INVESTIGATION INTO THE DIVERSITY OF ANTIFUNGAL AEROBIC ENDOSPORE-FORMING BACTERIA ASSOCIATED WITH BULK AND CROP RHIZOSPHERE SOIL

By

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"Lord I thank you for allowing me to meet everyone who has guided, encouraged, supported and assisted me in achieving this milestone in my life"

ABSTRACT

Members of the genus *Bacillus* are mainly Gram positive, aerobic rod shaped, endospore-forming bacteria that are increasingly being recognised for their ability to promote plant growth and antagonise fungal pathogens. From a biological control perspective, *Bacillus* spp. strains that produce antifungal compounds are of particular interest. In this study, aerobic endospore-formers were isolated from an undisturbed indigenous grassland soil and screened for antifungal activity and other plant growth promoting traits. Endospore-formers were also isolated from rhizosphere soil associated with the roots of maize, wheat and kale grown in pots containing soil from the same grassland site. Microbial diversity amongst isolates showing antifungal activity was investigated using different molecular fingerprinting methods, namely, intergenic transcribed spacer–PCR (ITS-PCR), random amplified polymorphic DNA-PCR (RAPD-PCR) and 16S rRNA gene amplification and sequencing. Characterization of the active antimicrobial compound(s) associated with selected isolates was also attempted.

Prior to isolating from bulk and rhizosphere soils, samples were pre-heated to eliminate heat sensitive vegetative cells. Mean endospore counts were; wheat rhizosphere, Log 6.03 c.f.u g^{-1} soil; maize rhizosphere, Log 5.88 c.f.u g^{-1} soil; kale rhizosphere Log 5.90 c.f.u g^{-1} soil; and bulk soil Log 5.67 c.f.u g^{-1}soil. A total of three hundred and eighty-four isolates were screened for antagonism towards *Rhizoctonia solani* using dual-culture plate bioassays. Thirty four of the isolates (~9%) mostly isolated from the bulk soil inhibited *R. solani* at varying degrees. Differences in antimicrobial interactions were apparent in *in vitro* bioassay; supposedly due to different concentrations and/or types of antimicrobial compounds. Biochemical tests for amylase, cellulase, chitinase, and proteinase activity, siderophore production and inorganic phosphate solubilisation were conducted. None of the isolates possessed all of these attributes and only a few showed multiple traits. Ninety-one percent of the isolates exhibited proteinase activity, 76% were able to hydrolyze starch whereas only four displayed cellulase activity. Only four isolates from the bulk-soil were capable of solubilising inorganic phosphate.

ITS-PCR and 16S rRNA gene sequence analysis showed high levels of genetic homology amongst isolates and the majority were closely associated with representatives of the *B. cereus* group. Isolate C76 was the exception, being closely matched with *B. subtilis*. ITS-PCR banding

profile was useful for distinguishing between species but did not distinguish within species. RAPD-PCR distinguished finer levels of genetic diversity between and within sample sets, with primer OPG-11 showing the greatest levels of heterogeneity. DNA extraction methods and the influence of template DNA dilution were investigated to determine their influence on RAPD-PCR analysis reproducibility. Prominent bands were comparable for crude template- and kit-extracted DNA but slight changes in band intensity and in some instances, additional faint bands were observed. At the highest DNA concentrations tested (7 µg/ml), further bands with molecular weights above 2.5 kbp were apparent. Strict standardization of PCR conditions greatly reduced variability of the RAPD-PCR analysis.

Isolates from the different sample sets were screened for the presence of genetic markers associated with the biosynthesis of zwittermicin A, an aminopolyol antibiotic produced by some members of the *B. cereus* group. In an initial screen only one isolate, W96, yielded PCR amplicons consistent with those previously reported in the literature for the zwittermicin A genes. Later a further sixteen isolates grouped with W96 on the basis of the RAPD-PCR fingerprinting profiles, were screened for the presence of these genes. Of these, only six showed PCR amplification products similar to W96. Sequence homology testing against the GenBank database confirmed the presence of the zwittermicin A genes in these isolates. Isolate W96 was selected for further extraction and characterization of its antifungal compound(s). However, after culturing in various broth media cell free supernatants of W96 failed to show antifungal activity *in vitro* even when the supernatants were concentrated 20-fold.

These findings provide a general overview of the diversity of aerobic endospore-forming bacteria present in an undisturbed indigenous grassland soil that exhibited antifungal activity *in vitro* and the limited influence tested crop rhizospheres have on this diversity. Combined use of ITS-PCR, 16S rRNA sequencing and RAPD-PCR techniques served as a rapid and effective means of grouping isolates for further investigations of their potential use as biocontrol agents and plant growth promoting rhizobacteria.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations and it has not been submitted for a higher degree at any other institution.

Jolly Musoke

C. Hunter (Supervisor)

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Chapter 1: Literature Review

1.1 Introduction

The ability of certain micro-organisms to act as environmentally friendly alternatives to synthetic fungicides been has been increasingly reported on in the literature (Ongena and Jacques, 2007). The attributes associated with these micro-organisms include biological control of fungal phytopathogens and plant growth-promotion (McSpadden Gardener, 2004; Carzorla *et al*., 2007). Biological control involves the use of native and/or non-native organisms to suppress the activity and survival of undesirable organisms, such as soil-borne phytopathogens (Siddiqui, 2006, Yobo, 2005, Avis *et al*., 2008). Biocontrol of these pathogens has largely been attributed to plant growth-promoting rhizobacteria (PGPR), which are found in close association with plant roots (Compant *et al*., 2005; Siddiqui, 2006). PGPR exhibit a number of direct or indirect inhibition mechanisms, such as antibiosis, siderophore production and induced systemic resistance that benefit plant growth and productivity. This can result in improved seed germination, seedling emergence, mineral utilization and healthier root systems (Siddiqui, 2006; Yobo, 2005). *Azospirillum*, *Bacillus* and *Pseudomonas* are some examples of PGPR that have been extensively studied (Antoun and Prevost, 2006; Avis *et al*., 2008). This review will focus on *Bacillus* spp. as biological control and plant growth-promoting agents.

Members of the genus *Bacillus* are regarded as promising plant growth-promoting agents, due to their ability to antagonise fungal pathogens and stimulate plant growth (McSpadden Gardener, 2004). These organisms exhibit various antifungal mechanisms, such as antibiosis and nutrient competition, which assist in controlling soil-borne phytopathogens (Mena-Violante and Olalde-Portugal, 2007; Ramarathnam *et al*., 2007). Additionally, their ability to produce endospores that are highly tolerant of harsh conditions facilitates the formulation of stable biocontrol products with prolonged shelf life viability and more environmental application options (Selim *et al*., 2005; Ongena and Jacques, 2007). *Bacillus* spp. are found ubiquitously in soil where they have adapted to complex soil conditions. They are also routinely isolated from rhizosphere environments where they may function as plant growth-promoting rhizobacteria (PGPR) (Silo-Suh *et al*., 1994; Selim *et al*., 2005). A number of *Bacillus* spp., such as, *B. subtilis*, *B.*

licheniformis, *B. amyloliquefaciens, B. cereus* and *B. thuringiensis* have been reported to exhibit PGPR potential (Daffonchio *et al*., 1998a; Fernando *et al*., 2006).

A major limitation of microbial inoculants, including *Bacillus* spp., is their inconsistency in efficacy when applied to field environments. As a result, only a few strains have been successfully commercialized (Nelson, 2004; Mohammadipour *et al*., 2009). This varied performance is attributed in part to poorly understood biological mechanisms which influence *in situ* levels of biological agents (Berg *et al*., 2002; Ongena and Jacques, 2007; Athukorala *et al*., 2009). Plant type is thought to play a key role in determining the composition of rhizosphere communities and therefore has an important impact on rhizobacteria population dynamics. Factors influencing an organism's ability to successfully colonize plants and function within diverse environments are still not fully understood (Emmert and Handelsman, 1999; Whipps, 2001). There is also limited information on the distribution and diversity of native *Bacillus* populations occurring in various ecosystems (Garbeva *et al*., 2004).

1.2 *Bacillus*

Members of the genus *Bacillus* are Gram Positive, mainly aerobic rod-shaped bacteria that are able to form dormant endospores that can survive harsh environmental conditions (Shoda, 2000; Freitas *et al*., 2008). They size from 0.5 - 2.5 μm × 1.2 – 10 μm and the cells are usually motile with peritrichous flagella (Holt *et al*., 2000; Garbeva *et al*., 2003; Freitas *et al*., 2008). *Bacillus* spp. are chemoorganotrophs and exhibit either aerobic or facultative anaerobic metabolism (Holt *et al*., 2000; Freitas *et al*., 2008). Within this genus high levels of physiological and genetic diversity have been detected using conventional morphological and phenotypic techniques (Tewelde, 2004). Recent advances in molecular phylogenetic methods such as 16S rRNA/DNA sequence analysis have shown that members of the genus need to undergo further taxonomic separation, due to the high levels of diversity observed within the genus (Ash *et al*., 1991; Logan *et al*., 2008).

The ability to form endospores is an important survival trait since it allows vegetative cells to differentiate into resting structures when conditions are unfavourable; for example, when nutrients are depleted or when cell densities reach a critical threshold (Mizumoto *et al*., 2006). Endospores are extremely resistant to a wide range of harsh environmental conditions, such as

heat, desiccation, UV and chemical extremes. This adaptation gives *Bacillus* PGPRs a distinct biotechnological advantage over other less robust biocontrol agents, such as the *Pseudomonadas* (Kavitha *et al*., 2005). The ability to form endospores assists in the isolation of *Bacillus* spp. from environmental samples, since only these structures will survive a heat pre-treatment step (80°C for 10-30 min) that eliminates all vegetative cells.

Most species of *Bacillus* are considered biologically safe to humans and the interaction of saprophytic bacilli with humans and plants are generally non-pathogenic (Shoda, 2000). Notable exceptions include *B. anthracis,* which can be fatal to humans and animals (Cherif *et al*., 2003). Additionally, certain strains of *B. cereus* can cause food poisoning and spoilage (Logan, 2008; Jensen *et al*., 2003; Manzano *et al*., 2009). Numerous *Bacillus* strains synthesize a variety of beneficial substances such as hydrolytic enzymes, antibiotics, probiotics and commercially important solvents (Wu *et al*., 2006). An example is *B. licheniformis* which has been used for the commercial production of enzymes such as α-amylase, proteases and Penicillinase as well as Bacitracin antibiotics (Daffonchio *et al*., 1998b). *Bacillus* strains are therefore important in both agricultural and biotechnological contexts (Cazorla *et al*., 2007).

Bacillus spp. are found in an array of terrestrial habitats which include close associations with plants where they occur either as epiphytes or endophytes (Logan, 2008; Garbeva *et al*., 2003). Many *Bacillus* spp. are ubiquitous in soil and are readily culturable from bulk soil, rhizosphere soil, rhizoplane and phytosphere (McSpadden Gardener, 2004; Cazorla *et al*., 2007; Avis *et al*., 2008). It is difficult to accurately predict the overall distribution, prevalence and diversity of *Bacillus* spp. in soil, due to the great variation in environmental conditions and complex biological interactions occurring in this habitat (McSpadden Gardener, 2004). Nonetheless, it has been suggested that the majority of culturable *Bacillus* spp. isolated from agricultural soil are phenotypically and phylogenetically related to *B. cereus* and *B. subtilis* (McSpadden Gardener, 2004). Other *Bacillus* species isolated from agricultural soils include *B. benzoevorans*, *B. lentus* and *B. coagulans* (Garbeva *et al*., 2003).

1.2.1 Bacillus ecology

Bacillus spp. play essential roles in ecosystem functioning; for example, their ability to utilize a wide range of organic materials originating from plants and animals allows recycling of carbon, nitrogen and phosphorous within the environment (Garbeva *et al*., 2003). These processes are facilitated through the production of extracellular enzymes such as cellulase, glucanases, proteases and chitinases that hydrolyse complex polymers within the soil environment. *Bacillus* spp., such as *B. macerans* and *B. azotofixans* can also fix atmospheric nitrogen from the inert molecular state (N_2) into compounds such as ammonia, nitrate or nitrogen dioxide (Tewelde, 2004). Ultimately, the ecological functions of *Bacillus* spp. contribute towards the composition and quality of soil (Garbeva *et al*., 2003). These mechanisms and interactions have been harnessed by mankind for improved crop yields and other biotechnological applications.

1.2.2 *Bacillus* in the rhizosphere environment

The rhizosphere is the narrow zone of soil directly influenced by plant roots (Berg and Smalla, 2009). It represents a complex environment where chemical, physical and biological interactions are distinct from those in the surrounding bulk soil. Up to 40% of photosynthates produced by plants are released as root exudates into the rhizosphere (Atlas and Bartha, 1998; Baudoin *et al*., 2002; Nelson, 2004). Root exudates are comprised of different sugars, amino acids, carbohydrates, vitamins and keto acids; hence, they serve as a source of substrates which can support large microbial populations (Atlas and Bartha, 1998; Whipps, 2001; Garbeva *et al*., 2004). Consequently, the rhizosphere is perceived as a region that supports a higher microbial mass than the surrounding bulk soil (Brimecombe *et al*., 2001).

Micro-organisms within the rhizosphere may arise from seed-borne populations or native residents in the adjacent bulk soil (Brimecombe *et al*., 2001). Interactions between microbes and plants can either be beneficial, harmful or neutral to plant health and productivity. *Bacillus* spp. can positively influence plant health directly or indirectly through various mechanisms such as increasing nutrient availability to the host plant, antagonising and suppressing phytopathogens, inducing systemic plant resistance and by producing plant growth regulators (McSpadden Gardener, 2004; Ongena and Jacques, 2007). Soil-borne phytopathogens are also attracted to the rhizosphere environment which usually serves as the infection court where disease interactions

take place. Ultimately, plant health and nutrition are influenced to a large extent by the competitive balance between PGPR and phytopathogens (Brimecombe *et al*., 2001). Biotechnological initiatives use this competiveness to harness environmentally friendly antimicrobial agents, such as antibiotics, biosurfactants and enzymes (Berg and Smalla, 2009).

Several biotic and abiotic factors influence the structural and functional diversity of bacterial communities within the rhizosphere (Figure 1.1), with plant species and soil type being the two main influences (Nelson, 2004; Berg and Smalla, 2009). It is not clear which of these is the key determinant and several reports suggest that either one can dominate depending on prevailing conditions. Berg *et al*. (2002) demonstrated the plant specificity of *Verticillium dahliae* Kleb. associated antagonists, showing that plant species were selective for specific microbial antagonists. Root exudates are thought to be the driving force of this plant specificity. The quality and quantity of root exudates vary with plant type, and are influenced by various factors such as cultivar, physiological maturity, the prevailing physiochemical conditions, as well as root morphology and location (Atlas and Bartha, 1998; Baudoin *et al*., 2002; Palomino *et al*., 2005; Hawkes *et al*., 2007). Consequently, root exudates will affect relative bacterial abundance, community and activity within the rhizosphere as the microbes respond differently and have unique metabolic requirements (Baudoin *et al*., 2002; Nelson, 2004). The distribution of rhizobacteria along the root surface is not uniform and they tend to congregate at sites with elevated root exudation. For example, root tips are considered an important site for exudation of simple sugars and organic acids, hence rhizobacteria aggregate in high densities in this region (Brimecombe *et al*., 2001). Further investigations into factors influencing the extent of plant-specificity and other underlying mechanisms of this interaction are needed, particularly for effective application of biocontrol agents.

It is essential for biological control agents to colonize, function and persist within the rhizosphere for sustainable plant growth-promotion to be achieved. In other words, PGPR need to be rhizosphere competent (Kumari and Srivastava, 1999; Choudhary and Johri, 2009). The colonization process involves recognition, adsorption, anchoring, colonization, growth, interactions with the host plant and an ability to genetically express their plant growth-promoting traits (Brimecombe *et al*., 2001). It is of great importance for the rhizobacteria to sense and respond to root exudates and/or chemotactants released by plants, to initiate colonization (Berg

and Smalla, 2009). Ultimately, factors influencing rhizodeposition will affect root colonization. Many *Bacillus* spp. are motile, which provides a competitive advantage over non-motile micro-organisms during early root colonization. This is essential in plant growth promotion as early establishment of these bacilli in high numbers assists in out-competing phytopathogens and has a positive influence on seed germination and early root growth. Zheng and Sinclair (2000) demonstrated the importance of motility using *B. megaterium* B153-2-2 non-motile mutants. They showed that there is a strong correlation between flagellation and an ability to colonize roots. Rhizobacteria should also be able to utilize root exudates and initiate plant-bacterium interaction by attaching themselves to the root surfaces. Root attachment can be achieved by cell-surface proteins, fimbriae or capsular polysaccharides (Berg and Smalla, 2009). In *Bacillus* spp. extracellular polysaccharide production and the ability to form biofilms assists their ability to attach to root surfaces. Other characteristics that assist *Bacillus* spp. in their rhizosphere competence are their ability to synthesise a spectrum of secondary metabolites, such as biosurfactants and antibiotics. Ongena and Jacques (2007) reported that the synthesis of surfactin by *B. subtilis* strains played an important role in facilitating microbial motility, biofilm formation and/or antagonistic activity towards competing micro-organisms. Although *Bacillus* spp. exhibit various traits that allow them to persist within the rhizosphere, variable field performances are often observed (Raupach and Kloepper, 1998; Emmert and Handelsman, 1999). This has been linked to factors that limit root colonization over a range of diverse environments as well as low cell densities and/or short-lived inocula (Whipps, 1997). Factors that regulate the utilization of available substrates and interactions with native organisms are still not fully understood (Whipps, 2001; Nelson, 2004; Avis *et al*., 2008). Further research into *Bacillus* colonization of plant roots is therefore needed in order to enhance biotechnological applications.

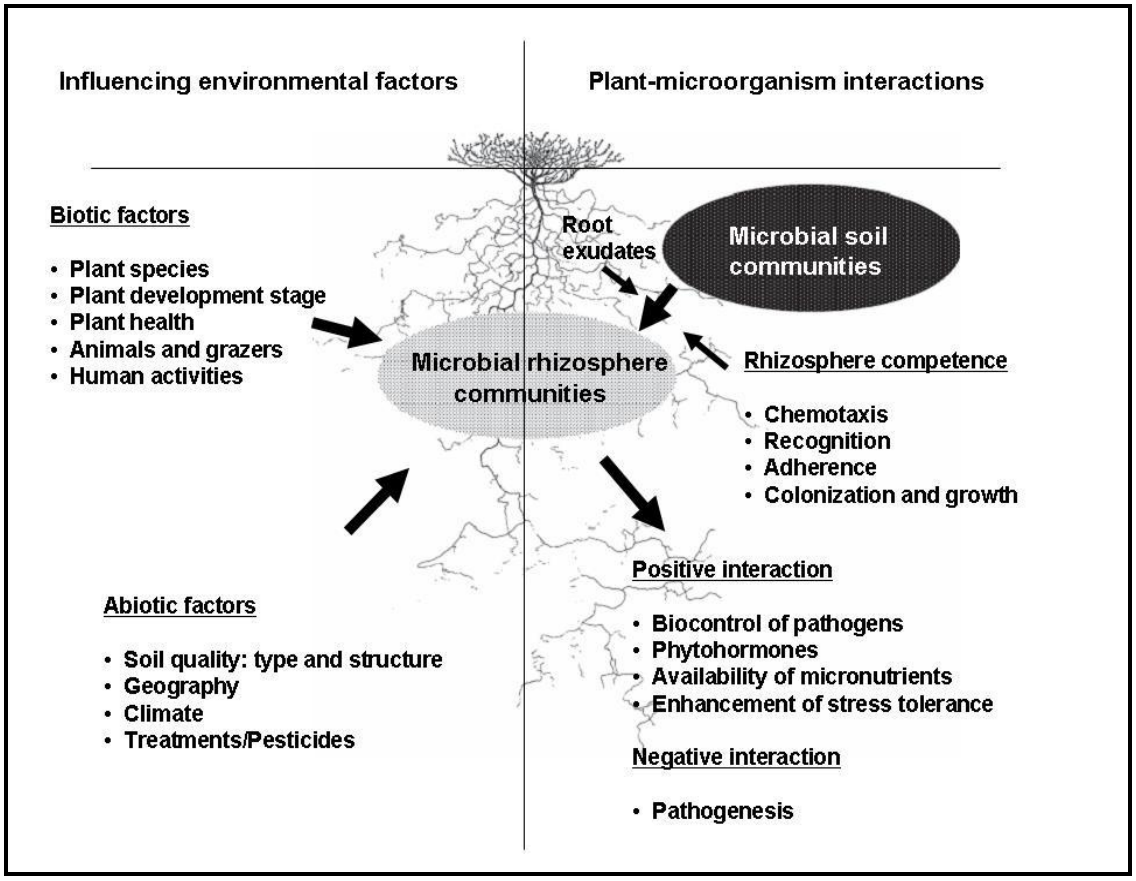


Figure 1.1: Factors influencing rhizosphere microbial competence (Source: Berg and Smalla, 2009).

1.2.3 Biotechnological importance

Various *Bacillus* spp. exhibit biotechnological potential; examples include *B. subtilis* which has been used as a biological fungicide, while *B. thuringiensis* serves as a biological insecticide and *B. mycoides* as a plant growth-promoting rhizobacterium (PGPR) (de Freitas *et al*., 1997; Daffonchio *et al*., 1998a; Nelson, 2004; Ongena and Jacques, 2007; Herman *et al*., 2008). As PGPR, *Bacillus* strains can enhance both plant health and productivity (Thomashow *et al*., 1997; Herman *et al*., 2008).

Direct plant growth-promotion may entail increasing nutrient availability to plants through the solubilisation of nutrients. For example, *B. subtilis* strains have been shown to solubilise organic and inorganic phosphates, thus increasing its availability to plants (Probanza *et al*., 2001; McSpadden Gardener, 2004; Stein, 2005). *Bacillus* spp. can also produce volatile compounds

such as 2, 3-butanediol and/or phytohormones such as gibberellins, auxins and cytokinins that have been shown to stimulate plant tissue growth and regulate plant growth, respectively (Atlas and Bartha, 1998; McSpadden Gardener, 2004; Mena-Violante and Olalde-Portugal, 2007).

Indirect mechanisms of plant growth-promotion may entail enhancing nutrient availability to plants through promotion of mycorrhizal/rhizobial symbioses with host plants. Examples include *B. cereus* strains which promote symbioses between *Bradyrhizobium japonicum* and soybeans (Kloepper *et al*., 2004). However, the most common mechanism of indirect plant growth is based on the biological control of phytopathogens which can result in healthier plants with increased yields (Nelson, 2004; Selim *et al*., 2005). *Bacillus* spp. exhibit a range of these biologically active mechanisms (Athukorala *et al*., 2009). Amongst them is nutrient competition, for example, association with the production of iron chelating compounds, siderophores. *B. subtilis* has been shown to produce the catecholic siderophore, 2, 3-dihdroxybenzotlglycine (Shoda, 2000; May *et al*., 2001). These compounds facilitate the scavenging and uptake of ferric iron (Fe^{3+}) when it is present in low concentration in the surrounding environment. This excludes iron from pathogens which require it for growth, thereby indirectly protecting the host plant (Nelson, 2004; Herman *et al*., 2008; Choudhary and Johri, 2009). *Bacillus* spp. can also stimulate plant defence through triggering an induced systemic response, enabling plants to resist subsequent pathogen infection (McSpadden Gardener, 2004). For example, *B. subtilis* FZB-6 has been shown to stimulate systemic resistance in tomatoes against *Fusarium* spp. (Ongena and Jacques, 2007). The production of inhibitory catabolic enzymes such as chitinase and protease can also antagonise phytopathogens through fungal cell wall degradation (McSpadden Gardener, 2004). However, antimicrobial mechanisms in *Bacillus* spp. have mainly been associated with antibiotic production (Romero *et al*., 2007a; Herman *et al*., 2008).

1.3 *Bacillus* antibiotics

Antibiotics are defined as compounds of microbial origin that inhibit or disrupt normal growth of other micro-organisms usually at low concentrations (Thomashow *et al*., 1997; Lancini and Demain, 1999). Members of the genus *Bacillus* can synthesize a wide range of antibiotics (Table 1.1). Some *Bacillus* strains produce multiple antibiotic compounds which are thought to be partly the reason for their broad spectrum of activity against phytopathogens (Garbeva *et al*., 2003; Ongena and Jacques, 2007). Peptide antibiotics appear to be the major group of antimicrobial compounds produced by members of the *Bacillus* genus. Antibiotics such as polyketides, aminopolyols and aminoglycosides have also been characterised (Silo-Suh *et al*., 1994; Stein, 2005; Ongena and Jacques, 2007).

