on Golden Delicious apples. *Journal of Applied Microbiology*, **92(2):** 247-255.

Okon, Y., Albrecht, S.L. and 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:** 86-88.

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

Okon, Y. and Labandera-Gonzales, C.A. 1994. Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. *Soil Biology and Biochemistry*, **26:** 1591-1601.

Olsen, G.J., Woese, C.R. and Overbeek, R. 1994. The winds of (evolutionary) change: breathing new life into microbiology. *Journal of Bacteriology*, **176:** 1-6.

Olsson, P and Wallander, H. 1998. Interactions between ectomycorrhizal fungi and the bacterial community in soils amended with various primary minerals. *FEMS Microbiology Ecology*, **27:** 195-205.

Omar, N., Berge, O., Shalaan, S.N., Hubert, J., Heulin, T. and Balandreau, J. 1992. Inoculation of rice with *Azospirillum brasilense* in Egypt. Results of five different trials between 1985 and 1990. *Symbiosis*, **13:** 281-289.

Omay, S.H., Schmidt, W.A. and Martin, P. 1992. Indoleactic acid production by the rhizosphere bacterium *Azospirillum brasilense* Cd under *in vitro* conditions. *Canadian Journal of Microbiology*, **39:** 187-192.

Omar, N., Heulin, T., Weinhard, P., Alaa El-Din, and Balandreau, J. 1989. Field inoculation of rice with *in vitro* selected plant-growth promoting-rhizobacteria. *Agronomie*, **9:** 803-808.

Page, A.L., Miller, R.H. and Keeney, D.R. 1982. Nitrogen-Inorganic forms. In *Methods of Soil Analysis Part 2. Chemical and Microbioological Properties.* Second edition. American Society of Agronomy Inc., Madison, USA.

Papendick, R.I. and Campbell, 1981. Theory and measurement of water potential. In: *Water potential relations in soil microbiology.* SSSA Special Publication Number 9. Proc Div. S-1 and S-3 of Soil Science Soc. of America. Chicago, Illinois, Dec 3-8, 1978. Soil Science Society of America.

Patriquin, D.G., Dobereiner, J. and Jain, D.K. 1983. Sites and processes of association between diazotrophs and grasses. *Canadian Journal of Microbiology,* **29:** 900-915.

Pederson, R.N., Black, I.D., Dyson, C.B. and Hannam, R.J. 1994. Effects of the herbicide metasulfuron-methyl on root length, nutrient levels, grain protein and yield of barley. *Australian Journal of Experimental Agriculture,* **34:** 499-504.

Pennanen, T., Fritze, H. and Bååth, E. 1996. Phospholipid fatty acid composition and heavy tolerance of soil microbial communities along heavy metal polluted gradients in coniferous forests. *Applied and Environmental Microbiology,* **62:** 420-428.

Rademaker, J.L.W. and de Bruijn, F.J. 1997. Characterisation and classification of microbes by rep-PCR genomic fingerprinting and computer-assisted pattern analysis. In: *DNA markers: Protocols, Applications and Overviews.* Eds. Caetano-Anollés G. and Gresshoff P.M. J.Wiley & Sons Inc.

Rahman, K.S.M., Tharira-Rahman, J., Lakshmanaperumalsamy, P. and Banat, I.M. 2002. Towards crude oil degrdation by a mixed bacterial consortium. *Bioresource Technology,* **85 (3):** 257-261.

Rajendran, N., Matsuda, O., Imamura, N. and Urushigawa, Y. 1992. Variation in microbial biomass community structure in sediments of eutrophic bays as determined by phospholipid ester-linked fatty acids. *Applied and Environmental Microbiology,* **58:** 562-571.

Ramsay, A.J. and Bawden, A.D. 1983. Effects of sterilization and storage on respiration, nitrogen status and direct counts of soil bacteria using acridine orange. *Soil Biology and Biochemistry*, **15:** 263-268.

Razi, S.S. and Sen, S.P. 1996. Amelioration of water stress effects on wetland rice by urea-N, plant growth regulators, and foliar spray of a diazotrophic bacterium *Klebsiella* sp. *Biology and Fertility of Soils*, **23(4):** 454-458.

Richie, N.J. Schutter, M.E., Dick, R.P. and Myrold, D.D. 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterise microbial communities in soil. *Applied and Environmental Microbiology*, **66:** 1668-1675.

Rodrigues, N.J., Malavolta Jr., V.A. and Victor, O. 1986. Meio simples para o isolamento e cultivo de *Xanthomonas campestris* pv. citri tipo B. *Summa Phytopathologica*, **12(1-2):** 16.

Rovira. A. D. 1965. Interactions between plant roots and soil microorganisms. *Annual Review of Microbiology*, **19:**241-266.

Ruppel, S., Hecht-Buchholz, C., Remus, R., Hartmann, U. and Schmelzer, R. 1992. Settlement of the diazotrophic, phytoeffective bacterial strain *Pantoea agglomerans* on and within winter wheat: an investigation using ELISA and transmission microscopy. *Plant and Soil*, **145:** 261-273.