1.3.1 Peptide antibiotics

Among the many peptide antibiotics studied, lipopeptides have received a great deal of attention due to their antifungal activity and association with biocontrol organisms. The main lipopeptide families associated with *Bacillus* spp. are iturin, surfactin and fengycin (Ongena and Jacques, 2007). These lipopeptides are largely amphiphilic with a cyclic structure, consisting of a β-amino or β-hydroxy fatty acid integrated into a peptide moiety, as seen in Figure 1.2 (Romero *et al*., 2007b). Lipopeptide antibiotics sometimes incorporate unusual constituents, such as D- amino acids (Stein, 2005).

1.3.1.1 Iturin

The iturin family which is commonly synthesised by *B. subtilis* and *B. amyloliquefaciens* is represented by iturin, bacillomycin and mycosubtilin (Ongena and Jacques, 2007; Guez *et al*., 2008). These antibiotics are composed of a heptapeptide linked to a β-amino fatty acid chain, as illustrated in Figure 1.2B (Aranda *et al*., 2005; Romero *et al*., 2007b). Iturins exhibit strong antifungal activity against a wide range of fungi, including *Fusarium oxysporum* Schlecht. and *Rhizoctonia solani* Kűhn (Fernando *et al*., 2006; Rodrigues *et al*., 2006; Romero *et al*., 2007b). These antibiotics mainly affect the membrane structure of fungal cells. The fatty acid component of iturin interacts with cholesterol molecules in the host's plasma membrane, resulting in the formation of ion-conducting pores which lead to osmotic imbalances (Ongena and Jacques,

2007; Romero *et al*., 2007b). Nuclear membranes and other cytoplasmic organelles are also disrupted by this compound. Iturin has also been shown to cause cytoplasmic aggregation and/or disruption of plasma membrane by formation of small vesicles (Rodrigues *et al*., 2006).

1.3.1.2 Surfactin

Surfactin antibiotics are comprised of heptapeptides linked to a β-hydroxy fatty acid chain (Figure 1.2A) and are commonly produced by *B. subtilis*, *B. coagulans*, *B. pumilus* and *B. licheniformis* (Maget-Dana and Ptak, 1995; Vollenbroich *et al*., 1997). These antibiotics are exceptional biosurfactants and are thought to contribute to rhizosphere competence through lowering surface tension; which assists in biofilm formation and motility towards favourable environments (Ongena and Jacques, 2007).

Surfactins have limited antifungal activity because cholesterol associated with eukaryote membranes seems to destabilise the antibiotic (Maget-Dana and Ptak, 1995; Romero *et al*., 2007b). Hence, surfactins' target range depends on membrane sterol content (Ongena and Jacques, 2007). Nonetheless, the antimicrobial action of surfactin relies on inserting its fatty acid chain into the host membrane's lipid bilayer, thereby causing permeability changes (Rodrigues *et al*., 2006). Unlike iturins which bring about osmotic imbalances caused by ion-conducting pores, surfactins disrupt the lipid membrane and at high concentrations can result in complete membrane disintegration (Vollenbroich *et al*., 1997; Ongena and Jacques, 2007). Additionally, the biosurfactant nature of surfactin also serves as an anti-adhesion agent inhibiting pathogen biofilm formation (Rodrigues *et al*., 2006; Romero *et al*., 2007b).

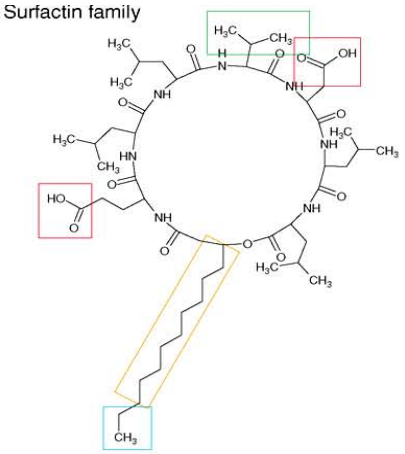
1.3.1.3 Fengycin

The fengycin family, also referred to as plipastatins, are biosynthesised mainly by *B. subtilis*, *B. amyloliquefaciens*, *B. cereus* and *B. thuringiensis* strains (Kim *et al*., 2004; Ongena and Jacques, 2007). These antibiotics are composed of a decapeptide linked to a β-hydroxy fatty acid chain, as shown in Figure 1.2C (Stein, 2005). In some instances, fengycin can have an unusual amino acid, L-ornithine, in the peptide moiety portion of its structure (Romero *et al*., 2007b). Fengycins exhibit strong antifungal activity, especially against filamentous fungi, where they interact with the host lipid membrane causing alterations in membrane structure and permeability (Deleu *et al*., 2005; Ongena and Jacques, 2007).

Table 1.1: Examples of *Bacillus* antibiotics

Producing organism(s)	Antibiotic	Structure	Range	Reference
B. cereus	Kanosamine	3-amino-3-deoxy-D-glucose	Antifungal, Antibacterial	Milner *et al*. 1996a
B. cereus/ B. thuringiensis	Zwittermicin A	Linear aminopolyol	Anti-oomycete Antifungal, Antibacterial, Insecticidal,	Fernando *et al*. 2006; Athukorala *et al*. 2009
B. cereus	Cerexins	Acylpeptide	Antibacterial	Tewelde, 2004
B. subtilis ATCC 6633	Rhizocticin A	Phosphono-oligopeptide	Antifungal	Kavitha *et al*. 2005
B. subtilis	Iturin	Cyclic lipopeptide	Antifungal, Antibacterial,	Tewelde, 2004; Rodrigues *et al*. 2006
	Surfactin	Acidic cyclic lipopeptide	Biosurfactant, Slightly antifungal Antibacterial, Antimycoplasma, Antiviral	Maget-Dana and Ptak, 1995; Rodrigues *et al*. 2006; Romero *et al*. 2007b
	Fengycin	Cyclic lipopeptide	Antifungal	Deleu *et al*. 2005
Bacillus licheniformis	Bacitracin	Cyclic polypeptide	Topical antibacterial, metal ion binding	Tewelde, 2004
Bacillus polymyxa	Polymyxin	Cyclic acylpeptide	Antibacterial	Katz and Demain, 1977

A

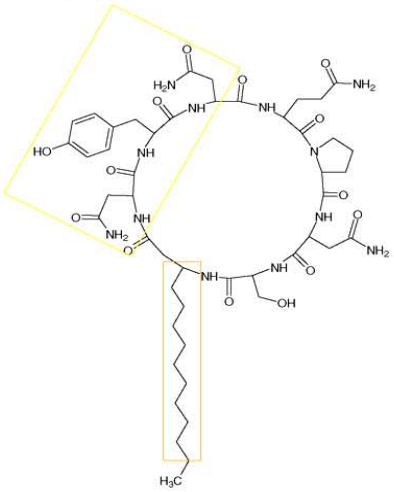


Variants		Lenght and branching of the acyl chain
Esperin**	**L-Glu-L-Leu**-D-Leu-**L-Val**-L-Asp-D-Leu-**L-Leu-COOH**	
Lichenysin***	**L-XL_1-L-XL_2**-D-Leu-**L-XL_4**-L-Asp-D-Leu-**L-XL_7**	*i*-C_{13}, *ai*-C_{13}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Pumilacidin	**L-Glu-L-Leu**-D-Leu-**L-Leu**-L-Asp-D-Leu-**L-XP_7**	
Surfactin	**L-Glu-L-XS_2**-D-Leu-**L-XS_4**-L-Asp-D-Leu-**L-XS_7**	*i*-C_{14}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}

** the β-carboxyl of Asp_5 is engaged in the lactone
*** or halobacillin
XL_1 = Gln or Glu ; XL_2 = Leu or Ile ; XL_4 and XL_7 = Val or Ile ;
XP_7 = Val or Ile ;
XS_2 = Val, Leu or Ile ; XS_4 = Ala, Val, Leu or Ile ; XS_7 = Val, Leu or Ile

n, linear
i, iso
ai, anteiso

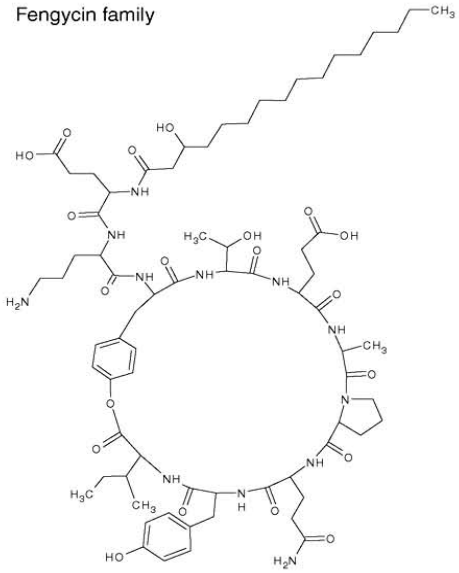
B



Bacillomycin D	**L-Asn**-D-Tyr-D-Asn-**L-Pro-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin F	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Thr**	*i*-C_{16}, *i*-C_{17}, *ai*-C_{17}
Bacillomycin L	**L-Asp**-D-Tyr-D-Asn-**L-Ser-L-Gln-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin LC*	**L-Asn**-D-Tyr-D-Asn-**L-Ser-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}, *i*-C_{16}
Iturin A	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Iturin A_L	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{16}, *i*-C_{16}
Iturin C	**L-Asp**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Mycosubtilin	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Ser-L-Asn**	*n*-C_{16}, *i*-C_{16}, *ai*-C_{17}

* or bacillopeptin

C



Fengycin A**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}
Fengycin B**	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}, C_{17}
Plipastatin A	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}
Plipastatin B	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}

** double bond between carbons 2-3, 3-4 or 13-14 were reported for some acyl chains

Figure 1.2: Structures of representative members and variants within the three *Bacillus* lipopeptide antibiotic families. A, represents the surfactin family; B, represents iturin family; and C, represents the fengycin family (Source: Ongena and Jacques, 2007).

1.3.2 Aminopolyol antibiotics

Zwittermicin A represents a unique structural class of linear aminopolyol antibiotics (Figure 1.3) and is mainly produced by *B. cereus* and *B. thuringiensis* strains (Raffel *et al*., 1996; Stohl *et al*., 1999a; Emmert *et al*., 2004). This antibiotic has structural features in common with peptide and polyketide antibiotics (Zhao *et al*., 2007). Zwittermicin A exhibits a broad range of antimicrobial activity. Its ability to suppress damping-off disease caused by the oomycete, *Phytophthora medicaginis* Hansen. is of particular significance and it has been reported to inhibit germ tube elongation during spore germination (Silo-Suh *et al*., 1998; Stohl *et al*., 1999a; Zhao *et al*., 2007). Zwittermicin A has been shown also to enhance *B. thuringiensis* insecticidal properties but the mechanisms involved have not been fully elaborated (Stohl *et al*., 1999a; Zhao *et al*., 2007).

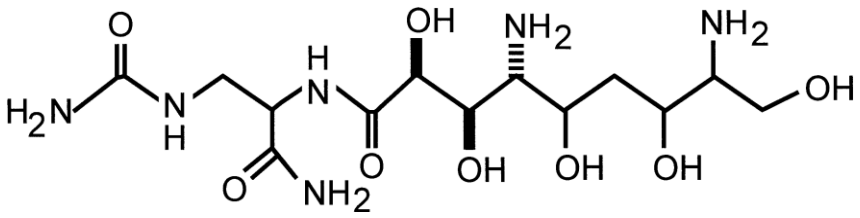


Figure 1.3: Structure of zwittermicin A (Source: Silo-Suh *et al*., 1998).

1.3.3 Importance of antibiotics to the producer organisms

Bacillus antibiotics are usually products of secondary metabolism and do not appear to have functions during vegetative growth (Kleinkauf and von Dohren, 1997). A number of hypotheses speculating antibiotic functions have been proposed. These include:

a) providing a competitive advantage by inhibiting and suppressing normal growth of competing organisms (Lancini and Demain, 1999);

b) acting as signalling molecules that regulate cellular differentiation from vegetative cells into spores and/or maintenance of dormancy within populations (Katz and Demain, 1977);

c) assisting in root colonization and biofilm formation (Ongena and Jacques, 2007);

d) assisting in motility through the swarming action linked to biosurfactant production (Ongena and Jacques, 2007);

e) facilitation of metal uptake into cells (Lancini and Demain, 1999);

f) detoxification of metabolites (Katz and Demain, 1977).

In order to prevent antibiotic compounds antagonizing the producing strains, *Bacillus* spp. have developed various self-protection mechanisms. These include: an ability to change cell permeability, thereby restricting re-entry of produced antibiotics; the presence of enzymatic compounds that detoxify antibiotics; modification of its own target site; and antibiotic cell compartmentalization (Katz and Demain, 1977; Lancini and Demain, 1999). For example edine, an antibiotic produced by *B. brevis*, is inactive intracellularly because it is bound to edine-synthesizing poly-enzyme compounds which in turn are linked to a complex of cytoplasmic membrane and DNA (Katz and Demain, 1977).

1.3.4 Antibiotic production – general principles

Antibiotics produced by *Bacillus* spp. are secondary metabolites which are commonly produced during transition from exponential to stationary growth phases (Yu *et al*., 2002; Ongena and Jacques, 2007). Their production is linked to factors influencing survival, for example, high cell densities, nutrient limitation and waste product accumulation (Katz and Demain, 1977; Mizumoto *et al*., 2006).

Bacillus lipopeptide antibiotics are generally synthesized non-ribosomally, by large multi-enzymes complexes, termed non-ribosomal peptide synthetases (NRPSs) (Hancock and Chapple, 1999; Ongena and Jacques, 2007). These synthetases are highly flexible (Stein, 2005). Antibiotic variations include changes in the type and sequence of amino acids, as well as in the nature, length and branching of the fatty acid side chains (Deleu *et al*., 1999; Stein, 2005). These variants or isoforms can have selective advantages and may result in the formation of novel antibiotics with different antimicrobial mechanisms/activities.

Aminopolyol antibiotics are biosynthesised by a hybrid of non-ribosomal peptide and polyketide synthesis pathways. Hence, they have structural features in common with both peptide and polyketide antibiotics (Emmert *et al*., 2004).

Regulation of *Bacillus* antibiotic production has not been fully established as it is complex and governed by a global regulatory system (Stein, 2005). This system appears to connect antibiotic synthesis with other starvation induced activities, such as sporulation and genetic competence (Marahier *et al*., 1993; Stein, 2005). Ultimately factors influencing this global regulatory system, including various environmental stimuli affect antibiotic production.

1.3.5 Antibiotic production *in situ*

The *in situ* production of antimicrobial metabolites by biocontrol agents is considered vital for effective phytopathogen control. Various studies have reported that biologically active metabolites arising from *Bacillus* spp. can be detected under field conditions (Thomashow *et al*., 1997). Romero *et al*. (2007b) for example detected the presence of the antibiotics bacillomycin, fengycin and iturin A on melon leaves that had previously been treated with *Bacillus* strains.

There are however, a number of challenges associated with the detection and extraction of antibiotics under field conditions. For instance, fundamental knowledge pertaining to *in situ* rates of antibiotic production and/or detectable threshold concentrations have not been fully established (Thomashow *et al*., 1997). Additionally, physiochemical conditions influencing the biological state of biocontrol organisms and their antibiotic production are not fully understood. However, new techniques such as monitoring the gene expression of antibiotic pathways with reporter genes and the use of mutant strains are among the methods used to investigate conditions affecting antibiotic production *in situ* and their field effects on phytopathogens (Thomashow *et al*., 1997; Whipps, 2001).

1.4 The advent of molecular techniques

1.4.1 Microbial community profiling

Traditionally, the structure and diversity of microbial populations within soil environments have mainly been investigated using culture-dependent techniques (Palomino *et al*., 2005). These techniques are largely based on isolating micro-organisms, identifying and characterising them through determination of their biochemical profiles, using kits such as API 50CH/B tests (Garbeva *et al*., 2004; Antoun and Prevost, 2006). Culturing is of importance since it facilitates a better understanding of specific organisms and their potential ecological functions. However, these classical techniques have some constraints since they do not take into account the activities of non-culturable organisms; thus, only a fraction of the total microbial biomass is represented (Nocker *et al*., 2007). Culture-dependent techniques are also laborious to use and the results obtained are often inconsistent (Brimecombe *et al*., 2001; Garbeva *et al*., 2004). Adequate handling of large number of samples is another problem. These shortcomings have led to a shift towards culture-independent molecular techniques.

Molecular techniques are generally based on analysing signature molecules using, for example, fatty acid profiling or more frequently, DNA-based techniques (Nocker *et al*., 2007). The latter can either directly clone and sequence DNA fragments (e.g. shotgun cloning) or amplify specific target sites using polymerase chain reaction (PCR) (Nocker *et al*., 2007). The PCR products are then cloned and/or sequenced or subjected to a variety of genetic profiling methods. Examples of molecular techniques that have been used to evaluate *Bacillus* community composition and diversity include: denaturing gradient gel electrophoresis (DGGE); restriction fragment length polymorphism (RFLP); and repetitive extragenomic palindromic PCR (REP-PCR) (Mavingui *et al*., 1992; Garbeva *et al*., 2003; Hoon *et al*., 2005; Manzano *et al*., 2009).

To determine levels of diversity within a sample set adequately, identification as well as characterization of potential candidate organisms through fingerprinting is recommended (Marten *et al*., 2000). Among the various DNA fingerprinting techniques, the predominant target for analysing has been the 16S Ribosomal RNA (rRNA) gene, as the rRNA gene is universally found in all living organisms (Xu and Cote, 2003; Han *et al*., 2005; Nocker *et al*., 2007; Freitas

et al., 2008). Rossler *et al*. (1991) for example successfully used 16S rRNA analysis to place nine *Bacillus* spp. into four distinct phylogenetic clusters.

Another genomic target commonly used for fingerprinting isolates is the intergenic transcribed spacer (ITS) region, located between the 16S and 23S ribosomal genes in prokaryotes (Figure 1.4) (Nocker *et al*., 2007). These spacer regions sometimes contain tRNA genes and the differences in number and type of tRNA gene inserts has been attributed to the higher heterogeneity in both length and nucleotide sequences within this region (Damiani *et al*., 1996; Xu and Cote, 2003). Although this technique can discriminate between species at an interspecific level, there is only limited discrimination between closely related strains (Daffonchio *et al*., 2003; Martinez and Sineriz, 2004; Freitas *et al*., 2008). Nonetheless, there are some molecular methods with improved ability to detect genetic diversity, such as Random amplified polymorphic DNA PCR (RAPD-PCR) (Atienzar and Jha 2006). Daffonchio *et al*. (1998b) demonstrated that RAPD-PCR was able to detect finer levels of genetic heterogeneity between *B. cereus* strains than was possible with ITS-PCR analysis.

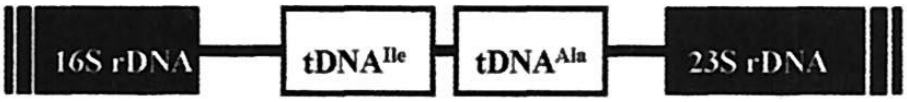


Figure 1.4: Schematic representation of the 16S-23S rDNA spacer region. The presence of tRNA genes is not universal and their type and number may vary between different species (Source: Cherif *et al*., 2003).

RAPD-PCR is a simple method based on random amplification of genomic sequences (Nilsson *et al*., 1998). Unlike the standard PCR, RAPD-PCR generally uses a single primer of 9 to 10 bases in length and amplification normally occurs at low annealing temperatures (Olive and Bean, 1999; Atienzar and Jha, 2006). A major advantage of RAPD-PCR is that no prior information on the genome under investigation is required, as it samples the whole genome (Daffonchio *et al*., 1998b; Atienzar and Jha, 2006). The technique is also highly sensitive and inexpensive as no specialized equipment is needed. RAPD-PCR is non-destructive which is especially useful for rare and/or valuable samples (Damiani *et al*., 1996). Though RAPD-PCR can be useful in discriminating between closely related species, there are limitations. The most frequently encountered is the lack of precise reproducibility, as the primers amplify randomly (Atienzar and Jha, 2006). Damiani *et al*. (1996) ascribed this to RAPD-PCR being highly

sensitive to even the slightest process changes, including the annealing conditions. This limitation can be overcome by adhering to precise reaction conditions; particularly, the PCR temperature profile, type of PCR machine, *Taq* polymerase, primers, $MgCl_2$ concentrations and type and concentration of DNA template (Brousseau *et al*., 1993). Another drawback of RAPD-PCR is the assumption that similar fragment sizes are a dependable indicator of genetic homology which is not always the case (Atienzar and Jha, 2006).

Consequently, appropriate molecular techniques or combination thereof should be considered for reliable and effective analysis of microbial diversity.

1.4.2 Phylogenetic analysis

Phylogenetic analysis entails determining the evolutionary history between a group of organisms through construction of a hierarchical diagram, called a cladogram or phlyogram (phylogenetic tree) (Xiong, 2006). This analysis is based on the assumption that organisms originate from a common ancestor.

Phylogenetic analysis consists of five steps. These include: selection of a specific molecular marker; sequence alignment; evolutionary modelling; tree construction; and assessing tree reliability (Xiong, 2006). The type of molecular marker used depends on the purpose of the study and the characteristics of the marker; commonly nucleotide or protein sequences are used. Thereafter, sequences are aligned, eliminating gaps and missing data. This step is critical in phylogenetic analysis as the topology of the evolutionary tree computed can be greatly affected. An example of an efficient alignment program is Clustal W (Tamura *et al*., 2007). Evolutionary relations are then inferred using these aligned sequences, either through character (Parsimony) or distance based methods (Xiong, 2006). This evolutionary distance can be estimated using an independent estimation approach (IE) or maximum composite likelihood (MCL) (Tamura *et al*., 2007). The latter is advantageous as it lowers errors when calculating evolutionary distances, as analysis of all sequence pairs is calculated simultaneously. Thus, a single set of parameters is used which is of importance in the construction of phylogenetic trees (Tamura *et al*., 2007). There are two main methods for constructing a tree using distance data. These are the unweighted pair gap method with arithmetic mean (UPGMA) and the neighbour-joining method. After computing the tree, it needs to be statistically evaluated for reliability and consistency.

Examples of programmes that can do this evaluation include Bootstrapping, Bayesian simulation and the Shimodiara-Hasegawa test (Xiong, 2006).

Phylogenetic analyses are computer based and there are several software programs available. For example: phylogeny inference package (PHYLIP); phylogenetic analysis by maximum likelihood (PAML); and molecular evolutionary genetic analysis (MEGA) (Tamura *et al*., 2007; Yang, 2007). MEGA is commonly used as it is freely available, user friendly and has multiple functions in a single computing program. Examples of these functions include web-based data acquisition, automatic and manual sequence alignment, various modes of estimating evolutionary distances, construction and visualization of phylogenetic trees, and testing molecular evolutionary hypotheses (Tamura *et al*., 2007). MEGA 4.1 has additional features such as, caption software which informs all underlying models/assumptions used in the analyses of the results obtained. The maximum composite likelihood programme used to estimate evolutionary distances between sequences have also been installed.

The main limitation of phylogenetic analysis is its inherent subjectiveness, as it is based on many assumptions that all the organisms are descendants of a common ancestor (Xiong, 2006).

1.4.3 Screening for antibiotic producers

Due to the inconsistent and unreliable field performances of recognised Plant Growth Promoting Rhizobacteria (PGPR), there is an ongoing need to search for new ones (Raupach and Kloepper, 1998). Ideally, they should exhibit a variety of plant growth-promoting traits and broad spectrum activity against phytopathogens (Ongena and Jacques, 2007). Additionally, these novel PGPRs must be able to grow and exhibit plant growth-promoting traits under varied environmental conditions. Focus has been mainly on isolating PGPRs from agricultural fields and greenhouse crop trials (Berg and Smalla, 2009). However, research needs to extend to native plants and natural ecosystems as promising candidate organisms could possibly be found within the untapped diverse microbial populations. An interesting area to investigate is the undisturbed indigenous grassland soils that have a large, uninterrupted turnover of a variety of organic matter arising from the extensive root system and leaf tissue occurring in such areas. This varied organic content is associated with microbial diversity since metabolically different micro-organisms are required to degrade the complex substrates to provide carbon sources, thus playing

essential roles in nutrient cycling (Alexander, 1977). Additionally, such sites seldom encounter plant pathogens; it would be of interest to investigate if antagonistic resident microbes are responsible for the low incidences of plant diseases (Gibson, 2009).

Traditionally, antibiotic producers are mainly screened for using culture-dependent techniques which entails isolation of candidate strains from various sources (Sadfi *et al*., 2002; Fernando *et al*., 2006; Hsieh *et al*., 2008; Athukorala *et al*., 2009). Pure cultures of these isolates are then obtained through the use of specific enrichments and/or selective media. Thereafter, their antagonistic ability is tested using various assays such as antibiosis through dual culture bioassays, or assays for siderophore production and extracellular enzymes such as chitinase and proteinase. Screens for plant growth-promotion traits include phosphorous solubilisation, nitrogen fixation and phytohormone production. These techniques are time consuming and laborious, which is disadvantageous as antibiotic producers should preferably be screened using rapid and reliable techniques (Mohammadipour *et al*., 2009). Detection of novel antibiotics is urgently needed as there is an increase in phytopathogen resistance to available fungicides. Additionally, there is an ongoing need for environmentally friendly biopesticides due to increasing awareners of environmental protection.