Ruppel, S. and Merbach, W. (1997). Effect of ammonium and nitrate on $^{15}N_2$-fixation of *Azosprillum* spp. and *Pantoea agglomerans* in association with wheat plants. *Microbiological Research*, **152:** 377-383.

Sadasivan, L. and Neyra, C. 1985. Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: exopolysaccharides and cyst formation. *Journal of Bacteriology*, **163:** 716-723.

Sait, M., Hugenholtz, P. and Janssen, P.H. 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environmental Microbiology*, **4 (11):** 654-666.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual.* Second Edition. Ed. Nolan, C., Cold Spring Harbor Laboratory Press.

Sarig, S., Blum, A. and Okon, Y. 1988. Improvement of water status and yield of field-grown grain sorghum (*Sorghum bicolor*) by inoculation *with Azospirillum brasilense. Journal of Agricultural Science*, **110:** 271-277.

Sarwar, M and Kremer R.I. 1995. Enhanced suppression of plant growth through production of L-tryptophan-derived compounds by deleterious rhizobacteria. *Plant and Soil*, **172(2):** 261-269.

Sattelmacher, B. and Thoms, K. 1989. Root growth and ^{14}C translocation into the roots of maize (*Zea mays* L.) as influenced by local nitrate supply. *Zeitschrift fur Pflanzenernahrung und Bodenkunde*, **152:** 7-10.

Searle, P.L. 1984. The Berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review. *Analyst*, **109:** 549-568.

Shi, Y. and Duke, T. 1998. Cooperative model of bacterial sensing. *Physical Review*, **A 58(5 part B):** 6399-6401.

Siciliano, S.D., Theoret, J.R., de Freitas, J.R., Hucl, P.J. and Germida, J.J. 1998. Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Canadian Journal of Microbiology*, **44:** 844-851.

Siciliano, S.D. and Germida, J.J. 1998. Biolog analysis and fatty acid methyl ester profiles indicate that Pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii. Soil Biology and Biochemistry*, 30(13): 171-1723.

Silveser, W.B. and Musgrave, D.R. 1991. Free-living diazotrophs. In: *Biology and Biochemistry of Nitrogen Fixation.* Eds. Dilworth, D.J. and Glenn, A.R. Elsevier Science Publishers. Netherlands.

Smida, J., Leibhard, S., Nickel, A.M., Eckardt-Schupp, F and Hieber, L. 1996. Application of repetitive sequence-based PCR (Inter-LINE PCR) for the analysis of genomic rearrangements and for the genome characterisation on different taxonomic levels. *Genetic Analysis: Biomolecular Engineering,* **13:** 95-98.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. and Scheifer, K. 1997. Phyogenetic analysis and *in situ* identification of bacteria in activated sludge. *Applied and Environmental Microbiology,* **63:** 2884-2896.

Soil Survey Staff, 1975 . *Soil taxonomy. A basic system for making and interpreting soil surveys.* Handbook 436, Soil Conservation Service, USDA, Washington DC.

Soil Survey Staff. 1990. Keys to soil taxonomy. 4th Edition. *Soil Management Support Services Technical Monograph No 6.* Virginia Polytechnic Institute and State University, Blacksburg, USA.

Sprent, J.I. 1979. The metabolic problems of biological fixation. In: *The Biology of Nitrogen-fixing Organisms.* McGraw-Hill Book Company (UK), Ltd. UK.

Sprent, J.I. and James, E.K. 1995. N_2-fixation by endophytic bacteria: questions of entry and operation. In: *Azospirillum VI and Related Microorganisms. Genetics-Physiology - Ecology.* Eds. Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer -Verlag. Germany.

Stace, H.C.T., Hubble, G.D., Brewer, R., Northcote, K.H., Sleeman, J.R., Mulcahy, M.J. and Hallsworth, E.G. 1968. *A Handbook of Australian Soils.* Rellim, Glenside, South Australia.

Stahl, D.A. and Flesher, B. 1987. *Applications of ribosomal RNA sequencing to studies of microbial ecology.* Gray Freshwater Biological Institute, Summer Course, July 6-10.

Steenhoudt, O. and Vanderleyden, J. 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiological Reviews*, **24:** 487-506.

Taylor, B.L. and Zhulin, I.B. 1998. In search of higher energy - metabolism-dependent behaviour in bacteria. *Molecular Microbiology*, **28(4):** 684-690.

Thirup, J., Johansen, A and Winding, A. 2003. Microbial succession in the rhizosphere of live and decomposing barley roots as affected by the antagonistic strain *Pseudomonas fluorescens* DR 54-BN14 or the fungicide imazalil. *FEMS Microbiology Ecology*, **43:** 383-392.

Thomas-Bauzon, D., Weinhard, P., Villecourt, P. and Balandreau, J. 1982. The spermosphere model. I. Its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. *Canadian Journal of Microbiology*, **28:** 922-928.