Recent advances in understanding antibiotic biosynthetic mechanisms and regulatory genes have lead to the development of specific primers that target these sites. These primers are increasingly being used for the detection of antibiotic producers, as this technique offers a simple, rapid, specific and culture-independent method (Fernando *et al*., 2006; Mohammadipour *et al*., 2009). An example of this is the detection of the *sfp* gene in *Bacillus* spp., which is considered essential to the biosynthesis of surfactin. The conserved distribution of *sfp* in *Bacillus* spp. has made it a useful marker gene from which primers can be designed to screen for surfactin producers (Abushady *et al*., 2005; Stein, 2005). Mohammadipour *et al*. (2009) illustrated this, using specific primers targeting the *sfp* gene and confirming antibiotic production by high performance liquid chromatography (HPLC). All isolates containing the *sfp* gene (675bp) produced surfactin at concentrations ranging from 55 to 1610 mg/L. Genetic regions *ItuD* and *lpa -14* are used for screening iturin producers as these genes are essential in the biosynthesis of this substance (Shoda, 2000; Stein, 2005; Hsieh *et al*., 2008). However, the use of genetic markers for antibiotic detection has its limitations, as some non-antibiotic producing strains may test positive for the

marker(s) possibly mutated in the biosynthetic and regulating genes (Athukorala *et al*., 2009). For instance, *itu* mutants containing the iturin biosynthesis genes but not producing the antibiotic have been identified due to the lack of the essential regulatory gene, *Ipa* (Stein, 2005). Thus, when using genetic detection of antibiotic producer's, precautions are necessary as strains may contain marker genes but lack the regulatory genes essential for the antibiotic production. Also, antibiotic gene primers might be too specific and may not detect variants or isoforms of an antibiotic family. Athukorala *et al*. (2009) illustrated this as fengycin produced by *B. amyloliquefaciens B36* was not detected using marker specific primers but was detected using matrix-assisted laser desorption ionization- time of flight-mass spectroscopy (MALDI-TOF-MS). Thus, methods such as MALDI-TOF-MS, HPLC and carbon-nuclear magnetic resonance (C-NMR) should be used to verify antibiotic production (Han *et al*., 2005; Tendulkar *et al*., 2007; Athukorala *et al*., 2009).

1.5 Overall aims and objectives

The search for novel biocontrol agents has mainly been based on agricultural soil; however, there is an untapped source of potentially diverse bacteria in indigenous grassland soil. The overall aim of this study was to investigate the presence and diversity of antifungal endospore-forming bacteria associated with an undisturbed indigenous grassland soil. The influence of crop rhizospheres on this diversity was also investigated.

The objectives were to:

1. isolate aerobic endospore-formers from bulk and rhizosphere soil and to screen for antagonism towards *R. solani in vitro*;
2. screen for possible antifungal and plant growth promoting mechanisms *in vitro*;
3. assess the suitability of several molecular methods (ITS-PCR, 16S rRNA sequencing and RADP-PCR) in determining the diversity between isolates; and
4. assess the use of a PCR based method to screen for the presence of specific antibiotic gene markers (zwittermicin A biosynthetic and resistance genes) and to attempt the isolation and characterization of active antifungal compound(s).

Chapter 2: *In vitro* screening for endospore-forming plant growth-promoting rhizobacteria (PGPR), isolated from bulk and maize, wheat and kale rhizosphere soil

2.1 Introduction

Over the past decades, there have been increased concerns regarding the overuse of chemical fungicides. These compounds are known to have harmful environmental impacts, to support the emergence of fungicide-resistant pathogens and to contribute to escalations in ongoing farming costs (San-Lang *et al*., 2002; Selim *et al*., 2005; Mizumoto *et al*., 2006). Thus, environmentally friendly measures to promote plant growth and protect crops against fungal pathogens have received a great deal of attention in recent years (Berg *et al*., 2002; Selim *et al*., 2005). In this regard members of the genus *Bacillus* have demonstrated their suitability for plant growth-promotion and biological control of phytopathogens (Probanza *et al*., 2001; Mena-Violante and Olalde-Portugal, 2007).

Plant growth-promoting *Bacillus* strains stimulate plant growth either directly and/or indirectly (Herman *et al*., 2008). Indirect plant growth-promotion usually involves pathogen suppression via mechanisms such as nutrient competition and/or production of antimicrobial metabolites. These antimicrobial metabolites include fungal cell-wall degrading enzymes, iron-chelating siderophores, and antibiotics (Berg *et al*., 2002; Selim *et al*., 2005). Direct plant growth-promotion can be achieved by improving nutrient availability to a host plant or through the production of growth-promoting phytohormones (Probanza *et al*., 2001; Mena-Violante and Olalde-Portugal, 2007).

The rhizosphere is a nutritionally rich environment that supports a variety of complex interactions between bacteria and plant roots (Probanza *et al*., 2001; Nelson, 2004). Relatively little is known about the influence of plant and soil type on the specificity of PGPR root colonization (Garbeva *et al*., 2004). This knowledge is crucial for the successful application of biological control agents, since environmental conditions have a profound influence on root colonization and on the composition and diversity of bacterial rhizosphere communities (Berg *et al*., 2002).

The aims and objectives of this study were to isolate aerobic endospore-formers from an undisturbed indigenous grassland soil and to screen them for antifungal activity against the phytopathogen, *Rhizoctonia solani*, using *in vitro* bioassays. Isolates were obtained from bulk soil and soils from the rhizosphere of maize (*Zea mays L.*), wheat (*Triticum aestivum*) and kale (*Brassica oleracea*) grown in pot trials. Isolates exhibiting antifungal activity were then subjected to various biochemical tests to screen for further PGPR attributes and to ascertain the level of functional diversity within the sample set.

2.2 Materials and methods

2.2.1 Isolation of aerobic endospore-forming bacteria

Mispah clay-loam soil (Krasilnikov and Arnold 2009), was collected from the top (0-10 cm) layer of an uncultivated indigenous grassland area, located at the Life Science and Agriculture campus, University of Kwa-Zulu Natal, Pietermaritzburg, South Africa (Co-ordinates 29°, 37'S and 30°, 24'E). A total of 15 kg soil was collected from four randomly selected sites, mixed and finely sieved (2 mm).

Kale (*Brassica oleracea*), maize (*Zea mays)* and wheat (*Triticum aestivum*) seeds were planted separately (5 seeds/pot) into replicate pots (20 cm diameter) containing approximately 1 kg of soil. Pots containing the same amount of soil but without seeds served as bulk soil controls. Pots were maintained at 26 ± 2°C under glass house conditions for 3 weeks. Seedlings were watered on every third day and no fertilizer or soil turning methods were used.

After three weeks, seedlings were gently pulled out of each pot and lightly shaken to remove loose soil from the roots. For each plant type, soil that remained attached to the roots was collected and placed in sterile plastic bags before being transported to the laboratory for processing.

One gram of bulk or rhizosphere soil was suspended in 9 ml of sterile quarter strength Ringer's solution (Merck). The suspension was vortexed for 10 min and then heated at 80°C for 15 min. The suspension was then serially diluted to 10^{-5} and plated in duplicate into 10% ($^{w}/_{v}$) Tryptone Soy Agar (TSA) (Merck), using the pour plate technique. After incubating the plates at 30°C for 24 h, the average number of colony forming units (c.f.u) per gram of soil (wet weight) were

determined. It was assumed that each colony had arisen from a single endospore. Four replicate samples were processed per soil. The means of the endospore counts were then separated using Fisher's unprotected least significant difference test (5% level) (GenStat, V12.1)

Using aseptic techniques, distinct colonies were randomly picked off replicate plates at the highest dilution using an inoculating needle. Ninety-six isolates per rhizosphere/soil sample were transferred to appropriately labeled micro-titer wells containing 100 µl of 10% ($^{w}/_{v}$) Tryptone Soy Broth (TSB) (Merck) before being subjected to further screening procedures.

2.2.2 *In vitro* screening for antifungal activity

Bacterial isolates were screened for antagonistic activity towards *R. solani* (Microbiology culture collection), using an *in vitro* dual-culture plate assay on Potato Dextrose Agar (PDA) (Merck) (Tewelde, 2004). Four sterile filter paper discs (10 mm diameter) were placed on the periphery of each PDA plate, approximately 10 mm from the edge. Thereafter, 20 µl of an overnight broth culture of a test isolate, incubated at 30°C, was aseptically applied to three of the paper discs. Agar plugs (3 mm × 3 mm) cut from PDA plates covered with *R. solani* mycelium were aseptically transferred to the middle of each bioassay plate. These were then incubated at 30°C and assessed for zones of inhibition at 72 h intervals. The width of each inhibition zone was measured (mm) from the edge of the filter disc to the leading edge of mycelium growth.

Isolates exhibiting antifungal activity were then aseptically transferred from the micro-titer wells onto 10% ($^{w}/_{v}$) TSA plates using the 3-way streak method, to check for purity. Plates were incubated at 30°C for 72 h. In the event of a mixed culture, separated individual type colonies were tested for antagonistic activity towards *R. solani* as previously described.

Selected isolates were maintained on 10% ($^{w}/_{v}$) TSA plates incubated at 30°C. Alternatively, ten percent ($^{v}/_{v}$) glycerol broth stock cultures were prepared by adding in appropriate amount of glycerol into cultures grown in TSB. These were then maintained at -80°C.

2.2.3 Morphological characterization

Colony characteristics and cell morphology of antifungal endospore-formers were determined on overnight cultures grown at 30°C on 10% ($^{w}/_{v}$) TSA and in 10% ($^{w}/_{v}$) TSB, respectively. Bright

field microscopy was used to confirm the Gram reaction, determine cell morphology and endospore location within cells. Cell dimensions were measured at 1000 × magnification using a bright field microscope (Olympus Ax70 Fluorescent microscope, light DIC) fitted with analysis® 3.0 imaging system software. Ten cells per strain were measured against a 20 µm scale bar and average cell dimensions calculated.

2.2.4 Biochemical assays

Biochemical assays were performed by inoculating each isolate onto respective media plates with a loopful of overnight culture grown on 10% TSA at 30°C. Assays were performed in duplicates, in each instance an isolate was inoculated onto the periphery of plate covering an area of approximately 10 – 15 mm in diameter. Four to eight isolates were inoculated per plate.

2.2.4.1 Amylase activity assay

Amylase production was assayed by culturing bacteria on a starch based agar medium (Atlas, 1993). The medium contained ($g.l^{-1}$ distilled water) beef extract, 3.0; soluble starch, 10.0; bacteriological agar (Merck), 12.0 and was autoclaved at 121°C (15 Kpa) for 15 min. Molten agar was allowed to cool to < 55°C before being dispensed into standard (90 mm) petri dishes to a depth of ± 4 mm.

Following incubation of inoculated plates at 30°C for 72 h, plates were flooded with iodine solution. Amylase activity was indicated by a zone of clearing around the test isolate on the iodine stained starch media.

2.2.4.2 Cellulase activity assay

Cellulase activity was determined by culturing isolates on modified *Bacillus* agar medium (Atlas, 1993). The medium contained ($g.l^{-1}$ distilled water) KNO_3, 1.0; K_2HPO_4, 1.0; KCl, 1.0; $MgSO_4.7H_2O$, 0.5; glucose, 1.0; bacteriological agar (Merck), 20.0. The medium was then supplemented with 5.0 $g.l^{-1}$ of CMC (carboxymethyl cellulose sodium salt) (Sigma) and thereafter autoclaved at 121°C for 15 min. The molten agar was then dispensed into 90 mm petri dishes as described previously above.

Inoculated plates were incubated at 30°C for 72 h. Subsequent to incubation, plates were flooded with 1% ($^{w}/_{v}$) Congo red solution for 15 min and de-stained immediately afterwards with 1 M sodium chloride solution for 1 min. Light pink zone around the test isolate was considered indicative of cellulase activity.

2.2.4.3 Chitinase activity assay

Chitinase activity was assessed by culturing the bacteria on chitin agar medium (Hsu and Lockwood, 1975). The medium contained ($g.l^{-1}$ of distilled water) colloidal chitin, 3.0 (wet weight); $FeSO_4.7H_2O$, 0.0075; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 0.7; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.0525; $MnSO_4$, 1.0; bacteriological agar (Merck), 15.0. After these ingredients were dissolved in 1 l distilled water, the medium was adjusted to pH 8 using NaOH and then autoclaved at 121°C (15 Kpa) for 15 min.

Colloidal chitin used above was prepared by dissolving 20.0 g of bleached chitin in 200 ml of 30% HCl. This solution was slowly added to 2 l of ice cold water, causing the chitin to precipitate as a colloidal suspension. The suspension was filtered through Whatman #1 filter paper, under vacuum. The resulting filtrate pellet was re-suspended in 2 l of H_2O and re-filtered. This washing step was repeated four times before the chitin was added to the agar basal medium.

Inoculated plates were incubated at 30°C for 168 h. After this time, a zone of clearing around the test isolate on the opaque chitin agar was considered an indication of chitinolytic activity.

2.2.4.4 Proteinase activity assay

Proteinase activity was assayed by culturing bacteria on 10% ($^{w}/_{v}$) skim milk agar medium (Atlas, 1993). The medium was made up of 38.0 g TSA dissolved in 1 l distilled water and autoclaved at 121°C (15 Kpa) for 15 min. When cooled to ± 50°C the medium was aseptically supplemented with 100 ml of fat free milk (Elite) that had been autoclaved at 121°C (15 Kpa) for 12 min.

Inoculated plates were incubated at 30°C for 72 h. After this incubation period, zone of clearing observed around the test isolate on the skim milk agar was considered to be indicative of proteolytic activity.

2.2.4.5 Phosphate solubilization assay

The ability of selected bacteria to solubilize phosphate was assayed by inoculating them onto Pikovskaya medium supplemented with tricalcium phosphate (Nibhagupta *et al*., 2007).

The medium contained ($g.l^{-1}$ distilled water) $Ca_3(PO_4)_2$, 5.0; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4$, 0.1; KCl, 0.2; $MnSO_4$, 0.04; $FeSO_4$, 0.04; glucose, 10.0; yeast extract 0.5; bacteriological agar, 15.0. After dissolving these ingredients in 1 l distilled water, the medium was adjusted to pH 7.2 using 1 M NaOH and thereafter autoclaved at 121°C (15 Kpa) for 15 min.

Inoculated plates were incubated at 30°C for 144 h. After this period, clear zone around the test isolate on the opaque Pikovskaya medium was considered to be indicative of phosphate solubilization.

2.2.4.6 Siderophore production assay

Bacterial siderophore production was determined by culturing isolates on modified chrome azurol S (CAS) agar medium (Barghouthi *et al*., 1989).

The CAS base medium was made up of NaOH, 5.3 g; piperazine-N-N'-bis (2-ethanesulfonic acid) (Merck), 30.0 g; bacteriological agar, 20 g; dissolved in 150 ml distilled water. 100 ml of stock salt solution was then added to the base medium and 750 ml distilled water was added to make up 1 l and thereafter autoclaved at 121°C (15 Kpa) for 15 min. The salt solution contained $g.l^{-1}$ distilled water: KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 10.0, in a total volume of 250 ml.

When the sterilized CAS base medium had cooled to ± 50°C, the following filter-sterilized (0.22 µm cellulose acetate filter) solutions were added: 30 ml of 10% ($^{w}/_{v}$) casamino acid (Oxoid); 10 ml of 20% ($^{w}/_{v}$) glucose; 10 ml of 200 $\mu g.ml^{-1}$ thiamine (Sigma); 10 ml of 200 $\mu g.ml^{-1}$ nicotinic acid (Sigma); 1 ml of 1M $MgCl_2$, and 1 ml of 1M $CaCl_2$.

The CAS-iron-hexadecyltrimethylammonium bromide solution was prepared by dissolving 60.5 mg of CAS into 50 ml distilled water, followed by the addition of 10 ml of 1mM $FeCl_3.6H_2O$ (Saarchem) (in 10 mM HCl). This CAS-$FeCl_3$ solution was then slowly added to a solution containing 73 mg of hexadecyltrimethylammonium bromide that had been dissolved in 40 ml distilled water. The resulting solution was autoclaved at 121˚C (15 Kpa) for 15 min and allowed

to cool. Thereafter, the sterilized solution was aseptically added to the CAS base medium containing the filter-sterilized solutions.

Inoculated plates were incubated at 30˚C for 120 h. After this time, a yellow-orange zone around the test isolate was considered indicative of siderophore production.

2.3 Results

2.3.1 Isolation and enumeration of aerobic endospore-forming bacteria from bulk and maize, wheat and kale rhizosphere soils

The lowest average endospore c.f.u counts were obtained from the bulk soil whereas the highest were obtained for the wheat rhizosphere soil (Table 2.1). The numerical differences between each soil were relatively small but nonetheless statistically significant in most cases (Table 2.1).

Table 2.1: Average endospore-former counts from bulk and rhizosphere soils

Source	Mean Endospore c.f.u g^{-1} soil	Log Endospore c.f.u $.g^{-1}$ soil
Bulk soil	$4.68 \times 10^{5\,c}$	5.67^{c}
Kale	$7.94 \times 10^{5\,b}$	5.90^{b}
Maize	$7.59 \times 10^{5\,b}$	5.88^{b}
Wheat	$1.07 \times 10^{6\,a}$	6.03^{a}

Endospore-former counts with the same letter(s) are not significantly different from each other using Fisher's LSD test, at 5% level (LSD = 1.46×10^{5})

2.3.2 *In vitro* screening for aerobic endospore-forming bacteria antagonistic towards *R. solani*

Of the 384 isolates screened for their ability to antagonize *R. solani* in dual culture bioassays, 34 isolates (~9%) inhibited mycelial growth at varying levels (Table 2.2). Forty-one percent of these antagonists were isolated from bulk soil, 35% from wheat, 12% from maize and another 12 % from kale rhizosphere soils.

Antagonistic activity was observed as inhibited or disturbed zones of mycelial growth after 72 h (Plate 2.1). In some instances, mycelial inhibition was only observed on one side of the inoculated disc in a region distal to the fungal inoculation site (Plate 2.1A). This was recorded as evidence of mycelial inhibition (+) rather than as an edge effect, as compared to the uninoculated control disc, mycelial growth had completely overgrown the control disc. In other instances,

dense mycelial growth was observed at the region adjacent to the inhibition zone (Plate 2.1B) whereas only one isolate, C76 caused the mycelium to brown in the region adjacent to the inhibition zone (Plate 2.1C).

Antifungal activity was ranked according to the average width of the inhibition zones per isolate. The majority of the organisms screened showed average inhibition zones ranging from 2 - 4 mm, after 72 h; while a few exhibited inhibitions zones greater than 5 mm. After 168 h incubation, the majority of isolates were completely overgrown by *R. solani*, with the exception of five isolates (C27, C28, C37, C76 and C92) originating from bulk soil and three (K39, K51 and K53) from kale rhizosphere soil. These seven isolates showed antifungal activity even after 3 week incubation (Plate 2.2).

Table 2.2: Results for *in vitro R. solani* antagonism by endospore-forming bacteria isolated from bulk and maize, wheat and kale rhizosphere soil

Isolation location♦	Isolate Number	*R. solani* antibiosis (72 h)♣	*R. solani* antibiosis (168 hrs)♣	Comments on *R .solani* antibiosis¤ (72 h)
Bulk soil	C15	+	-	
Bulk soil	C16	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C17	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C27	++	++	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C28	++	++	
Bulk soil	C37	++	++	
Bulk soil	C40	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C54	++	-	
Bulk soil	C56	+	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C74	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C76	++	+	Browning of mycelium
Bulk soil	C78	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C91	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Bulk soil	C92	++	++	
Maize rhizosphere soil	M12	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Maize rhizosphere soil	M15	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Maize rhizosphere soil	M27	+	-	
Maize rhizosphere soil	M60	++	-	
Wheat rhizosphere soil	W3	++	-	
Wheat rhizosphere soil	W13	+	-	
Wheat rhizosphere soil	W15	++	-	
Wheat rhizosphere soil	W33	++	-	
Wheat rhizosphere soil	W39	++	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Wheat rhizosphere soil	W41	+	-	
Wheat rhizosphere soil	W50	+	-	
Wheat rhizosphere soil	W75	+	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Wheat rhizosphere soil	W90	++	-	
Wheat rhizosphere soil	W92	+	-	Dense mycelial growth adjacent to the edge of the inhibition zone
Wheat rhizosphere soil	W95	+++	-	
Wheat rhizosphere soil	W96	+	-	
Kale rhizosphere soil	K39	+++	+	
Kale rhizosphere soil	K51	+++	++	
Kale rhizosphere soil	K53	++	+	
Kale rhizosphere soil	K89	++	-	

♦Bulk soil was collected from the top (0-10 cm) layer of an uncultivated indigenous grassland area, located at the Life Science and Agriculture campus, University of Kwa-Zulu Natal, Pietermaritzburg, South Africa. Rhizosphere soils were collected from the soil adhering to the roots on each crop type.

♣ *In vitro R. solani* antibiosis: dual-plate bioassays on PDA plates after 72 h and 168 h incubation at 30°C, (-) represents no inhibition, (+) represents < 2 mm wide inhibition zone, (++) represents 2 – 4 mm wide inhibition zone, (+++) represents ≥ 5 mm wide inhibition zone.

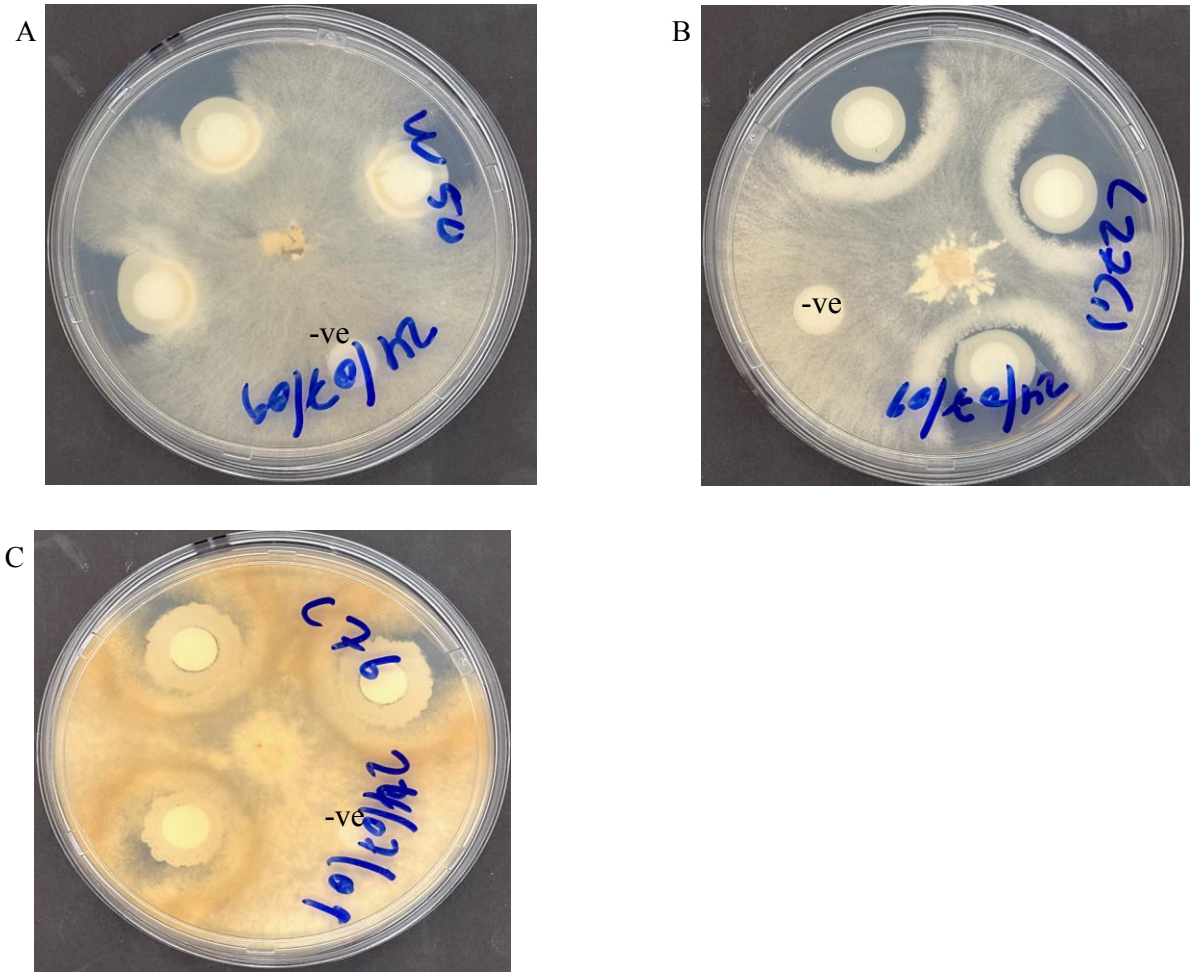


Plate 2.1: Dual culture bioassays showing inhibition of *R. solani* by Isolates W50 (A), C27 (B) and C76 (C) after 72 h. Bioassays were performed on PDA plates incubated at 30°C. (-ve) is an uninoculated control disc.

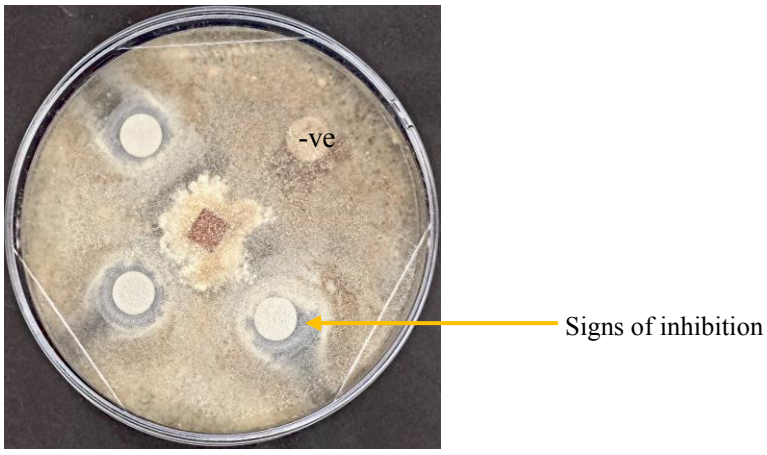


Plate 2.2: Signs of inhibition against *R. solani* by the bulk soil Isolate C28 evident after 3 week incubation on PDA plates at 30°C. (-ve) is an inoculated control disc.

2.3.3 Morphological characterization

The morphological characteristics of isolates from bulk and rhizosphere soils were very similar (Table 2.3). Most colonies were cream white, flat and had irregular colony form and undulate colony margins on 10% ($^{w}/_{v}$) TSA plates, after 24 h incubation at 30°C. Isolate C76 differed in that it had a mucoid colony texture and convex colony elevation.

Microscopic observations confirmed that all the antagonistic isolates were Gram positive and rod shaped, with the majority having rounded cell ends (Plate 2.3A-F). These cells had average cell dimensions of 3 - 4 µm in length and 1 – 1.5 µm in width (Table 2.3). Endospore location was mainly sub-terminal mixed with terminal (Plate 2.3B), with few isolates having centrally located endospores.

Table 2.3: Morphological characterization of bacterial isolates antagonistic to *R. solani*

	Colony morphology ♦		Cell morphology ♣			Spores
Isolate No.	Form	Margin	Length	Width	Cell Shape	Endospore position
C15	Irregular	Lobate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped ,Squared ends	Sub-Terminal/Terminal
C16	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Squared ends	Sub-Terminal/Terminal
C17	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Squared ends	Sub-Terminal/Terminal
C27	Irregular	Undulate	2 - 3 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C28	Irregular	Undulate	3 - 4 µm	1 µm -1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C37	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C40	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C54	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C56	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C74	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C76	Circular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C78	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Squared ends	Sub-Terminal/Terminal
C91	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
C92	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Squared ends	Sub-Terminal/Terminal
M12	Irregular	Undulate	5 - 6 µm	1.5 µm - 2 µm	Rod shaped, Squared ends	Sub-Terminal/Terminal
M15	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
M27	Irregular	Undulate	2 - 3 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
M60	Irregular	Undulate	2- 3 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Central/Sub-terminal
W3	Irregular	Lobate	4 - 5 µm	1.5 µm - 2 µm	Rod shaped, Rounded ends	Central/Sub-terminal
W13	Irregular	Undulate	4 - 5 µm	1.5 µm - 2µm	Rod shaped, Rounded ends	Central/Sub-terminal
W15	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W33	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W39	Irregular	Undulate	2 - 3 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W41	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W50	Irregular	Undulate	5 - 6 µm	1.5 µm - 2 µm	Rod shaped, Rounded ends	Central/Sub-terminal
W75	Irregular	Undulate	3 - 4 µm	1.5 µm - 2 µm	Rod shaped, Square ends	Central/Sub-terminal
W90	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W92	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
W95	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Square ends	Central/Sub-terminal
W96	Irregular	Undulate	3 - 4 µm	1.5 µm - 2 µm	Rod shaped, Square ends	Central/Sub-terminal
K39	Irregular	Undulate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
K51	Irregular	Lobate	3 - 4 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal
K53	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Square ends	Central/Sub-terminal
K89	Irregular	Undulate	4 - 5 µm	1 µm - 1.5 µm	Rod shaped, Rounded ends	Sub-Terminal/Terminal

♦ Colony morphology of cultures grown on 10% ($^{w}/_{v}$) TSA after 72 h incubation at 30°C.

♣ Cell morphology and the cell dimensions recorded are the mean measurements of ten cells per Gram stain observed using bright field microscopy at 1000× magnification.

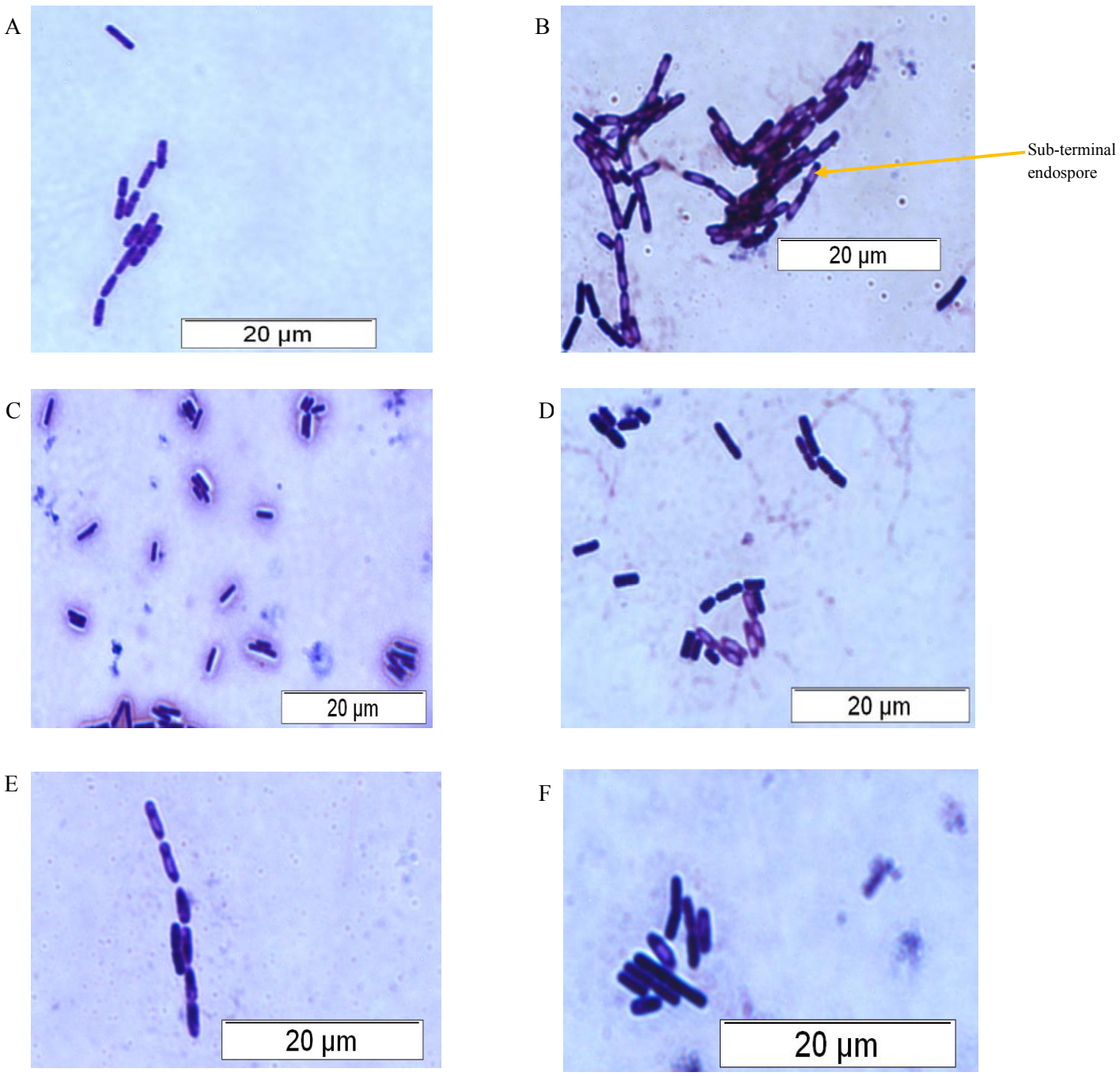


Plate 2.3: Gram stains showing cell morphology and endospore location in Isolate C15, C54 and C76 from bulk soil (A, B and C, respectively); M60 from maize rhizosphere soil (D); W41 from wheat rhizosphere soil (E); and K39 from kale rhizosphere soil (F).

2.3.4 Biochemical assays

The biochemical and PGPR assay results are presented in Table 2.4 and illustrated in Plates 2.4 - 2.7. A large portion of the isolates (26/34) hydrolyzed starch to varying degrees, while only 4/34 isolates hydrolyzed cellulose. Chitinolytic activity occurred in 13/34 of the isolates, with the highest proportion coming from the bulk soil. A majority (31/34) of the isolates exhibited proteinase activity. Only 4/34 isolates were able to solubilize phosphate, all of them also from the bulk soil. Siderophores were produced in 14/34 isolates, the number of positive isolates being divided between the bulk and the three rhizosphere soils.

Table 2.4: Biochemical and plant growth promoting assay results of aerobic endospore-formers isolated from bulk and maize, wheat and kale rhizosphere soils

Isolate Number	Amylase♣	Cellulase♣	Chitinase♣	Proteinase♣	Phosphate Solubilization♣	Siderophore♣
C15	+	-	-	+++	-	-
C16	++	-	++	++	-	+
C17	+++	-	++	+++	+	-
C27	++	-	-	++	-	-
C28	+	-	++	++	-	-
C37	++	-	+	++	-	-
C40	++	-	+	++	-	-
C54	++	-	+	+++	-	-
C56	+++	-	+	++	-	-
C74	+	-	-	+	-	-
C76	-	+++	-	+++	-	-
C78	+++	-	-	+++	++	++
C91	++	-	++	+	+	+++
C92	-	-	-	++	+	-
M12	+	+++	+++	-	-	+
M15	+	-	-	+++	-	-
M27	+	-	-	++	-	-
M60	+	-	++	++	-	+
W3	+	-	+	++	-	+
W13	+	-	-	-	-	++
W15	+	-	-	++	-	-
W33	+	-	-	+++	-	++
W39	++	-	-	-	-	-
W41	-	-	-	++	-	++
W50	-	+	-	+++	-	+++
W75	-	+	-	++	-	+
W90	-	-	-	++	-	-
W92	-	-	-	++	-	++
W95	+	-	-	+++	-	-
W96	-	-	-	++	-	-
K39	++	-	++	+++	-	+
K51	+	-	-	+++	-	-
K53	+++	-	-	+++	-	+
K89	++	-	++	+++	-	-

♣Bioassays plates were examined for zones of clearing and rated as: (–) no visible zone of clearance, (+) minimal zone of clearance, (++) moderate zone of clearance, and (+++) complete hydrolysis of the substrate. All values are means of duplicate tests.

Plate 2.4: Detection of amylase activity by Isolates C27, C40, C54 and C56 after 72 h. Amylase activity was seen as clear zones surrounding colonies growing on iodine stained starch agar plates incubated at 30°C.

Plate 2.5: Cellulose hydrolysis by Isolate M12 growing on cellulose agar after 72 h incubation at 30°C. Cellulase activity was indicated by the presence of light pink zones around the colony.

Plate 2.6: Isolates M12 and M60 growing on chitin agar after 168 h incubation at 30° C. Chitinolytic activity is indicated by clear zones surrounding colonies on opaque chitin agar plates.

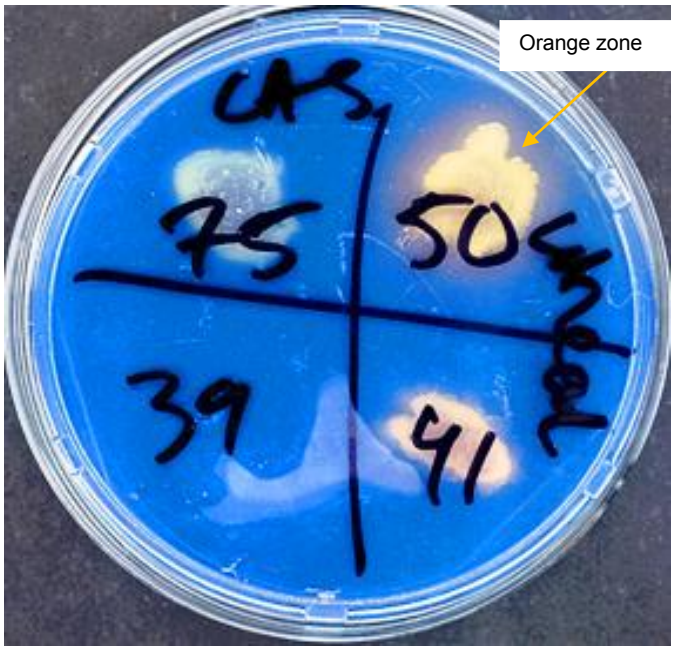


Plate 2.7: Siderophore production by Isolates W41, W50 and W75 growing on CAS medium after 120 h incubation at 30°C. Siderophore production is indicated by an orange zone surrounding the colonies.

2.4 Discussion

Native grassland soil ecosystems are considered to be fairly stable environments that are inherently resistant to soil-borne pathogen infestations (Gibson, 2009). This in part, has been attributed to the resident microbial communities which are thought to out-compete or suppress fungal pathogens. Undisturbed indigenous grassland soils are often associated with elevated levels of complex organic substrates arising from root exudates and reoccurring inputs of plant residues (Beare *et al*., 1997). As such, microbial communities within these environments are thought to differ from those associated with agricultural lands which are intensively managed (Alexander, 1977). As agricultural practices such as tilling can promote soil aeration leading to the rapid degradation and turnover of organic matter within soil; thus possibly lowered levels of microbial diversity (Alexander, 1977). Hence, undisturbed native grassland environments represent an untapped source of potentially useful microorganisms that warrant screening for biocontrol or PGPR potential.

A heating step was used in this study to select for heat stable aerobic endospore-forming bacteria namely, *Bacillus* spp. and other closely related taxa. A shortcoming of this isolation method is that only dormant endospores are selected for. This infers that they were present but does not necessarily indicate whether the *Bacillus* isolates play an active role within the habitat in which they were isolated from. However, since the bacterial isolates used in this study were isolated from agar plates at high dilutions (10^{-4} to 10^{-5}), it can be assumed that they were a well represented group within the indigenous grassland microbial community. If these organisms were not of significance within the habitat, it would be expected that they would not occur in high numbers and thus not obtained at high dilutions.

Bacillus spp., particularly *B. cereus* and *B. subtilis* are saprophytic micro-organisms commonly isolated from soil and a wide range of crop rhizospheres (Valain *et al*., 2006). Populations ranging from Log 3 to Log 6 c.f.u $.g^{-1}$ soil have been reported in the literature (McSpadden Gardener, 2004; Choudhary and Johri, 2009). Endospore-former counts obtained in this study compared closely with the upper range of these data (Table 2.1). The wheat rhizosphere yielded the highest counts of Log 6.03 c.f.u $.g^{-1}$soil and the bulk soil the lowest count of Log 5.67 c.f.u $.g^{-1}$ soil. This suggests that a rhizosphere effect might have occurred due to the deposition of plant derived nutrients arising primarily from root exudates (Atlas and Bartha, 1998; Nelson,

2004; Choudhary and Johri, 2009). Thus, increased levels of organic carbon arising largely from crop root exudates might explain the higher *Bacillus* counts associated with the rhizospheres.

Rhizoctonia solani is a destructive fungal phytopathogen that infects a wide range of crops (Yobo, 2005). It can drastically reduce crop yield and exhibits a range of symptoms which include pre- and post emergence damping-off in seedlings, root rot, stem canker and black scurf in potatoes (Choudhary and Johri, 2009). *Rhizoctonia* spp. are difficult to control and crop resistance is not widespread. Only a few effective chemical fungicides are registered for use against this pathogen (Duffy *et al*., 1996). Several reports in the literature have indicated that selected strains of *Bacillus* spp. have shown some success in controlling *R. solani* when applied as biocontrol agents (Ryder *et al*., 1998; Yu *et al*., 2002). Examples include *B. cereus* UW 85, *B. subtilis* GB03 and *B. subtilis* RB14 (Asaka and Shoda, 1996; Silo-Suh *et al*., 1998; Yu *et al*., 2002).

In the present study, 34 of the 384 (~9%) isolates tested antagonized *R. solani* to varying degrees after 72 hours (Table 2.2). Most of these were isolated from bulk and wheat rhizosphere soil. The lowest incidence of antifungal activity was obtained from maize and kale rhizosphere samples. These findings confirmed that native aerobic endospore-formers exhibiting antifungal activity were present in the soil type under investigation. In addition, rhizosphere soil did not appear to have a stimulatory effect on the number of antagonistic organisms isolated. The higher number of antagonists obtained from the wheat rhizosphere sample may reflect rhizosphere conditions that were more conducive for colonization.

The isolates selected in this study were found to inhibit *R. solani* to different extents (Table 2.2). The majority of isolates (86%) showed low levels of antifungal activity with inhibition zones of 2 - 4 mm; only Isolates W95 (wheat rhizosphere), K39 and K51 (kale rhizosphere) produced larger inhibition zones (~ 5 mm). These levels of antifungal activity are not as great as those recorded by other investigators using similar protocols. For example, Tewelde (2004) obtained inhibition zones greater than 12 mm wide. The low levels of antifungal activity observed in the present study could be due to the type of compound(s) produced (ie. low levels of antagonistic activity), low concentrations of antibiotic(s) being produced, or poor diffusion of the antifungal compound(s) within the agar medium. Further research into optimizing production of the antifungal agents should be undertaken to ascertain whether or not the use of different culture

media would improve antibiotic production. The effect of pH and temperature on antifungal compound(s) production should also be investigated.

Suppression of fungal pathogens can be due to various biocontrol mechanisms, with antibiotic activity being the most reported (Berg *et al*., 2002; Romero *et al*., 2007a). However, other microbial metabolites such as, biosurfactants, siderophores and chitinase are also reported to have antifungal activity (McSpadden Gardener, 2004). In the present study, different antimicrobial interactions were apparent *in vitro* (Table 2.2). Some isolates were found to induce dense mycelial growth at the periphery of the inhibition zone, which may have resulted from the mycelium continuing to grow at the interface with the inhibition zone. Isolate C76 on the other hand elicited a browning effect on the *R. solani* mycelium adjacent to the produced inhibition zones (Plate 2.1C). This could be due to activation of pigment production in mycelia as a result of the stress induced conditions. These varied responses of *R. solani* to the different isolates may reflect differences in the antifungal compound(s) produced. Thus, further research is needed into the underlying antimicrobial mechanisms to better understand the varied fungal response and factors influencing these mechanisms.

Several isolates (viz., C15, M27, W13, W41, W50, W96) only showed evidence of fungal inhibition on one side of the inoculated disc in the region distal to the fungal inoculums (Plate 2.1 A). This was attributed to the concurrent inoculation of the test isolate and test fungus which possibly resulted in mycelial colonization occurring at a faster rate than at which the antifungal compound(s) was produced. Since the distal region was furthest from the source of mycelial growth, it may be that there was insufficient time for the antibiotics produced to diffuse into the agar. A possible solution to this is that in future bioassays, a test isolate should be inoculated prior to the fungal pathogen, at least overnight in order to allow even compound diffusion throughout the agar plate.

Although the isolates showed inhibitory activity after 72 h 72% of these isolates were overgrown by *R. solani* after 168 h incubation (Table 2.2). This suggests that the compound(s) produced by these isolates were fungistatic rather than fungicidal. Possible reasons for the fungistatic action could be that low concentrations of the antifungal compound(s) were produced or alternatively, the antifungal compound(s) produced might not have been stable and lost activity over time (Tewelde, 2004). However, in some instances several isolates (e.g. Isolate C28) showed

fungicidal effects against *R. solani*, producing inhibition zones that persisted over a 3 week incubation period (Plate 2.2).

Isolates from the different sample sets showed very similar morphological characteristics. Their cell and colony morphologies are similar to those described for members of the *B. cereus* group and closely related species in Bergey's Manual of Systemic Bacteriology (Holt *et al*., 2000). The *B.cereus* group comprises *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. anthracis*, *B. pseudomycoides* and *B. weihenstephanensis* (Raffel *et al*., 1996; Vilain *et al*., 2006). Members of this group are commonly isolated from soil and the rhizosphere of certain plants (Jensen *et al*., 2003; McSpadden Gardener, 2004; Vilain *et al*., 2006). They have been reported to play important roles in the biocontrol of plant diseases, thus indirectly promoting plant growth (Ryder *et al*., 1998). Some *B. cereus* strains produce antibiotics such as zwittermicin A and kanosamine that can suppress various fungal phytopathogens (Milner *et al*., 1995; Silo-Suh *et al*., 1998; Nair *et al*., 2004). Additionally, *B. thuringiensis* is considered one of the most successful biological insecticides (Ongena and Jacques, 2007). However, some strains of *B. cereus* can cause food poisoning and spoilage, while *B. anthracis* is a pathogen to humans and animals (Logan, 2008; Cherif *et al*., 2003; Jensen *et al*., 2003). Thus, careful identification and characterization of strains associated with *B. cereus* is essential before establishing them commercially as plant growth promoting agents.

A number of *Bacillus* spp. have been shown to produce chitinase, an enzyme that degrades chitin, a major structural component for certain fungal cell walls (Huang *et al*., 2005). Thus, chitinase producing organisms can potentially be used as antagonistic agents against fungal phytopathogens (Tewelde, 2004). In this study, 13/34 (38%) of the isolates tested showed evidence of chitinase activity (Table 2.4). No direct relationship between chitinase production and antifungal activity could be established. Isolates exhibiting chitinase activity were isolated from all the different soil samples tested. This suggests the presence of chitin substrates in the grassland soil, which would select for and favor the growth of chitin degraders. Within soil environments major sources of chitin include the exoskeletons of insects and cell wall components of certain types of fungi (Tewelde, 2004).

Siderophores are iron chelating compounds which facilitate the scavenging and uptake of ferric ions (Fe^{3+}) from the surrounding environment when iron is present at low concentrations (Stein,

2005; Wilson *et al*., 2006). This scavenging action has been linked to antimicrobial activity, as phytopathogens are deprived of the iron they require for growth (Herman *et al*., 2008). *Bacillus* strains have been shown to produce siderophores, including the catecholate siderophores petrobactin and bacillibactin produced by *B. anthracis* and *B. cereus* (Wilson *et al*., 2006). In this study, 14/34 (41%) of the isolates displaying antifungal action showed evidence of siderophore production. Further research into the underlying mechanisms involved in the antagonistic ability of siderophores is warranted to establish their effectivness for use in biocontrol applications.

A large portion of isolates, 26/34 (76%) were able to utilize starch as a carbon source indicating that they were able to produce extracellular amylases (Table 2.4). Production of amylase(s) in *Bacillus* spp. is common; examples include *B. cereus* and *B. coagulans* (Anto *et al*., 2006). The majority (91%) of isolates used in this study were also found to hydrolyze milk through the production of extracellular proteinase. Both these traits are important in nutrient cycling of carbon sources within the environment, as these enzymes breakdown complex substrates into simpler forms (Anto *et al*., 2006). These traits can be used for plant growth promotion which would assist plants by increasing nutrient availability.

Of the 34 isolates tested, only 4 were able to utilize carboxymethyl cellulose (CMC) (Table 2.4) for growth. These low counts were not unexpected since fungi, in most cases are responsible for cellulose degradation in soil (Dix and Webster, 1995). The cellulase activity detected in the isolates probably does not have a major bearing under natural grassland soil conditions, as most of the cellulose found in these environments would be present as a lignocellulose complex (Kuhad *et al*., 2007). Only a few bacteria can breakdown this complex compound and CMC which has an amorphous structure is not a good representative of native cellulose which occurs in a crystalline state.

Bacillus strains have been shown to increase phosphorous availability to plants, through the solubilization of both organic and inorganic sources (Probanza *et al*., 2001; McSpadden Gardener, 2004). Organic phosphate which accumulates in soil as insoluble phytate is converted to the soluble form by extracellular phytase, whereas solubilization of inorganic phosphate is associated with the production of organic acids. In this study, only four isolates from bulk soil were able to solubilize inorganic phosphate (Table 2.4). These isolates have potential as plant growth promoting agents through increasing phosphorous availability to plants.