Turner, G.L. and Gibson, A.H. 1980. Measurement of nitrogen fixation by indirect means. In: *Methods of Evaluating Biological Nitrogen Fixation*. Ed. Bergersen, F.J. John Wiley and Sons Pty. Ltd.

Ueckert, J., Niemann, E.G. and Fendrik, I. 1992. Mixed continuous suspended and immobilised culture of diazotrophic isolates from root-free soil and the endorhizosphere of *Leptochlea fusca L.* Kunth. *Symbiosis*, **12:** 75-84.

Ueckert, J. and Fendrik, I. 1995. Continuous culture application in physiological investigations on diazotrophic bacteria. In: *Azospirillum VI and Related Microorganisms. Genetics- Physiology - Ecology.* Eds. Fendrik, I., del Gallo, M., Vanderleyden, S.J. and de Zamaroczy, M NATO ASI Series Vol. G 37. Springer - Verlag. Germany.

Umali-Garcia, M., Hubbell, D.H., Gaskins, M.H. and Dazzo, F.B. 1980. Association of *Azospirillum* with grass roots. *Applied and Environmental Microbiology,* **39:** 219-226.

Umesha, S., Dharmeshi, S.M., Shetty, S.A., Krishnappa, M and Shetty, H.S. 1998. Biocontrol of downy mildew disease of pearl millet using *Pseudomonas fluorescens. Crop Protection,* **17(5):** 387-392.

Urquiaga, S., Botteon, P.B.L. and Boddey, R.M. 1989. Selection of sugar cane cultivars for associated biological nitrogen fixation using ^{15}N -labelled soil. In: *Nitrogen fixation with non-legumes.* Eds. Skinner, F.A., Boddey, R.M. and Fendrik, I. Kluwer Academic Publishers, The Netherlands.

van Tran, V., Omar, N., Heulin, T., Berge, O. and Balandreau, J. 1994. Selection of bacteria for enhanced plant growth and results of field tests. In: *Improving plant productivity with rhizosphere bacteria.* Proc.3rd International workshop on Plant Growth-Promoting Bacteria. Adelaide, South Australia. Eds. Ryder, M., Stephens, P.M. and Bowen, G.D.

van Tran, V., Berge, O., Balandreau, J., Ke, S.N. and Heulin, T. 1996. Isolation and nitrogenase activity of *Burkholderia vietnamiensis,* a nitrogen-fixing bacterium associated with rice (*Orza sativa* L) on a sulphate acid soil of Vietnam. *Agronomie,* **16(8):** 479-491.

van Tran, V., Ngôkê, S., Berge, O., Faure, D., Bally, R., Hebbar, P. and Heulin, T. 1997. Isolation of *Azospillum lipoferum* from the rhizosphere of rice by a new, simple method. *Canadian Journal of Microbiology,* **43:** 486-490.

Vázquez, M.M., César, S., Azcón, R. and Barea, J.M. 2000. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. *Applied Soil Ecology,* **15:** 261-272.

Vidhyasekaran,P, Rabindran, R., Muthamilan, M., Nayar, K., Subramaruan,N. and Vasumathi, K. 1997. Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. *Plant Pathology*, **46(3):** 291-297.

Volpon, G.T., De Polli, H. and Dobereiner, J. 1981. Physiology of nitrogen fixation in *Azospirillum lipoferum* Br17 (ATCC29709). *Archives of Microbiology,* **128:** 371-375.

Weaver, P.K., Wall, J.D. and Gest, H. 1975. Characterisation of *Rhodopseudomonas capsulata*. *Archives of Microbiol*ogy, **105**: 207-216.

Whipps, J.M. and Lynch, J.M. 1983. Substrate flow and utilisation in the rhizosphere of cereals. *New Phytologist*, **95:** 605-623.

Woese, C.R. 1987. Bacterial evolution. *Microbiological Reviews*, **51(2):** 221-271.

Wright, S.A.I., Zumoff, C.H., Schneideer, L. and Beer, S.V. 2001. *Pantoea agglomerans* strain EH 318 produces two antibiotics that inhibit *Erwinia amylovora in vitro*. *Applied and Environmental Microbiology*, **67(1):** 284-292.

Zadoks, J.C., Chang, T.T., Korzak, C.F. 1974. A decimal code for the growth stages of cereals. *Weed Research*, **14:** 415-421.

Zimmer, W. and Bothe, H. 1989. The phytohormonal interactions between *Azospirillum* and wheat. In *Nitrogen Fixation with Non-legumes*. p.137-145. Ed. Skinner, A. Kluwer Academic Publishers, The Netherlands.

Zimmer, W., Hundesgagen, B. and Niederau, E. 1994. Demonstration of the indolepyruvate decarboxylase gene in different auxin producing species of the *Enterobacteriaceae*. *Canadian Journal of Microbiology*, **40(12):** 1072-1076.