Overall findings indicated that antifungal activity was present in some members of the aerobic endospore-forming community isolated from an indigenous grassland soil. This supports the notion that native habitats harbor organisms with potential biocontrol traits. The rhizosphere environment did not appear to encourage widespread root colonization by the antagonistic endospore-forming isolates under investigation. A slight rhizosphere effect was evidenced as slightly increased microbial numbers within the rhizosphere of all the crops investigated was observed compared to the bulk soil. Low levels of antifungal activity against *R. solani* by the 34 endospore-forming isolates were observed *in vitro*. However, to evaluate their potential as biocontrol agents, *in vivo* trials would need to be performed. Some strains showed additional plant growth promoting traits, such as, the ability to increase nutrient availability to plants through solubilization of phosphate, sequestration of Fe^{3+} and degrading starch for nutrient recycling. These isolates would also need to be tested *in vivo* to determine their PGPR potential through increasing nutrient availability to plants. Based on the morphological and phenotypic tests performed, it was difficult to distinguish and identify isolates from the different sources. Also phenotypic/biochemical tests proved laborious, time-consuming and difficult to use effectively with the large sample numbers under investigation. Alternative approaches using culture-independent techniques which are becoming increasingly popular for the rapid and finer distinction between isolates were subsequently employed to determine the level of genetic diversity within the group of organisms under investigation.

Chapter 3: Determining the genetic diversity of antifungal aerobic endospore-formers from native grassland and maize, wheat and kale rhizosphere soil, using ITS-PCR, RAPD-PCR and 16S rRNA sequencing

3.1 Introduction

As indicated in the previous chapter, in recent years there has been a shift towards molecular techniques to identify *Bacillus* species and to determine their strain/community diversity. These methods include: fatty acid profiling; denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments; repetitive extragenomic palindromic PCR (REP-PCR); random amplified polymorphic DNA PCR (RAPD-PCR); amplification and sequencing of 16S rRNA and *gryB* genes; and, intergenic transcribed spacer region PCR (ITS-PCR) (Marten *et al*., 2000; Berg *et al*., 2002; Garbeva *et al*., 2003; Manzano *et al*., 2009).

The 16S rRNA genomic region is commonly targeted for amplification in order to distinguish between species and strains (Garcia-Martinez *et al*., 1999; Han *et al*., 2005; Nocker *et al*., 2007). However, closely related *Bacillus* species are known to exhibit high levels of homology in this region (Daffonchio *et al*., 1998a). Therefore, ITS-PCR has been proposed as a fingerprinting method as it is more discriminative compared to 16S rRNA sequence analysis (Damiani *et al*., 1996). ITS-PCR amplifies the internal transcribed spacer region separating the 16S and 23S ribosomal RNA gene. This region is not subjected to the same evolutionary pressure as the adjacent rRNA genes and hence tends to be more variable (Martinez and Sineriz, 2004). Another molecular method that has proved useful to discriminate inter- and intraspecific diversity in *Bacillus* is RAPD-PCR (Atienzar and Jha 2006). It involves the random amplification of DNA fragments from the whole genome using a single short (usually 10 nucleotide) arbitrary primer, under low stringency amplification conditions (Nilsson *et al*., 1998; Olive and Bean, 1999). Daffonchio *et al*., (1999) successfully used this technique to distinguish pathogenic *B. anthracis* strains from strains of the closely related *B. cereus*. RAPD-PCR is highly sensitive to even the slightest amplification condition changes, which affect its reproducibility (Atienzar and Jha, 2006). This sensitivity can however be overcome by strict standardization of the amplification

conditions, including the equipment used and the method of DNA preparation (Brousseau *et al*., 1993; Atienzar and Jha, 2006).

The aim of this study was to determine the genetic diversity of antifungal aerobic endospore-formers isolated from indigenous grassland bulk soil and to compare them with isolates obtained from the rhizospheres of 3 crop types using ITS-PCR, RAPD-PCR and 16S rRNA sequence analyses.

3.2 Materials and methods

3.2.1 Bacterial strains

A total of 34 *Bacillus* isolates, antagonistic towards *R. solani* were investigated for heterogeneity (Table 3.1). These isolates were obtained from bulk soil of native grassland and rhizosphere soil of three crop types: kale (*Brassica oleracea*), maize (*Zea mays)* and wheat (*Triticum aestivum*). Eight *Bacillus* reference strains were also used in the study for comparative purposes (Table 3.2).

Table 3.1: *Bacillus* isolates screened for heterogeneity

Source	**Isolates**
Bulk soil	C15, C16, C17, C27, C28, C37, C40, C54, C56, C74, C76, C78, C91, C92
Maize rhizosphere soil	M12, M15, M27, M60
Wheat rhizosphere soil	W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96
Kale rhizosphere soil	K39, K51, K53, K89

Table 3.2: *Bacillus* reference strains used in the diversity study

Species	Strain designation
B. amyloliquefaciens	DSM 7[b]
B. cereus	R105[c]
B. licheniformis	DSM 13[b]
B. pumilus	DSM 27[b]
B. subtilis	R16[c]
B. subtilis	ATCC 6633[a]
B. subtilis	DSM 3258[b]
B. subtilis. subtilis	DSM 10[b]

[a]ATCC, American Type Culture Collection, Rockville, MD, USA. [b]DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig Germany. [c]Microbiology culture collection, University of KwaZulu-Natal, Pietermaritzburg, KZN, RSA.

Each isolate was 3-way streaked onto 10% ($^{w}/_{v}$) Tryptone Soy Agar (TSA) (Merck) and incubated at 30°C for 72 h, to check for purity. These were then sub-cultured on 10% ($^{w}/_{v}$) TSA plates at 72 h intervals and incubated at 30°C. Long term storage was achieved by growing cultures overnight in Tryptone Soy Broth (TSB) (Merck), thereafter adding 20% ($^{v}/_{v}$) glycerol and freezing at − 80°C (Nilsson *et al*., 1998).

3.2.2 DNA extraction

3.2.2.1 Kit DNA extraction

From an overnight culture grown in TSB (30°C at 150 rpm), 1 ml was dispensed into a sterile microfuge tube and centrifuged at 8.000 ×g for 5 min. The supernatant was discarded and the pelletized cells subjected to DNA extraction and purification using a Nucleospin® DNA extraction kit (Macherey-Nagel), following the protocol recommended by the manufacturer. After extraction, the presence of DNA was checked for using gel electrophoresis (3.2.5). In addition, the quantity of DNA was determined by UV absorbance at A_{260}. DNA concentration was estimated using the equation: A_{260} × dilution factor × 50 = χ µg/ml DNA. The DNA sample was stored at -20°C.

3.2.2.2 DNA extraction from colony pick-off

A single colony from an overnight culture grown on 10% ($^{w}/_{v}$) TSA at 30˚C, was picked-off using a sterile 200 µl pipette tip and re-suspended in 50 µl sterile TE buffer (10 ml of 1M Tris and 2 ml 0.5M Na_2EDTA made up to 1 l with distilled water), in a 200 µl PCR tube (Brousseau *et al*., 1993). To extract DNA, tubes were subjected to two cycles of heating at 94˚C for 10 min and cooling at 4˚C for 5 min, using a thermocycler (Gene Amp PCR system, 2400). Cell debris were then pelleted by centrifugation at 10 000 rpm (Hermle Z160M) for 1 min, at room temperature. The resultant supernatant was kept at -20°C and used directly for PCR.

3.2.3 Intergenic transcribed spacer-PCR (ITS-PCR)

ITS-PCR was performed in a Bioer thermal cycler, using the reverse and forward primers shown in Table 3.3 (Daffonchio *et al*., 1998b). Amplification reactions were carried out in sterile 200 µl thin walled PCR tubes. Each reaction (50µl) consisted of 4.5 µl of 25 mM $MgCl_2$ (Promega), 10 µl of 5× GoTaq® Flexi buffer (Promega), 1 µl PCR nucleotide mix (10mM dNTP's each) (Promega), 1 µl of 10 mM reverse primer, 1 µl of 10 mM forward primer, 0.25 µl of GoTaq® DNA polymerase (1U) (Promega), 2 µl of crude template DNA or 1 µl of kit extracted DNA, made up to volume with sterile nuclease-free water. Controls were included for each set of PCR reactions performed. Kit extracted template DNA of *B. subtilis* ATCC 6633 was used as a positive control, whereas, PCR reactions lacking template DNA were used as negative controls.

The ITS-PCR temperature profile was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 2 min, and an elongation step at 72°C for 2 min. This was then followed by a final extension step at 72°C for 7 min, after which samples were cooled to 4°C and refrigerated at this temperature until electrophoresis analysis could be performed (Daffonchio *et al*., 1998b).

Table 3.3: Primers* used for ITS-PCR, RAPD-PCR, and 16S rRNA-PCR analyses

Primer	Sequence (5´- 3´)	Nucleotides	References
ISR-35 (ITS-PCR)	GTCGTAACAAGGTAGCCGTA	20	Martinez and Sineriz, 2004
ISR-1494 (ITS-PCR)	CAAGGCATCCACCGT	15	Martinez and Sineriz, 2004
OPG-5 (RAPD-PCR)	CTGAGACGGA	10	Daffonchio *et al*., 1998b
OPG-8 (RAPD-PCR)	TCACGTCCAC	10	Daffonchio *et al*., 1998b
OPG-11 (RAPD-PCR)	TGCCCGTCGT	10	Daffonchio *et al*., 1998b
OPG-16 (RAPD-PCR)	AGCGTCCTCC	10	Daffonchio *et al*., 1998b
Bac-F (16S-PCR)	GGGAAACCGGGGCTAATACCGGAT	24	Garbeva *et al*., 2003
1378-R (16S-PCR)	CGGTGTGTACAAGGCCCGGGAACG	24	Garbeva *et al*., 2003

*Primers were synthesized and supplied by Inqaba Biotec™, Hatfield, Pretoria, South Africa.

3.2.4 Random amplified polymorphic DNA-PCR (RAPD-PCR)

RAPD-PCR reactions were carried out in sterile 200 µl thin walled PCR tubes. Each amplification reaction consisted of 1.5 µl of 25 mM $MgCl_2$, 5 µl of 5× GoTaq® buffer, 0.5 µl PCR nucleotide (10mM dNTP's each), 1 µl of 10 mM RAPD primer, 0.25 µl of GoTaq® DNA polymerase (1U), 1 µl of crude template DNA, made up to a total volume of 25 µl with sterile nuclease-free water. Four primers OPG-5, OPG-8, OPG-11 and OPG-16 (Table 3.3) were individually assessed using the RAPD-PCR protocol described above. Positive and negative controls were performed for each set of PCR reactions using the same template DNA for the positive control as described previously in 3.2.3.

The RAPD-PCR amplifications were carried out in a Bioer thermal cycler with the temperature profile of: initial denaturation at 94°C for 4 min, followed by 40 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 35°C for 1 min and an elongation step at 72°C for 2 min. This was followed by a final extension step at 72°C for 5 min, after which samples were maintained at 4°C until electrophoretic analysis could be performed (Daffonchio *et al*., 1998b).

The influence of the DNA extraction method used on RAPD-PCR reproducibility was evaluated using crude or kit extracted DNA from *B. subtilis* ATCC 6633 and strain K51 isolated from kale

rhizosphere soil. The effect of diluting template DNA on RAPD-PCR reproducibility was also investigated using the same two strains. For this purpose, kit extracted DNA from each isolate was diluted 1:1, 1:10, 1:100 and 1:1000 with sterile nuclease-free water prior to RAPD-PCR.

Based on the range of heterogeneity achieved primer OPG-11was used to compare 22 environmental isolates (Table 3.4) with six of the *Bacillus* reference strains (*B. subtilis* ATCC 6633, *B. amyloliquefaciens* DSM 7, *B. subtilis. subtilis* DSM 10, *B. licheniformis* DSM 13, *B. pumilus* DSM 27, *B. subtilis* DSM 3258, and *B. cereus* R105). The 22 environmental isolates were selected as representatives of all the distinct banding patterns previously discerned in the first RAPD-PCD investigation

Table 3.4: Environmental isolates selected for comparative purposes and sequencing

Source/Origin	Isolates
Bulk soil	C15, C16, C27, C40, C54, C56, C76, C91
Maize rhizosphere soil	M12, M15, M60
Wheat rhizosphere soil	W13, W33, W39, W41, W75, W90, W92, W95
Kale rhizosphere soil	K39, K51, K53

3.2.5 Gel electrophoresis

Samples were prepared for electrophoresis by mixing 7 µl of DNA extraction products or PCR products with 3 µl of loading dye (Promega). These were then loaded on a 1.5% ($^{w}/_{v}$) agarose gel and ran in 1 × Tris-acetate buffer (TAE) (0.04M Tris-acetate, 0.001M EDTA) for 5 min at 100 V, followed by 80 V for 80 min. The gels were then stained with ethidium bromide (conc. 25 µg/ml) for 10 min. Banding patterns were viewed using a UV trans-illuminator and the images captured with a Versadoc imaging system (Bio-Rad) using gel documentation software (Quantity One, Bio-Rad).

3.2.6 Groupings based on banding patterns

Fingerprint banding patterns arising from ITS-PCR and RAPD-PCR (Figure 3.2 and Figure 3.9, respectively) were analyzed using G-BOX® imaging software (Syngene). Band sizes were extrapolated from DNA ladder standards (1 kb, Promega) and the matrix data generated was then

subjected to clustering, using the Neighbor-Joining method. A dendrogram was then generated using the same software and the tolerance level was set at 0.05%.

3.2.7 16S rRNA-PCR and sequencing

16S rRNA-PCR was performed with a *Bacillus* specific forward primer (Bac-F) and a universal reverse primer (1378-R) (Table 3.3) (Garbeva *et al*., 2003).

Reactions were carried out in sterile 200 µl thin walled PCR tubes. Each amplification reaction consisted of: 3 µl of 25 mM $MgCl_2$, 10 µl of 5× GoTaq® Flexi buffer, 1 µl PCR nucleotide mix (10mM dNTP's each), 1 µl of 10 mM forward primer, 1 µl of 10 mM reverse primer, 0.25 µl of GoTaq® DNA polymerase (1U), 1 µl of crude template DNA, made up to a final volume of 50 µl with nuclease-free water. Positive and negative controls were performed for each PCR reaction as described in 3.2.3, using kit-extracted DNA template from *B. subtilis* R16 as the positive control.

The 16S rRNA-PCR amplifications were carried out in a Bioer thermal cycler using the following temperature profile: 94°C for 5 min initial denaturation, followed by 35 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 65°C for 90 sec, and an elongation step at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. All samples were kept at 4°C until electrophoretic analysis could be performed (Garbeva *et al*., 2003).

The resulting 16S rRNA PCR products were purified and then sequenced by Inqaba Biotech™, Hatfield, Pretoria, using an ABI 3130 XL sequencer and the Sanger sequencing method. The sequence data acquired was edited using Chromas LITE software (Version 2.01) and subjected to a NCBI database blast search to identify sequence homologies. Subsequently, sequences were aligned using Clutal W software and compared with 16S rRNA sequence data of selected *Bacillus* strains sourced from the GenBank database (www.ncbi.com). Thereafter, the evolutionary distance between strains was computed by the Maximum Composite Likelihood method using MEGA 4.1 software. A Neighbour-Joining phylogenetic tree was then formulated with Boot strapping analysis (Tamura *et al*., 2007).

3.3 Results

3.3.1 ITS-PCR

ITS-PCR banding patterns for each of the 34 strains used in the study are shown in Figure 3.1. With very few exceptions, isolates displayed a high degree of homology in terms of band numbers and sizes. For these isolates, three prominent bands were visible with approximate sizes of 200 bp, 450 bp and 550 bp. Isolate C76 (lane 11) yielded a banding pattern that closely resembled that of the positive control *B. subtilis* ATCC 6633 (lane 16). Isolate W3 (lane 17) was unique in that it yielded six different bands.

ITS-PCR was repeated with 21 local isolates that were randomly selected from the different soil types and were compared with known *Bacillus* spp. reference strains (Figure 3.2). Isolates still showed similar banding patterns obtained in Figure 3.1. *Bacillus cereus* R105 (lane 7) was clearly distinguishable from the rest of the reference strains (lane 1-6) based on band size, and showed high levels of homology with most of the environmental isolates, particularly those obtained from the indigenous bulk soil (Figure 3.2).

A Neighbor-Joining dendogram was constructed to compare the ITS-PCR banding profile obtained in Figure 3.2 (Figure 3.3). At a level of ~ 93.5% relatedness, three main clusters were distinguished between the 21 local isolates. The bulk soil isolates formed a tight group (cluster II^a) distinct from the rhizosphere isolates (cluster I^a). Exceptions include wheat rhizosphere isolates, W41 and W13 which were grouped closely with isolates from the bulk soil. Bulk soil isolate C76 was distantly related (cluster III^a) from any of the local isolates but was closely related with the *B. subtilis* reference group, more particularly *B. subtilis* ATCC 6633 strain. The local isolates, predominantly from the bulk soil were confirmed to be closely related to the *B. cereus* R105 strain as had been observed in Figure 3.2. Within the reference strains, clear distinction between *B. cereus* R105 and *B. subtilis* group was observed. Further distinctions within the latter strains were detected, as the following strains were closely grouped: *B. subtilis* DSM 3258 and *B. amyloliquefaciens* DSM 7; *B. subtilis* ATCC 6633; *B. pumilus* DSM 27, *B. subtilis*. *subtilis* DSM 10 and *B. licheniformis* DSM 13, with the latter 2 more closely related.

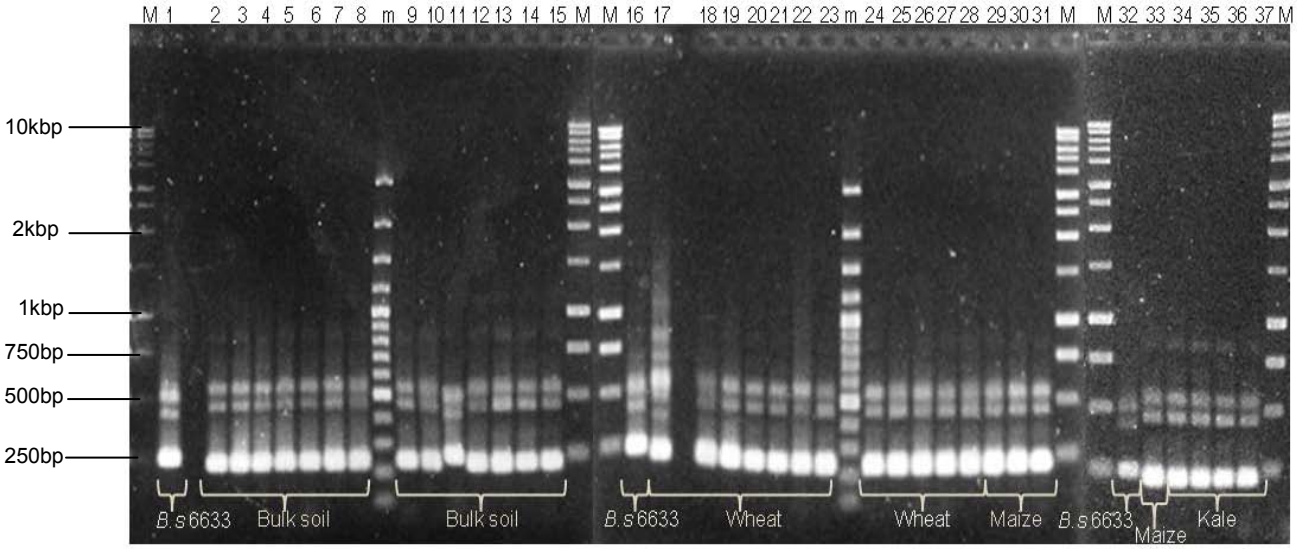


Figure 3.1: Intergenic transcribed spacer-PCR (ITS-PCR) analysis of antifungal aerobic endospore-forming bacteria on 1.5% agarose gels. Lanes: M, 1 kbp ladder; m, 100 bp ladder; 1, *B. subtilis* ATCC 6633; 2-15, bulk soil isolates C15, C16, C17, C27, C28, C37, C40, C54, C56, C76, C74, C78, C91, C92; 16, *B. subtilis* ATCC 6633; 17-28, wheat rhizobacteria W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96; 29-31, maize rhizobacteria M12, M15, M27; 32, *B. subtilis* ATCC 6633; 33, maize rhizobacteria M60; 34-37 kale rhizobacteria K39, K51, K53, K89.

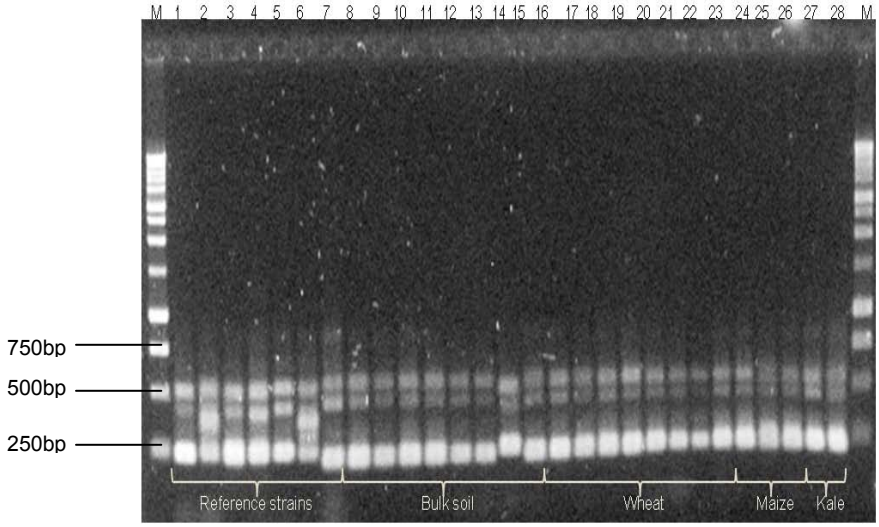


Figure 3.2: ITS-PCR comparison of local endospore-forming isolates with *Bacillus* reference strains, on a 1.5% agarose gel. Lanes: M, 1kbp ladder; 1, *B. subtilis* ATCC 6633; 2, *B. amyloliquefaciens* DSM 7; 3, *B. subtilis. subtilis* DSM 10; 4, *B. licheniformis* DSM 13; 5, *B. pumilus* DSM 27; 6, *B. subtilis* DSM 3258; 7, *B. cereus* R105; 8-15, bulk soil isolates C15, C16, C27, C40, C54, C56, C76, C91; 16-23, wheat rhizobacteria W13, W33, W39, W41, W75, W90, W92, W95; 24-26, maize rhizobacteria M12, M15, M60; 27-28 kale rhizobacteria K51, K53.

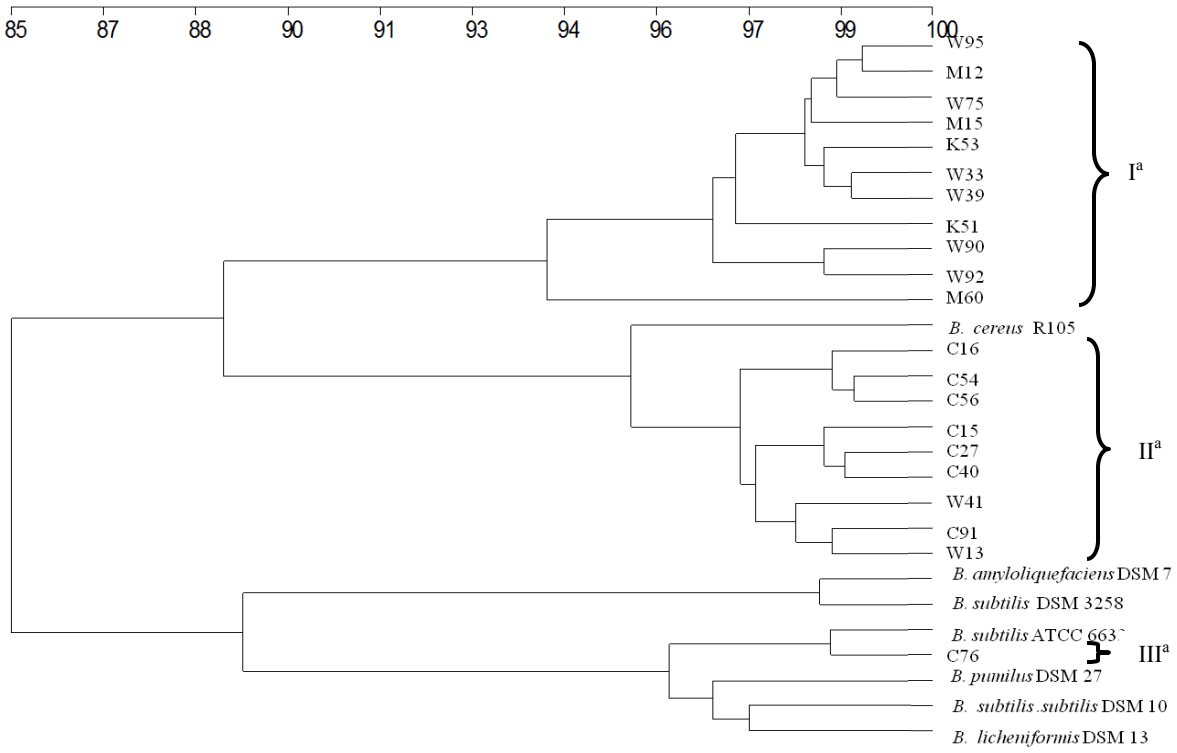


Figure 3.3: Neighbour-Joining dendrogram of local endospore-forming isolates from different soil types, showing their relationship to *Bacillus* spp. reference strains, inferred from ITS-PCR band patterns in 1.5% agarose gels, using G-BOX software.

3.3.2 RAPD-PCR analysis

3.3.2.1 Influence of DNA preparation and dilution

The influence of the DNA extraction method used and the effect of diluting template DNA on RAPD-RCR reproducibility are shown in Figure 3.4. RAPD profiles for crude- and kit-extracted template DNA for each strain were very similar. However, band intensities varied with the level of dilution. When the original DNA concentration (approximately, 7 µg/ml for *B. subtilis* ATCC 6633 and 5 µg/ml for Isolate K51) was at low dilutions of 1:1 and 1:10, additional bands greater than 2.5 k bp were apparent in both strains but these became fainter or disappeared at higher dilutions of 1:100 and 1:1000.

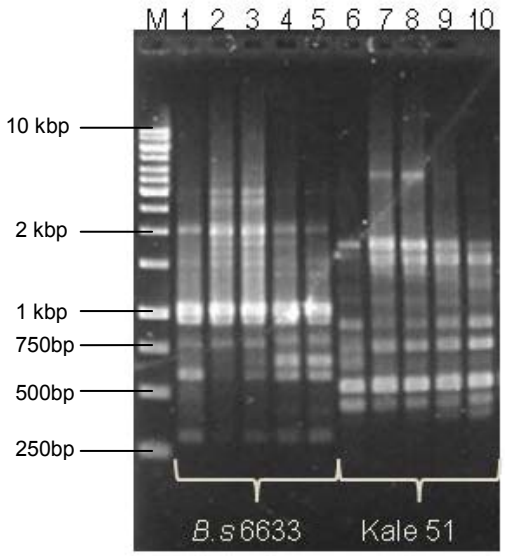


Figure 3.4: Influence of template DNA preparation method and dilution on RAPD-PCR reproducibility, with OPG-11 as primer. Lanes: M, 1 kbp ladder; 1, *B. subtilis* ATCC 6633 crude DNA extract; 2-5, *B. subtilis* ATCC 6633 kit extracted DNA, dilution ratios 1:1, 1:10, 1:100, and 1:1000; 6, Kale 51 crude DNA extract; 7-10, Kale 51 kit extracted DNA, dilution ratios 1:1, 1:10, 1:100 and 1:1000.

3.3.2.2 Primers

RAPD banding patterns for each of the primers tested are shown in Figures 3.5 to 3.8. Primer OPG-5 yielded unsatisfactory results as many isolates were not amplified, particularly those obtained from bulk and kale rhizosphere soil (Figure 3.5). This data was therefore not analyzed further.

Primers OPG-8, OPG-11 and OPG-16 were more successful in producing amplified bands from the different soil isolates, as seen in Figure 3.6, Figure 3.7 and Figure 3.8, respectively. Based on visual comparison of prominent band patterns and band sizes, these primers grouped bulk soil isolates into four main clusters (Table 3.5). Isolate C15 was not amplified when primer OPG-16 was used.

Amplified wheat strains were grouped into three similar clusters by OPG-8 and OPG-11, whereas OPG-16 distinguished four groups. Wheat isolate W15 was not amplified by OPG-8 or OPG-11, whereas, Isolates W3 and W95 were not successfully amplified by primers OPG-11 and OPG-16. Primer OPG-11 did not amplify Isolates W75 and W96; whereas, OPG-16 was unsuccessful at amplifying Isolate W90.

Amplified maize isolates were grouped into two similar clusters by OPG-11 and OPG-16, whereas OPG-8 yielded only one observable grouping. Maize Isolates M27 and M60 were not successfully amplified by OPG-11 and OPG-16, respectively.

Amplified kale isolates showed similar groupings when OPG-8 and OPG-16 were used, whereas OPG-11 distinguished between

Isolates K51 and K53 the only two strains amplified, as shown in Table 3.5. Isolate K89 was not amplified by OPG-11 or OPG-16, whereas Isolate K39 was not successfully amplified only by primer OPG-11.

Though primer OPG-11 was unsuccessful at amplifying all isolates (26/34), it was still used for further analyses as clearer distinctions between the different groupings were observed and more clusters were obtained.

Table 3.5: Groupings of antifungal aerobic endospore-formers based on visual comparison of band patterns and sizes acquired from RAPD-PCR using primers OPG-8, OPG-11 and OPG-16

Sample set	RAPD-PCR Primers		
	OPG-8	**OPG-11**	**OPG-16**
	No. of groupings	**No. of groupings**	**No. of groupings**
Bulk soil	1) C15 2) C16, C17, C27, C28, C37, C40, C74, C78, C92 3) C54, C56, C91 4) C76	1) C15 2) C16, C17, C27, C28, C37, C40, C74, C78, C92 3) C54, C56, C91 4) C76	1) C16, C17, C27, C28, C37, C40, C74, C78, C92 2) C54, C56, C91 3) C76 (C15) *
Wheat	1) W3, W13, W33, W39, W50, W75, W92, W95, W96 2) W41 3) W90 (W15) *	1) W13, W33, W39, W50, W92 2) W41 3) W90 (W3, W15, W75, W95, W96) *	1) W13 2) W15, W39, W75 3) W33, W50, W92, W96 4) W41 (W3, W90, W95) *
Maize	1) M12, M15, M27, M60	1) M12, M60 2) M15 (M27) *	1) M12 2) M15, M27 (M60) *
Kale	1) K39, K51, K53, K89	1) K51 2) K53 (K39, K89) *	1) K39, K51, K53 (K89) *

* Enclosed isolate number(s) were not amplified by respective primer.

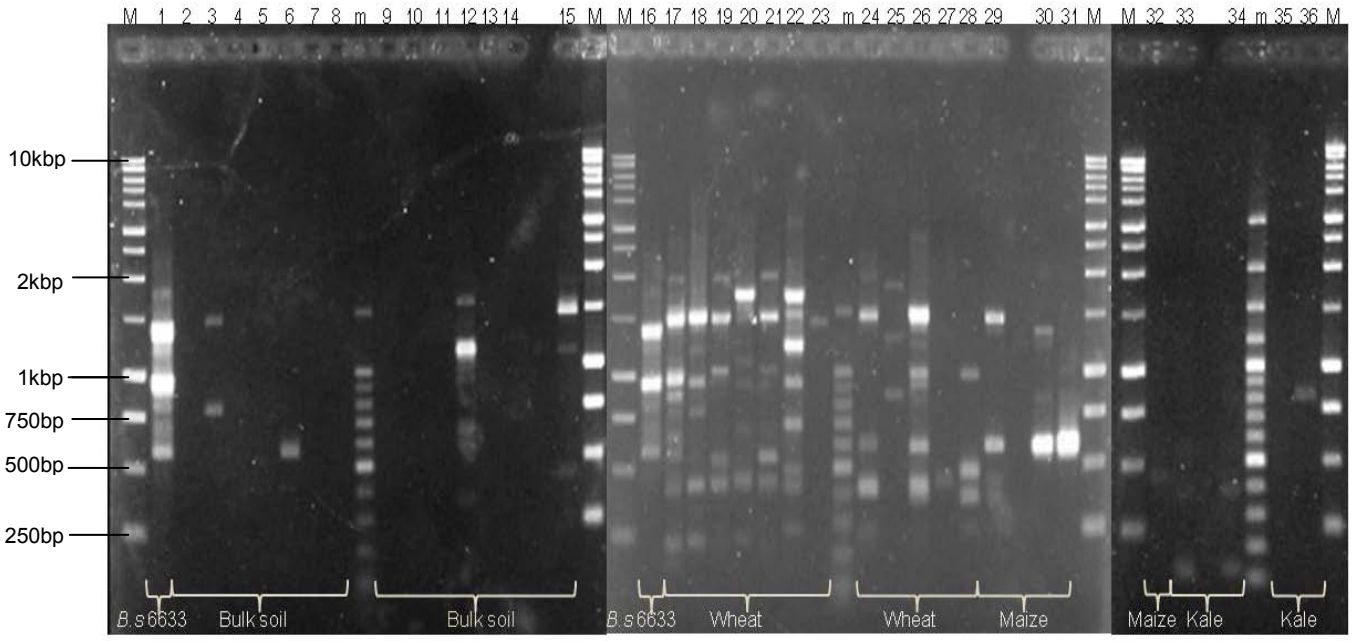


Figure 3.5: Random amplified polymorphic DNA-PCR (RAPD-PCR) analysis of antifungal aerobic endospore-forming bacteria on 1.5% agarose gels, with OPG-5 as primer. Lanes: M, 1kbp ladder; m, 100bp ladder; 1, *B. subtilis* ATCC 6633; 2-15, bulk soil isolates C15, C16, C17, C27, C28, C37, C40, C54, C56, C74, C76, C78, C91, C92; 16, *B. subtilis* ATCC 6633; 17-28, wheat rhizobacteria W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96; 29-32, maize rhizobacteria M12, M15, M27, M60; 33-36, kale rhizobacteria K39, K51, K53, K89.

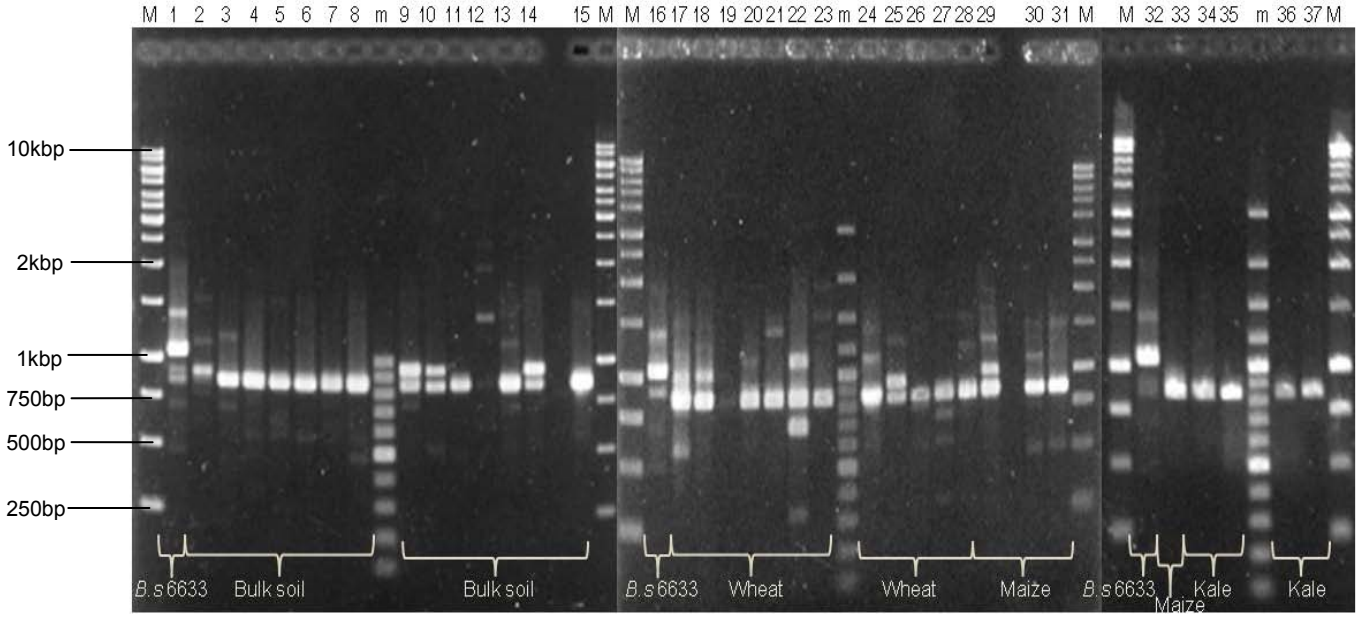


Figure 3.6: RAPD-PCR analysis of antifungal aerobic endospore-forming bacteria on 1.5% agarose gels, with OPG-8 as primer. Lanes: M, 1kbp ladder; m, 100bp ladder; 1, *B. subtilis* ATCC 6633; 2-15, bulk soil isolates C15, C16, C17, C27, C28, C37, C40, C54, C56, C74, C76, C78, C91, C92; 16, *B. subtilis* ATCC 6633; 17-28, wheat rhizobacteria W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96; 29-31, maize rhizobacteria M12, M15, M27; 32, *B. subtilis* 6633; 33 maize rhizobacteria M60; 34-37, kale rhizobacteria K39, K51, K53, K89.

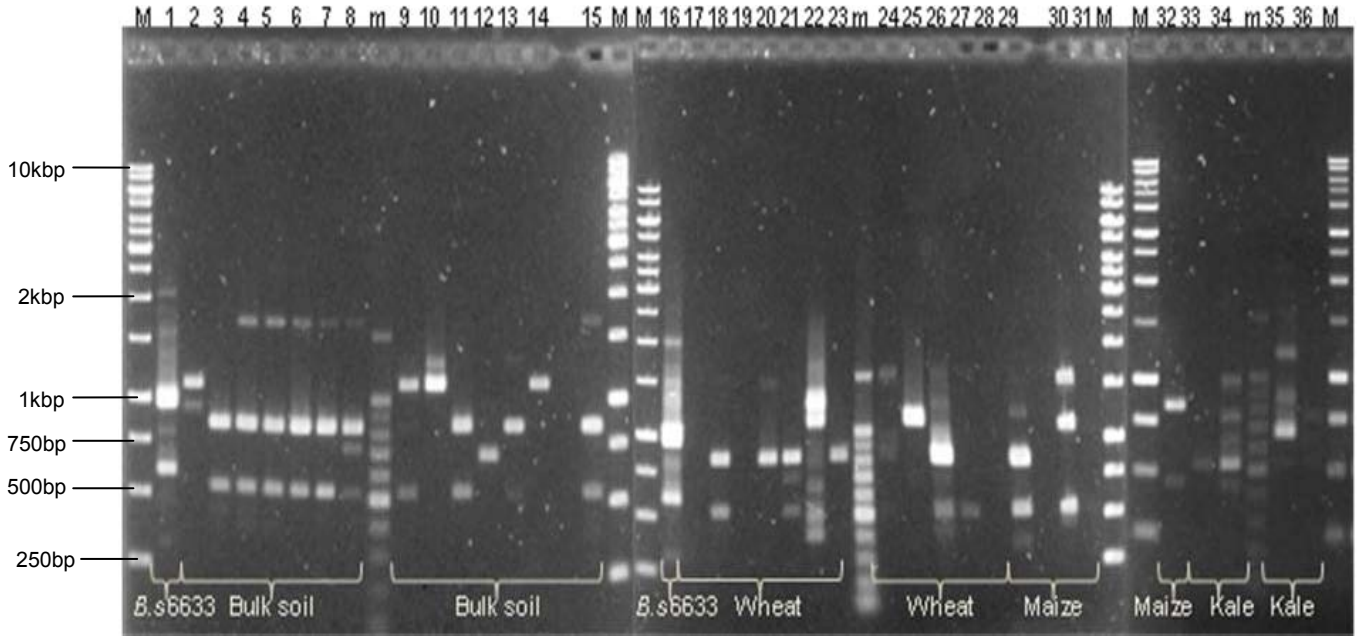


Figure 3.7: RAPD-PCR analysis of antifungal aerobic endospore-forming bacteria on 1.5% agarose gels, with OPG-11 as primer. Lanes: M, 1kbp ladder; m, 100bp ladder; 1, *B. subtilis* ATCC 6633; 2-15, bulk soil isolates C15, C16, C17, C 27, C 28, C 37, C 40, C 54, C 56, C 74, C 76, C 78, C 91, C 92; 16, *B. s ubtilis* ATCC 6633; 17 -28, w heat rhizobacteria W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96; 29-32, maize rhizobacteria M12, M15, M27, M60; 33-36, kale rhizobacteria K39, K51, K53, K89.

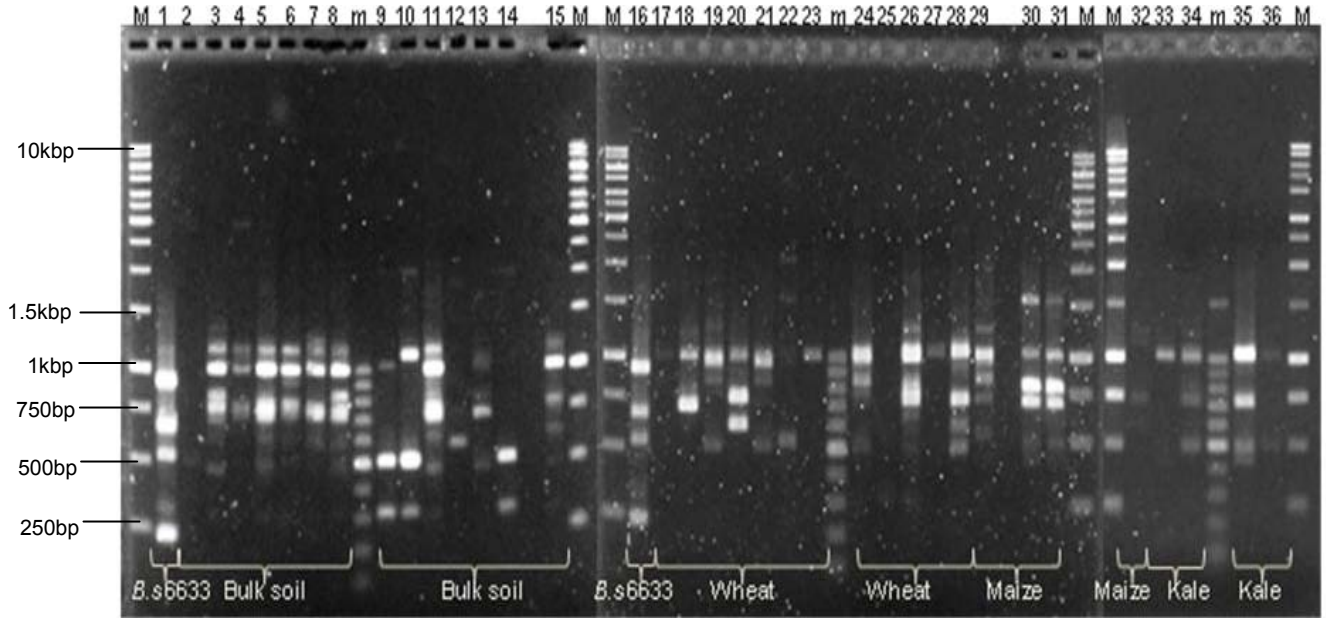


Figure 3.8: RAPD-PCR analysis of antifungal aerobic endospore-forming bacteria on 1.5% agarose gels, with OPG-16 as primer. Lanes: M, 1kbp ladder; m, 100bp ladder; 1, *B. subtilis* ATCC 6633; 2-15, bulk soil isolates C15, C16, C17, C 27, C 28, C 37, C 40, C 54, C 56, C 74, C 76, C 78, C 91, C 92; 16, *B. s ubtilis* ATCC 6633; 17 -28, w heat rhizobacteria W3, W13, W15, W33, W39, W41, W50, W75, W90, W92, W95, W96; 29-32, maize rhizobacteria M12, M15, M27, M60; 33-36, kale rhizobacteria K39, K51, K53, K89.

3.3.2.3 Reproducibility of RAPD-PCR and comparison of local isolates with known *Bacillus* strains

The reproducibility of RAPD-PCR was tested on 22 local isolates and compared with *Bacillus* spp. reference strains, using OPG-11 as primer (Figure 3.9). The banding patterns for each isolate were similar to those obtained in the earlier analysis (compare Figures 3.7 and 3.9). In some instances, viz. Isolates C54, W13, W75, W95 and M12 had additional faint bands that were discerned and variation in individual band intensities was evident. The reference strains had different RAPD-PCR banding profiles within the group and the 22 local isolates tested.

A Neighbour-Joining phylogenetic tree was constructed to infer the relationship between the 22 local isolates and the *Bacillus* reference strains, based on their RAPD-PCR banding profiles (Figure 3.10). At a level of ~80% relatedness, isolates were grouped into 6 clusters (Figure 3.10). Within some of these (i.e. clusters I^b and III^b), strains obtained from both bulk and rhizosphere soils were apparent. At higher levels of relatedness (i.e >86%), further groupings were evident in which isolates from the same source were grouped. Wheat isolate, W41 (cluster IV^b) was distinct from the other isolates, with relatedness of less than 63%. No close relationship between the reference strains and local isolates was evident.

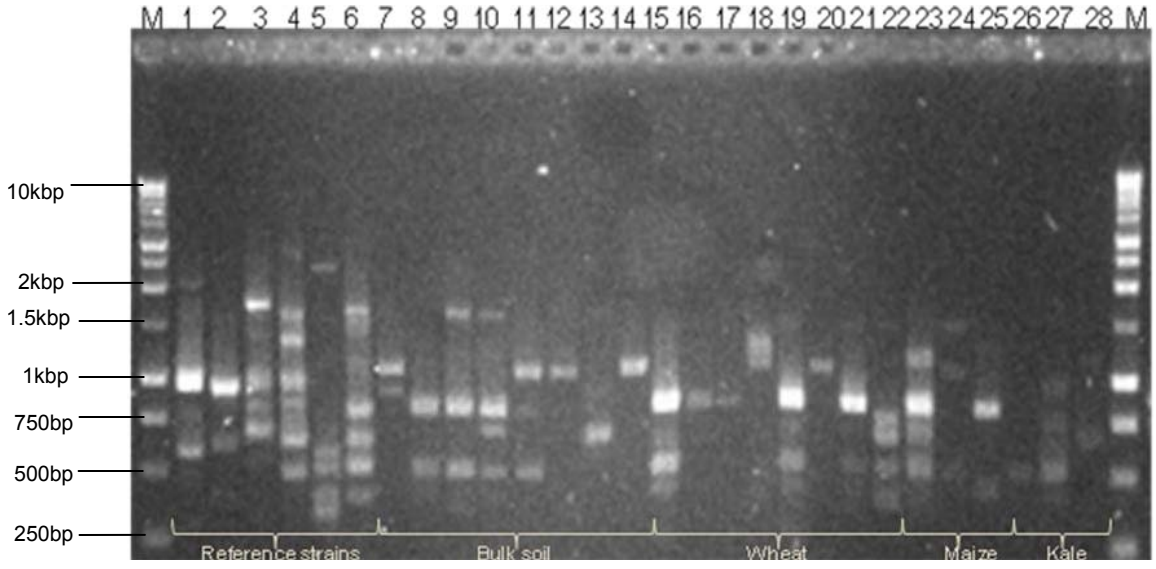


Figure 3.9: RAPD-PCR analysis of the local isolates and reference *Bacillus* strains on 1.5% agarose gel, with OPG-11 as primer. Lanes: M, 1 kbp ladder; 1, *B. subtilis* ATCC 6633; 2, *B. amyloliquefaciens* DSM 7; 3, *B. subtilis. subtilis* DSM 10; 4, *B. licheniformis* DSM 13; 5, *B. pumilus* DSM 27; 6, *B. subtilis* DSM 3258; 7 - 14, bulk soil isolates C15, C16, C27, C40, C54, C56, C76, C91; 15-22, wheat rhizobacteria W13, W33, W39, W41, W75, W90, W92, W95; 23-25, maize rhizobacteria M12, M15, M60; 26-28 kale rhizobacteria K39, K51, K53.

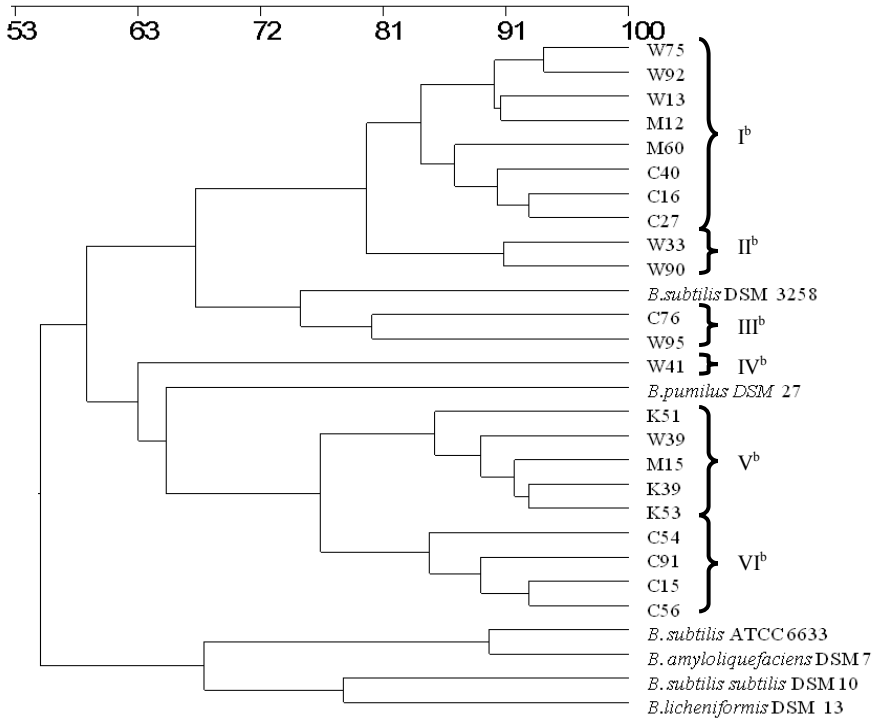


Figure 3.10: Neighbour-Joining dendrogram of the local isolates and reference *Bacillus* strains inferred from band patterns on 1.5% agarose acquired in RAPD-PCR analysis, with OPG-11 as primer.

3.3.3 16S rRNA sequencing

Amplified PCR fragments of the 16S rRNA gene from the 22 environmental isolates and the reference strain *B. subtilis* R16 are shown in Figure 3.11. All isolates had a band size of approximately 1.25 kbp.

All the 22 environmental isolates, with the exception of Isolate C76 were matched with the *B. cereus* group (99% to 100% similarity) when 16S rRNA sequence data was compared on the NCBI/BLASTN database. Isolate C76 was found to be closely associated with members of the *B. subtilis* group (100% similarity). The evolutionary distances between these sequences and 10 *Bacillus* spp. reference strains acquired from GenBank (www.ncbi.com) were then used in inferring a Neighbour-Joining phylogenetic tree computed in MEGA 4.1, as seen in Figure 3.12. The majority of isolates were grouped as being identical to the *B. cereus* reference strains R105, 5YW6 and BBD-217-1a. Isolate C76 was shown as being closely related to *B. subtilis* strains SKVTC-12 and ATCC 6633.

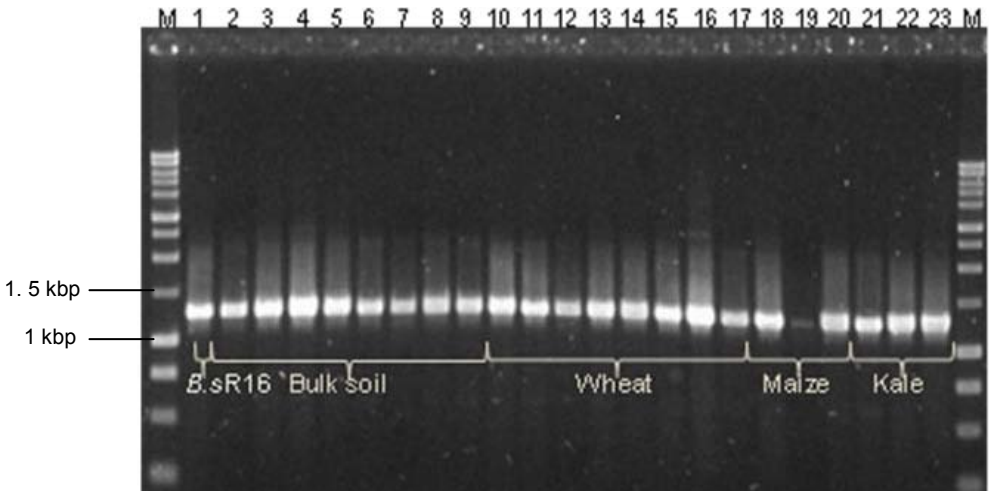


Figure 3.11: 16S rRNA analysis using *Bacillus* specific forward primer (Bac-F) and a universal reverse primer (1378-R) on 1.5% agarose gel. Lanes: M, 1 kbp ladder; 1, *B. subtilis* R16; 2-9, bulk soil isolates C15, C16, C27, C40, C54, C56, C76, C91; 10-17, wheat rhizobacteria W13, W33, W39, W41, W75, W90, W92, W95; 18-20, maize rhizobacteria M12, M15, M60; 21-23 kale rhizobacteria K39, K51, K53.

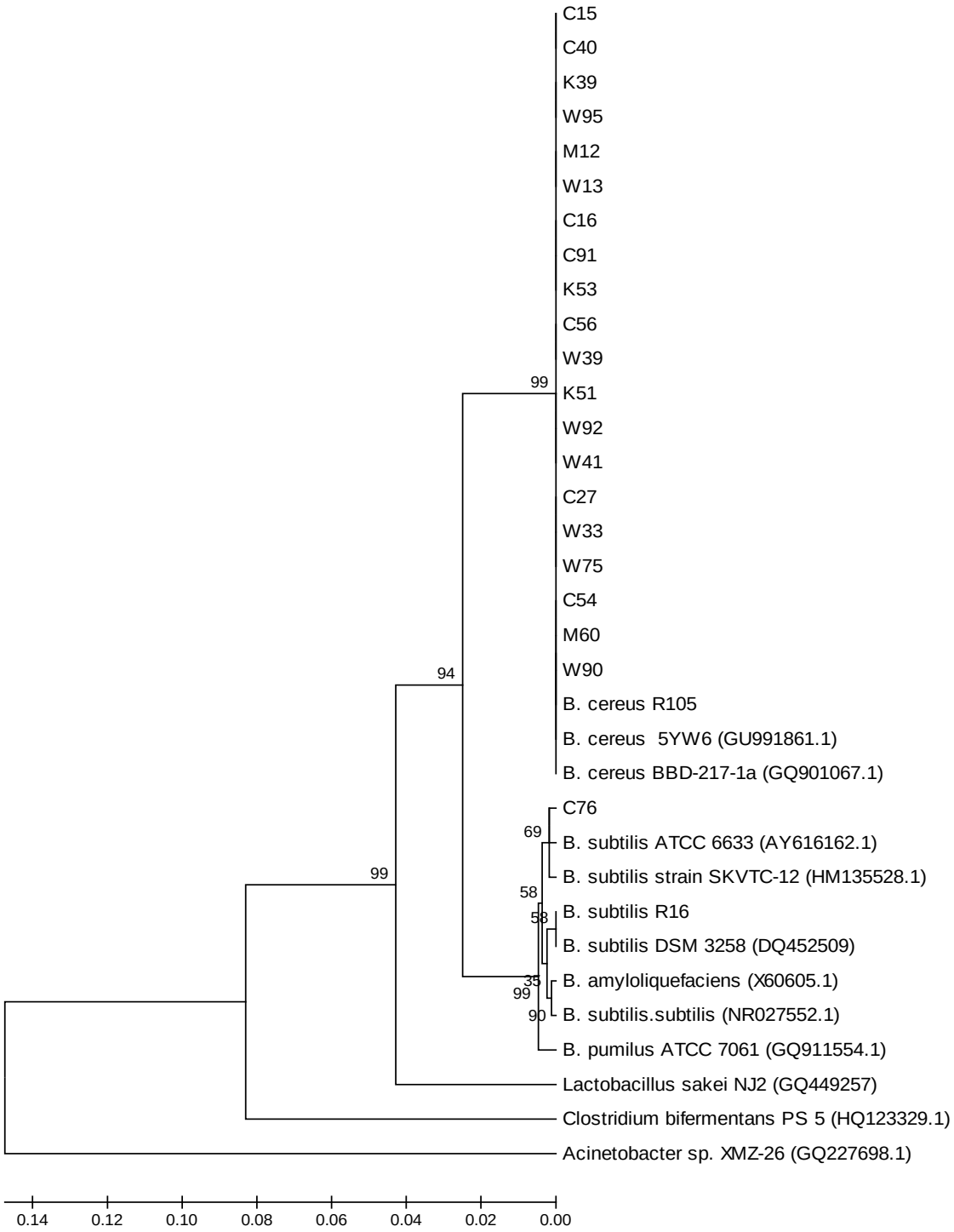


Figure 3.12: Neighbour-joining tree showing the phylogenetic affiliation of local isolates obtained from bulk soil and maize, wheat and kale rhizosphere soil to 10 *Bacillus* reference strain sequences. Relationships were inferred using MEGA 4.1 (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method. Percentages given at nodes indicate calculated bootstrap values. Letters represent the origin of isolates: C= bulk soil, W = wheat rhizosphere soil, M = maize rhizosphere soil, K = kale rhizosphere soil. Accession numbers are indicated in (). Bacterial strains of *Acinetobacter*, *Clostridium* and *Lactobacillus* served as the out groups.

3.4 Discussion

Various researchers have proposed ITS-PCR as a useful technique for distinguishing between *Bacillus* species and strains (Daffonchio *et al*., 2003; Martinez and Sineriz, 2004). This technique entails the amplification of intergenic regions separating 16S and 23S ribosomal RNA genes (Freitas *et al*., 2008). This region is not highly conserved like the adjacent rRNA genes and thus microorganisms including the members of the genus *Bacillus* and closely related taxa, exhibit differences in ITS length and/or ribosomal operon organization (Martinez and Sineriz, 2004). The multiple bands observed in the ITS-PCR analyses (Figures 3.1 and 3.2) were attributed to the fact that members of the genus *Bacillus* contain multiple copies of the 16S rRNA gene (Daffonchio *et al*., 1998b). These copies differ in the size of the ITS region and may or may not have tRNA genes interspersed, both of which will result in different band patterns when amplified (Cherif *et al*., 2003; Daffonchio *et al*., 2003). In this study, the majority of isolates from the bulk and rhizosphere soil exhibited high levels of ITS homology, since very similar band patterns and sizes were observed (Figure 3.1). This homology suggests that the isolates had similar ribosomal operon organizations, indicating their close relationships and possibly a recent common ancestor (Daffonchio *et al*., 1998b). When ITS-PCR banding patterns were compared using the Neighbour-Joining dendogram (Figure 3.3), it was evident that with the exception of Isolate C76, the isolates were clustered into two groups both of which showed no less than 88.6 % similarity with *B. cereus* R105. Conversely Isolate C76 showed 98.5% similarity with *B. subtilis* ATCC 6633. These findings were further corroborated by the BLAST search results which matched most of the isolates with the *B. cereus* group, whereas Isolate C76 was closely associated to the *B. subtilis* group, as seen in Figure 3.12. ITS-PCR was able to detect heterogeneity between strains within the *B. subtilis* reference group of micro-organisms (Figure 3.3). This fine distinction between the *B. subtilis* strains was attributed to the intergenic regions separating 16S and 23S rRNA region genes not experiencing the same selective evolutionary pressure as the adjacent rRNA regions (Martinez and Sineriz, 2004). These evolutionary changes may arise from random mutations or horizontal gene transfer (Stein, 2005).

Although ITS-PCR analysis revealed visually similar banding patterns in *B. cereus* and *B. subtilis* isolates, they were distinguishable when band sizes were considered (Figure 3.2). These results are similar to those obtained by Daffonchio *et al*. (2000) who found that *B. cereus* and *B.*

subtilis harbor similar ribosomal operon organization but different ITS lengths, hence they display similar banding patterns but have different band sizes (Daffonchio *et al*., 2000). Based on *B. subtilis* reference group banding profiles (Figure 3.2), closely related species were not easily distinguished (e.g. *B. subtilis*. *subtilis* DSM 10 (lane 3) and *B. pumilus* DSM 27 (lane 5)), whereas, in other cases intraspecific strain variation was evident (e.g. *B. subtilis* ATCC 6633 (lane 1) and *B. subtilis* DSM 3258 (lane 6)). Overall, these results suggest that ITS-PCR is a useful means for establishing preliminary differentiation between isolates and grouping closely related species into clusters for further analysis. Daffonchio *et al*. (2003) proposed that improved differentiation of species and strains can be achieved using ITS-homoduplex-heteroduplex (HHP) analysis. This technique entails separating ITS-PCR products on polyacrylamide based gels or mutation detection enhancement (MDE) gels instead of agarose gels, resulting in increased band numbers and more complex banding patterns being resolved. The detection of additional bands arises from the cross-hybridization of different ribosomal operons that can result in heteroduplex molecules being formed; which can be differentiated by their slow migration under electrophoretic conditions (Daffonchio *et al*., 2003). Thus, ITS-HHP analysis may be used to enhance the sensitivity of ITS-PCR in determining the diversity of species and strains within a population.

RAPD-PCR is another useful technique for distinguishing between closely related micro-organisms (Olive and Bean, 1999; Atienzar and Jha, 2006). Daffonchio *et al*. (1998b) illustrated this by showing that this procedure distinguished a greater level of diversity between *B. cereus* strains than did ITS-PCR and tDNA-PCR. RAPD-PCR is based on the amplification of certain segments of the whole genome, including extrachromosomal elements that match with a randomly chosen single short primer (Damiani *et al*., 1996; Atienzar and Jha, 2006). In this study, the influence of DNA extraction method on RAPD-PCR reproducibility was evaluated using crude and kit extracted template DNA. As seen in Figure 3.4, both methods yielded reproducible banding patterns that were comparable for the individual *B. subtilis* ATCC 6633 and K51 strains tested. Similarly, dilution of kit extracted DNA (Initial concentration 7 µg/ml) yielded consistent banding profiles even at the thousand-fold dilution. However, at high template concentrations, additional bands with molecular weights greater than 2.5 kbp were visible for both *B. subtilis* ATCC 6633 and K51 strains tested. This was attributed to high DNA template concentrations increasing the likelihood a non-specific amplification, particularly when an

annealing temperature with low levels of stringency is used (Roux, 1995). Nilsson *et al*. (1998) also observed these phenomenons and recommended that bands with molecular weight greater than 2 kbp be excluded from data analyses.

When kit-extracted DNA was diluted 100× and 1000×, similar banding patterns to those obtained using the colony pick-off method were observed (Figure 3.4). These results probably reflected the influence of low concentrations of template DNA on the RAPD procedure. Template DNA derived from this simple, fast and reproducible extraction method was suitable for RAPD-PCR. Additionally, this method promises to be a cost effective means of screening large numbers of isolates without the laborious purification steps associated with obtaining chromosomal DNA (Nilsson *et al*., 1998). Thus, this crude DNA extraction procedure was included in the standard protocol for routine DNA preparation in this study.

Of the four arbitrary primers (Table 3.3) used to investigate the diversity within the 34 antifungal aerobic endospore-forming bacteria, primer OPG-5 did not amplify the DNA of all of all the isolates being evaluated (Figure 3.5), and thus was excluded from further investigations. The remaining primers: OPG-8, OPG-11 and OPG-16 were useful for generating molecular fingerprinting profiles (Figures 3.6 – 3.8). The amplicons produced by these primers grouped the isolates within each sample set into several clusters, as seen in Table 3.5. The different primers did not show totally consistent clustering of all the isolates tested; however, some similarities were observed. For example, the amplified DNA from bulk soil isolates were clustered into similar groups by the 3 primers. Overall, it was difficult to accurately determine which primer detected the greatest level of heterogeneity as not all isolates were equally amplified. This is a commonly encountered problem which has led to the recommendation that multiple primers be used in heterogenic detection, as the amplification of different genomic regions might enhance distinctions between closely related species and strains (Daffonchio *et al*., 1998b). Notwithstanding this, primer OPG-11 was chosen for further studies because from the primers used, it provided the greatest degree of heterogenic identification allowing more clusters to be distinguished.

RAPD-PCR banding patterns of the 22 local isolates from the different soil types were compared with those obtained for *Bacillus* spp. reference strains, using OPG-11 as primer (Figure 3.9). No direct relationship between the local isolates and the reference strains was evident. However, a

clear distinction between the closely related reference strains could be made. This includes those strains with identical ITS-PCR banding patterns (e.g. *B. subtilis. subtilis* DSM 10 and *B. pumilus* DSM 27). These findings suggest that RAPD-PCR is more effective than ITS-PCR in distinguishing between closely related strains.

To test the reproducibility of the RAPD-PCR protocol using template DNA extracted from colony pick-offs, 22 of the 34 isolates from previous analysis were used. The 22 isolates were grown separately and crude template DNA obtained was used to perform RAPD-PCR. The banding patterns produced were compared with those obtained earlier to check reproducibility (compare Figure 3.7 and 3.9), having used OPG-11 as primer in both instances. The corresponding RAPD profiles were comparable with matching prominent bands. However, in some instances individual bands varied in intensity and additional faint bands were apparent (e.g. Isolates C54, W13, and M12). This could be attributed to variations in template DNA concentration, as it proved difficult to standardize the amount of template DNA extracted when using the colony pick-off method. Such variations could lead to inconsistencies in grouping patterns. Notwithstanding these problems, standardization of amplification processes including DNA-preparation, amplification conditions and the equipment used minimized the short-comings encountered with RAPD-PCR reproducibility (Damiani *et al*., 1996; Manzano *et al*., 2009). Nilsson *et al*. (1998) also found that faint bands were variable and recommended their exclusion from analyses as their inclusion could lead to false groupings.

The results of the ITS-PCR analyses and 16S rRNA sequencing indicated that the majority of isolates from both the bulk and rhizosphere soil were closely related to the *B. cereus* group. RAPD-PCR gave a finer indication of the level of strain diversity among these isolates. Similarly, antifungal and biochemical assays indicated a degree of phenotypic strain diversity within this group of organisms (Table 2.2 and 2.4). However, it proved difficult to match phenotypic traits with specific RAPD-PCR groupings (Table 3.5). Based on the RAPD-PCR banding profiles, a greater degree of genetic diversity was observed between isolates from the bulk soil compared to isolates obtained from the three rhizosphere soils (Figures 3.6 - 3.8). Of the rhizosphere isolates, only W41 was unique to the rhizosphere soil. The remaining rhizosphere isolates produced RAPD-PCR profiles comparable to several of the bulk soil isolates. Whether this indicates rhizosphere competence in the isolates from the bulk soil or that

there was insufficient time for a rhizosphere effect to develop still needs to be established. The former would be advantageous since it suggests broad-based plant growth promoting rhizobacteria that might prove profitable.

The *B. cereus* group consists of saprophytic micro-organisms commonly isolated from agricultural soil (McSpadden Gardener, 2004). Various strains including *B. cereus* UW 85 have exhibited plant growth promoting properties and production of several types of antibiotics (Zhao *et al*., 2007; Emmert *et al*., 2004). The latter include: aminopolyol antibiotics, zwittermicin A; and 3-amino-3deoxy-D-glucose antibiotic, kanosamine (Milner *et al*., 1995; Emmert *et al*., 2004). Zwittermicin A is of particular interest as its range of antimicrobial activity is broad and includes the important fungal phytopathogens, *Fusarium solani*, *Sclerotinia sclerotiorum*, *Pythium aphanidermatum* and *R. solani* (Milner *et al*., 1995; Silo-Suh *et al*., 1998). Hence, detection and extraction of zwittermicin A from the experimental isolates was attempted and the results are presented in Chapter 4.

Chapter 4: Detection and extraction of antifungal metabolites from aerobic endospore-forming bacteria

4.1 Introduction

Several *Bacillus* spp. are known for their ability to antagonize a range of fungal phytopathogens (McSpadden Gardener, 2004; Carzorla *et al*., 2007). Antibiotic production is considered to be the main mechanism of inhibiting these pathogens and has been positively linked to biological control of plant diseases (Asaka and Shoda, 1996; Sadfi *et al*., 2002).

Many *Bacillus* antibiotics exhibiting antifungal activity have been identified as peptide antibiotics composed mainly of amino acids and other residues (Stein, 2005; Kavitha *et al*., 2005). Examples include: iturin A produced by *B. subtilis*; fengycin M4 produced by *B. licheniformis*; and plipastatins produced by *B. cereus* (Kavitha *et al*., 2005; Rodrigues *et al*. 2006). Another unique class of *Bacillus*-produced antibiotics is the linear aminopolyol type, of which zwittermicin A produced by *B. cereus* and *B. thuringiensis* strains is probably the best known (Milner *et al*., 1995; Zhao *et al*., 2007). Zwittermicin A inhibits a wide variety of prokaryotes and eukaryotes, the latter including oomycetes (e.g. *Phytophthora medicaginis* and *Pythium torulosum*) and other fungal phytopathogens (such as, *Alternaria alternata* and *Botrytis cinerea*) (Silo-Suh *et al*., 1998; Stohl *et al*., 1999a). Zwittermicin A has also been shown to enhance the insecticidal activity of toxic proteins produced by *B. thuringiensis*, thereby increasing the mortality levels in insects that are typically resistant to the toxin (Emmert *et al*., 2004; Zhao *et al*., 2007).

The ability to characterize and identify specific types of antibiotics could be useful for selecting potential biocontrol agents (Athukorala *et al*., 2009). Traditionally, antibiotic production is assayed using culture-dependent techniques coupled with analytical methods such as thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) (Tewelde, 2004; Tendulkar *et al*., 2007). Recent advances in molecular cloning techniques have facilitated the characterization of genes associated with antibiotic biosynthesis and regulation in *Bacillus* spp. This has allowed for the development of specific primers and probes for PCR based detection of specific antibiotic-producing bacteria (Ramarathnam *et al*., 2007). This technique is less laborious and time consuming than the culture-dependent techniques.

The following study was undertaken to ascertain whether the local isolates aligned with *B. cereus* produce zwittermicin A. PCR detection of the zwittermicin A gene was based on the use of specific primers that amplify the zwittermicin A biosynthetic and resistance gene fragments. Antifungal metabolite production was also assayed using non-molecular techniques on different culture media such as half strength Tryptone Soy Broth ($^1/_2$ TSB) and Potato Dextrose Broth (PDB).

4.2 Materials and methods

4.2.1 Bacterial strains

Eleven aerobic endospore-forming bacterial isolates (Table 4.1), each randomly selected from the different RAPD-PCR groupings having used OPG-8, OPG-11 and OPG-16 as primers (Table 3.5), were used in a preliminary study to screen for isolates harboring zwittermicin A associated gene fragments. Subsequently, investigation into whether 17 bacteria isolates (Table 4.2) with RAPD-PCR banding profiles comparable to the positive isolate that was detected to house the zwittermicin A genes, were also screened for the targeted genes. Isolates were previously obtained from bulk and maize, wheat and kale rhizosphere soils, and were shown to exhibit antifungal properties. All of the isolates screened, with the exception of C76, were closely affiliated with *B. cereus* group. Isolate C76 was closely related to *B. subtilis* (Figure 3.12) and used as a negative control as zwittermicin A production has only been associated with members of the *B. cereus* group (Stohl *et al*., 1999a; Emmert *et al*., 2004). Pure cultures of each isolate were maintained on 10% Tryptone Soya Agar (TSA) incubated at 30°C. For long term storage, overnight cultures grown in Tryptone Soya Broth (TSB) were supplemented with 20% glycerol and kept at -80°C.

Table 4.1: Bacterial isolates used in the initial screening for zwittermicin A gene markers

Isolates	Origin
C15	Bulk soil
C54	Bulk soil
C76	Bulk soil
C91	Bulk soil
W41	Wheat rhizosphere soil
W90	Wheat rhizosphere soil
W96	Wheat rhizosphere soil
M15	Maize rhizosphere soil
M60	Maize rhizosphere soil
K51	Kale rhizosphere soil
K53	Kale rhizosphere soil

Table 4.2: Bacterial isolates with a similar RAPD-PCR profile having used either: OPG-8, OPG-11 or OPG-16 as primer

Isolates	Source
C16	Bulk soil
C17	Bulk soil
C27	Bulk soil
C28	Bulk soil
C37	Bulk soil
C74	Bulk soil
C78	Bulk soil
C92	Bulk soil
W33	Wheat rhizosphere soil
W50	Wheat rhizosphere soil
W92	Wheat rhizosphere soil
W95	Wheat rhizosphere soil
W96	Wheat rhizosphere soil
M27	Maize rhizosphere soil
M60	Maize rhizosphere soil
K39	Kale rhizosphere soil
K89	Kale rhizosphere soil

4.2.2 DNA extraction and detection of zwittermicin A genes using PCR

Crude DNA extracts were obtained for each isolate as previously described in section 3.2.2.2.

Initially, the 11 isolates that were randomly selected from the different RAPD-PCR groupings (Table 3.5) were examined for the presence of zwittermicin A biosynthetic and resistance genes using PCR amplification. The zwittermicin A biosynthetic gene was amplified using reverse primer, ZWITR1 and forward primer, ZWITF2 (Table 4.3), while the zwittermicin A resistance

gene was amplified using reverse primer, ZSRG-R and forward primer, ZSRG-F (Table 4.3). These primers were synthesized and supplied by Inqaba Biotec™, Hatfield, Pretoria, South Africa.

Amplifications were performed in sterile 200 µl thin walled PCR tubes. Each reaction (25 µl) consisted of 1.5 µl of 25 mM $MgCl_2$ (Promega), 5 µl of 5× GoTaq® Flexi buffer (Promega), 0.5 µl PCR nucleotide mix (10 mM dNTP's each) (Promega), 1 µl of 10 mM reverse primer, 1 µl of 10 mM forward primer, 0.25 µl of GoTaq® DNA polymerase (1U) (Promega), 1 µl of crude template DNA, made up to 25 µl with sterile nuclease-free water. PCR reactions lacking DNA template were used as negative controls, whereas crude DNA obtained from Isolate W96 was used as positive controls.

PCR amplifications were carried out in a Bioer thermal cycler with the following temperature profile: initial denaturation at 94°C for 4 min, followed by 38 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 57°C for 30 sec and an elongation step at 72°C for 1 min. Followed by the final extension step at 72°C for 10 min, after which samples were cooled to 4°C until electrophoretic analysis. Amplified DNA fragments were separated based on size using electrophoresis as described in section 3.2.5.

Subsequently, 16 isolates (Table 4.2) which were considered similar to the positive isolate based on their comparable RAPD-PCR banding patterns obtained using OPG-8, OPG-11 and OPG-16 as primer, were also analyzed for the presence of the zwittermicin A genes fragments. The positive isolate referred to, was the only isolate from the initial 11 screened that tested positive for the zwittermicin A gene fragments, and was thus subsequently used as the positive control (Figure 4.1). From the amplified PCR products, the positive control and randomly chosen isolates (C16 and W96 for the biosynthetic gene; C92 and W96 for the resistant gene) were then sequenced by Inqaba Biotec™, Hatfield, Pretoria, South Africa. Thereafter, specific sequence homologies were searched for on the NCBI database, n-BLAST search.

Table 4.3: Specific primer sequences used for the amplification of zwittermicin A biosynthetic and resistance genes

Zwittermicin A region	Primer	Sequence (5´- 3´)	Nucleotide	References
Biosynthetic gene	ZWIT-R1	GACCTTTTGAAATGGGCGTA	20	Athukorala *et al*. (2009)
	ZWIT-F2	TTGGGAGAATATACAGCTCT	20	Athukorala *et al*. (2009)
Resistance gene	ZSRG-R	TAAAGCTCGTCCCTCTTCAG	20	Athukorala *et al*. (2009)
	ZSRG-F	ATGTGCACTTGTATGGGCAG	20	Athukorala *et al*. (2009)

4.2.3 Extraction of antifungal compounds

4.2.3.1 Test Organism

Isolate W96 which originated from the wheat rhizosphere soil was selected for further antifungal metabolite extraction, on the basis that zwittermicin A biosynthetic and resistance genes were detected using PCR specific amplification.

4.2.3.2 Extraction from antibiotic production medium

Defined antibiotic production broth medium (Sadfi *et al*., 2002) was prepared by dissolving the following compounds in distilled water and bringing the final volume to 1 liter: 15 g, D-glucose; 5 g, DL-glutamic acid; 1.02 g, $MgSO_4.7H_2O$; 1.0 g, K_2HPO_4; 0.5 g, KCl and 1 ml of trace element solution. The trace element solution was prepared by dissolving 0.5 g, $MnSO_4.H_2O$; 0.16 g, $CuSO_4.5H_2O$; and 0.015 g, $FeSO_4.7H_2O$; in 100 ml of distilled water. The pH of the medium was adjusted to 6.0 with 5 N NaOH. Twenty ml aliquots were dispensed into 100 ml Erlenmeyer flasks and 200 ml aliquots into 500 ml flasks and autoclaved at 121°C (15psi) for 15 min.

A single colony of W96 was inoculated into 20 ml antibiotic production medium to establish a starter culture, which was incubated overnight at 30°C in a shaker incubator at 150 rpm. Two milliliter of the starter culture was inoculated into 200 ml of medium and incubated at 30°C, with shaking at 150 rpm for 72 h. Thereafter, the culture was centrifuged at 8, 000 ×g for 45 min. The resultant supernatant was divided into six 30 ml aliquots and then frozen overnight at -20°C before being lyophilized for 72 h. Duplicate freeze dried residues were then re-suspended in 1.5

ml of methanol, acetyl-acetate or water respectively, to achieve a 20-fold concentration for each solvent. Each extract was filter-sterilized (0.22 µm cellulose acetate filter) and then tested for antifungal activity against *R. solani* as previously described in section 2.2.2, using 50 µl of filter-sterilized supernatant per assay disc.

4.2.3.3 Extraction from liquid culture media grown in the presence of *R. solani* metabolites

Antifungal compound production was also assessed in Potato Dextrose Broth (PDB) and half strength TSB. Potato Dextrose Broth was prepared according to the manufactures instructions and the TSB by weighing out half of the recommended weight of the dehydrated powder. Both media were dispensed separately in 50 ml aliquots into 250 ml Erlenmeyer flasks and autoclaved for 15 min at 121°C (15 psi).

Rhizoctonia solani metabolites were obtained by cutting out agar plugs (10 x 10 mm) covered with *R. solani* mycelium on Potato Dextrose Agar (PDA) and aseptically transferred to 50 ml PDB and incubated at 30°C for 72 h with shaking at 150 rpm. One ml aliquots were withdrawn and centrifuged in sterile minifuge tubes at 8, 000 ×g for 10 min. The resultant supernatant was filter-sterilized using 0.22 µm cellulose acetate membrane filters.

To investigate whether *R. solani* metabolites stimulate aerobic endospore-forming bacterial isolates to produce antifungal compounds, Isolate W96 was grown in the presence of filter sterilized *R. solani* liquid culture. Single colonies of Isolate W96 were inoculated separately into 50 ml PDB and $^{1}/_{2}$ strength TSB, in duplicate. Thereafter, 2 milliters of filter sterilized *R. solani* metabolites were added to each of these media, which were then incubated at 30°C with shaking at 150 rpm. These cultures were incubated for 120 h until widespread sporulation of Isolate W96 was evident (as determined by peridiodic Gram staining). Thereafter, each culture was centrifuged at 8, 000 ×g for 15 min and filter-sterilized (0.22 µm cellulose acetate filter). Cell-free supernatants were tested for antifungal activity against *R. solani* as previously described in section 2.2.2. This was performed using 50 µl and 1 ml of filter-sterilized culture supernatants per assay disc. The controls were Isolate W96 cultures grown in the 2 different media as described above, except no *R. solani* metabolites were added. Antifungal controls were the same cultures as above, except they were not centrifuged and filter sterilized.

Larger amounts of Isolate W96 cultures (50 ml) were prepared using the same media, viz. PDB and $^{1}/_{2}$ strength TSB, in order to concentrate Isolate W96 metabolites. The cultures were incubated for the same period of time under identical conditions as above, except no *R. solani* metabolites were added. Bacterial cells were removed by centrifugation at 8,000 × g for 20 min. The resulting supernatants were each decanted into 30 ml bottles and frozen overnight at -20°C. Frozen samples were lyophilized for 72 h and the resulting dried residues were re-suspended in a total of 2.5 ml of distilled water for each media used, to achieve 20-fold concentration each. The re-suspended residues were filter-sterilized and tested for antifungal activity against *R. solani* as described previously in section 2.2.2, using 50 µl per assay disc.

4.3 Results

4.3.1 Screening for zwittermicin A genes and BLAST search

Of the eleven isolates screened, only one isolate, W96 obtained from wheat rhizosphere soil yielded PCR amplicons for both the zwittermicin A biosynthetic and resistance gene (Figure 4.1 A and B). A band size of ~779 bp was observed for the biosynthetic gene fragment, and one of ~1 kbp was observed for the resistance gene.

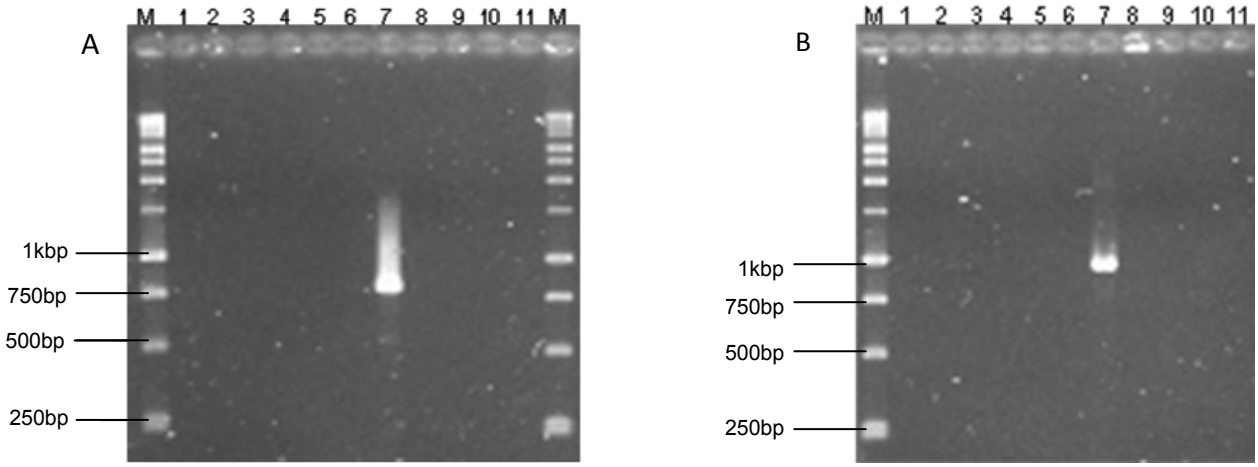


Figure 4.1: PCR amplification of zwittermicin A biosynthetic (A) and resistance (B) genes in local isolates from different soil types. Lanes: M, 1 kbp ladder; 1-4, bulk soil isolates C15, C54, C76, C91; 5-7, wheat rhizobacteria W41, W90, W96; 8-9, maize rhizobacteria M15, M60; 10-11, kale rhizobacteria K51, K53.

Isolates that were similar to positive strain W96 based on RAPD-PCR banding profiles were also tested for zwittermicin A biosynthetic and resistance gene markers. Of the 16 isolates tested, only 6 (C16, C27, C28, C37, C74 and C92) showed amplifications with similar band sizes to Isolate W96 for both genomic regions (Figure 4.2 A and B). However, additional band patterns were observed, approximately 500 bp for the biosynthetic gene and 250 bp for the resistance gene. The 1 kbp band obtained from the amplification of the resistance gene in the 6 isolates was fainter in comparison to that of Isolate W96.

Sequenced products of the Isolates C16 and W96 zwittermicin A biosynthetic region showed a high similarity (99%) to GenBank accession No. FJ430564.1 (zwittermicin A biosynthetic gene cluster), whereas sequenced PCR products of Isolates C92 and W96 zwittermicin A resistance region showed high correspondences (99%) to GenBank accession No. AY083683.1 (zwittermicin A resistance gene). This confirms the presence of zwittermicin A biosynthetic and resistance gene in other isolates (C27, C28, C37, and C74) where corresponding amplification bands were observed (Figure 4.2 A and B).

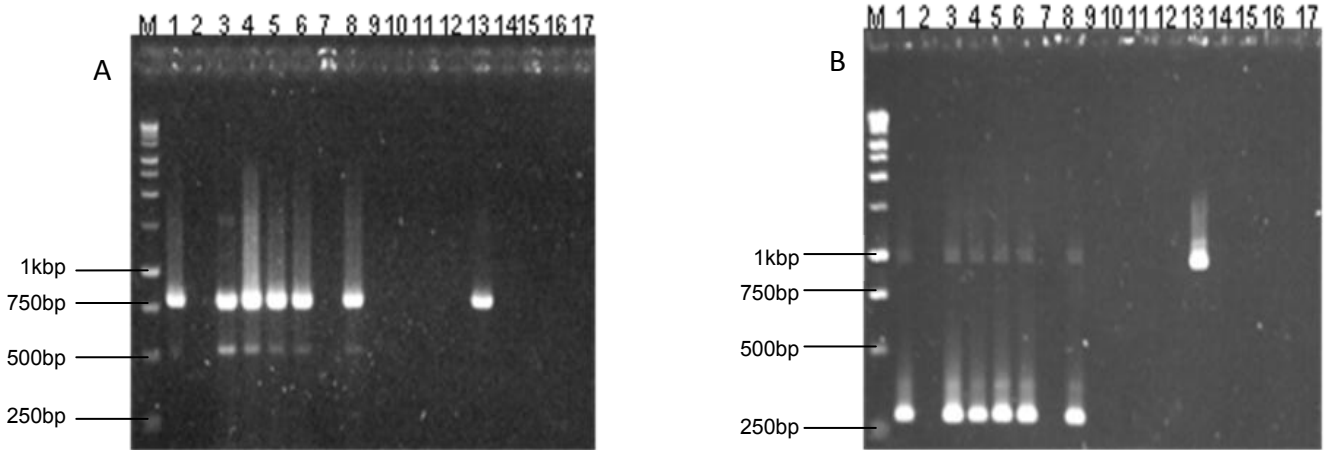


Figure 4.2: PCR amplification of zwittermicin A biosynthetic (A) and resistance (B) gene fragments in similar isolates according to their RAPD-PCR profiles. Lanes: M, 1 kbp ladder; 1-8, bulk soil isolates C16, C17, C27, C28, C37, C74, C78, C92; 9-13, wheat rhizobacteria W33, W50, W92, W95,W96; 14-15, maize rhizobacteria M27, M60; 16-17, kale rhizobacteria K39, K89.

4.3.2 Isolation of antifungal compounds

Antifungal activity tests on filter-sterilized supernatants from W96 cultures grown in antibiotic production media and concentrated supernatant (20-fold) showed no evidence of *R. solani* inhibition. Using a range of different solvents to extract active compound(s) likewise failed to produce an antifungal inhibitory response. Water proved more effective than acetyl- acetate and methanol in dissolving the lyophilized culture filtrates, suggesting the hydrophilic, polar nature of the residues.

Antifungal activity tests using filter-sterilized supernatants of W96 cultures grown in the presence of *R. solani* metabolites showed no signs of activity regardless of the culture media used, i.e. PDB and $^{1}/_{2}$ strength TSB. Similar findings were observed for filter-sterilized culture controls grown in the absence of *R. solani* metabolites. Antifungal activity was only evident in culture samples where the bacterial cells were not removed prior to testing.

4.4 Discussion

Members of the *B. cereus* group are known to produce a number of different types of antibiotic compounds that can inhibit fungi; examples include zwittermicin A, kanosamine and fengycin (Milner *et al*., 1999b; Emmert *et al*., 2004; Ongena and Jacques, 2007). The aminopolyol antibiotic, zwittermicin A is considered important since it had a broad range of activity against a variety of prokaryotes and eukaryotes. The latter include the fungal phytopathogens *R. solani*, *Fusarium graminearum* and *Sclerotinia sclerotiorum*, as well as oomycetes such as *Aphanomyces euteiches* and *Pythium toralosum* (Silo-Suh *et al*., 1998; Athukorala *et al*., 2009). It is therefore important to establish effective screening procedures for zwittermicin A production among members of the *B. cereus* group when investigating their potential as biocontrol agents. PCR based detection of specific antibiotic-producing bacteria is increasingly being used instead of the laborious and time-consuming culture-dependent isolation and screening route (Athukorala *et al*., 2009).

In this study, 11 different aerobic endospore-forming bacterial isolates that were shown to possess antifungal activity against *R. solani* were investigated for zwittermicin A production through PCR based detection of the biosynthetic gene. Each of these isolates was randomly selected from different RAPD-PCR groupings (Table 3.5) based on differences in banding patterns.

Of the 11 isolates tested, only Isolate W96 was detected positive for the zwittermicin A biosynthetic gene and resistance gene markers (Figure 4.1 A and B). Bands sizes of ~779 bp and ~ 1 kbp were obtained for respective gene markers. These band sizes were in agreement with the findings of Athukorala *et al*. (2009) who successfully detected zwittermicin A gene markers through specific PCR amplification in *B. cereus* L-07-01, *B. thuringiensis* BS8 and *Bacillus mycoides* S-07-01. Subsequently, Athukorala *et al*. (2009) was able to confirm zwittermicin A production using MALDI-TOF mass spectrometry. Other methods that have been used for the preliminary detection of zwittermicin A include testing the sensitivity of strains towards phage 7 and determining strains ability to inhibit *Erwinia herbicola* and *Phytophthora medicaginis* (Stabb *et al*., 1994).

From the *in vitro* dual plate bioassay tests, Isolate W96 exhibited relatively low levels of antagonism towards *R. solani* (Table 2.2) compared to findings reported in the literature. For example, Silo-Suh *et al*. (1998) reported that a zwittermicin A producing strain of *B. cereus* inhibited *R. solani* mycelial growth by up to 50% compared to a water control.

Interestingly, isolates harboring the zwittermicin A biosynthetic gene were the only isolates that also reflected the presence of the zwittermicin A resistance gene marker. This finding supports the assertion that it is important for antibiotic-producing organisms to have self-resistance mechanisms to protect themselves from the action of their own toxic metabolites (Stohl *et al*., 1999a). Zwittermicin A self-resistance gene in *B. cereus* UW85 is designated *zmaR* and has been shown to inactivate zwittermicin A by catalyzing its acetylation, hence zwittermicin A is rendered inactive (Milner *et al*., 1996b; Stohl *et al*., 1999b). It would be of interest to investigate whether *Bacillus* strains that do not produce zwittermicin A are sensitive to the antibiotic. This would suggest that zwittermicin A production in certain strains offers a competitive advantage within the aerobic endospore-forming bacterial community.

Isolates that showed antifungal activity and were similar to W96 based on their RAPD-PCR banding profile, were also tested for zwittermicin A biosynthetic and resistance gene markers. Of the 16 isolates screened, only 6 yielded the targeted amplicon fragments (Figure 4.2 A and B). These results indicate that closely affiliated strains grouped according to RAPD-PCR do not necessarily produce the same type of antibiotics. Athukorala *et al*. (2009) reported similar findings, where related *Bacillus* strains were found to produce different types of antibiotics. Thus, PCR based detection of specific antibiotic encoding sequences could be used to further distinguish between closely related PGPR strains. Conversely, this technique could be used to group isolates from different origins on the basis of the antimicrobial metabolites they potentially could produce.

As seen in Figures 4.2 A and B, additional bands were evident for each of the six isolates compared to W96. Sequence analysis of amplified PCR products from representatives of these Isolates (C16 for biosynthetic and C92 for resistance genes) and from W96, confirmed their similarity to zwittermicin A gene sequences lodged in the GenBank database. A possible explanation for the additional bands is that the PCR conditions used for these 6 strains were not optimal and requires further optimization. Alternatively, the additional bands could be attributed

to the presence of zwittermicin A isoforms or other closely related compounds, which could result in gene sequences with regions of homology to the zwittermicin A gene (Silo-Suh *et al*., 1994). Further investigation into possible reasons for the additional bands is warranted; for example, excising these bands from electrophoresis gels and cloning or sequencing them directly to identify and characterize them.

BLAST findings confirmed the presence of the zwittermicin A biosynthetic gene in Isolate W96. Thus, it was speculated that the antifungal activity shown by W96 could be attributed to this antibiotic compound. Zwittermicin A has previously been extracted from cultures of *B. cereus* strains grown in half strength TSB for 3-7 days (Milner *et al*., 1995; Stohl *et al*., 1999a; Kevany *et al*., 2009). Antibiotics such as zwittermicin A are produced as secondary metabolites and their production has been positively correlated with sporulation (Yu *et al*., 2002). Thus, in this study antibiotic extraction was only attempted once evidence of widespread sporulation in the culture was apparent.

In the present study, antifungal metabolites, particularly zwittermicin A were first assayed for in defined antibiotic production media. No activity was detected in cell-free extracts, even after 20-fold concentration. These findings were consistent with those of Sadfi *et al*. (2002) who suggested that antifungal metabolite(s) production in *B. cereus* X16 was induced in part by the presence of a fungal host during strain growth, as well as being influenced by the characteristics of the medium. Milner *et al*. (1995) also reported on the "medium effect" and found that zwittermicin A accumulation was highest in $^1/_2$ strength TSB supernatant, whereas the lowest antibiotic concentration was obtained in TSB supplemented with 50 mM phosphate buffer. With these recommendations in mind, W96 was grown in the presence of filter-sterilized *R. solani* metabolites in PDB and $^1/_2$ strength TSB. Unfortunately no antifungal activity was evidenced from cell-free supernatants of W96 culture grown in the presence of *R. solani* metabolites, regardless of media used. Antifungal activity was only observed when the culture media tested contained culture's bacterial cells. This suggests that in the present study, factors other than medium composition and presence/absence of *R. solani* metabolites (at low concentrations) might be the determining factors in antibiotic production. The failure to detect antifungal activity was possibly due to low concentrations of antifungal metabolite(s) being produced, as reflected in the small size inhibition zones recorded in the dual culture bioassays (Chapter 2, Table 2.2).

Alternatively, antifungal compound(s) produced could possibly bind to membrane surfaces during filter sterilization thereby being removed from the supernatant. Further research into effectively extracting antifungal metabolites, particularly from *B. cereus* is warranted. In addition, factors influencing zwittermicin A production, activity and efficient extraction are needed as the compound has great potential as a novel biocontrol agent.

Zwittermicin A is commonly characterized using paper electrophoresis, HPLC and matrix-assisted laser desorption ionization-time of flight-mass spectroscopy (MALDI-TOF MS) (Silo-Suh *et al*., 1998; Athukorala *et al*., 2009; Kevany *et al*., 2009). This compound is a linear aminopolyol formed from ethanolamine and glycol moieties which are rarely seen in natural products (Kevany *et al*., 2009). However, as no antifungal activity against *R. solani* was detected in any of the filter-sterilized extracts obtained, even the polar water extracts, characterization was not possible.

Chapter 5: General overview

Concerns regarding the detrimental impact chemical fungicides have on the environment has prompted a drive to find environmentally friendly alternatives for controlling plant diseases and plant growth promotion. Biological control agents and plant growth promoting rhizobacteria have shown potential in this regard (Siddiqui, 2006; Ongena and Jacques, 2007). The search for these bacterial agents has mainly focused on agricultural soils; however, there is a prospective untapped source in native soil environments, including undisturbed indigenous grassland soils (Berg and Smalla, 2009). These environments are thought to support a great diversity of microbial populations, attributed to the wide variety of complex organic matter found therein (Alexander, 1977). Additionally, undisturbed soil environments are fairly stable and rarely encounter soil borne plant diseases, possibly due to the resident microbial populations out-competing or suppressing phytopathogens (Gibson, 2009). Thus, research into biological control and/or plant growth promotion potential of organisms found in such environments is warranted.

The research presented in this thesis was undertaken to screen for biological control and/or plant growth promotion traits amongst aerobic endospore-forming bacteria isolated from a mispah clay-loam soil sourced from an undisturbed indigenous grassland soil environment. In addition, to select for rhizosphere competent bacteria, aerobic endospore-formers were also isolated from rhizosphere soil associated with three crop types (maize, wheat and kale) grown in the same soil. A major aim of this study was to determine the levels of genotypic diversity between isolates using ITS-PCR, 16S rRNA sequencing and RAPD-PCR. Additionally, the feasibility of using PCR methods to screen for strains that produce specific antibiotics (viz., zwittermicin A) was also assessed. From the main findings of this study it was established that:

- Approximately 9% of the aerobic endospore-formers isolated exhibited some level of antifungal activity towards *R. solani*. The majority were obtained from bulk and wheat rhizosphere soil, whereas the least antagonistic isolates were obtained from maize and kale rhizosphere soils. The levels of antifungal activity apparent in the *in vitro* assays did not appear to be very high which may count against their efficacy in future plant trials. Several types of antifungal interactions were discerned which suggested that different antibiotic compounds were possibly produced by certain strains. It would be of interest to

characterise these compounds to see if any new or novel compounds were present. An investigation into factors influencing antibiotic production and concentration may also assist in understanding the varied antimicrobial interactions observed.

- It became evident that majority of the isolates showing antifungal activity were morphologically indistinct and that the dual culture bioassays, when viewed in isolation, were of limited value for rationalizing isolates into smaller groups for further screening. The enzyme and biochemical assays performed gave some indication of the functional diversity between isolates and some insight into plant growth promoting traits (e.g., siderophore production, phosphate solubilisation, chitinase production) associated with certain strains. Very few of the isolates showed a broad spectrum of activity in this regard. It would be interesting to ascertain whether those strains exhibiting multiple PGPR traits out perform other isolates in plant growth promotion trials, particularly in the field.

- Genotypic characterization/fingerprinting of isolates were deemed more useful for distinguishing between isolates and determining the levels of genotypic diversity within and between the sample sets. ITS-PCR and 16S rRNA sequencing was found to be useful for distinguishing isolates at the species level. It was established that majority of the isolates were closely associated with the *B. cereus* group of bacteria. This finding suggests that this group of organisms were the predominant endospore-formers producing antifungal compound in the soil environment investigationed.

- RAPD-PCR was found to be suitable for further distinguishing between closely associated isolates. Several distinct clusters of strains were distinguished based on RAPD fingerprinting profiles. This technique showed its potential for rationalizing isolates into more manageable numbers for further analysis which is of importance when screening large numbers of isolates for biological control potential. Based on RAPD-PCR analysis, the majority of isolates did not appear to be specific to any of the rhizosphere soil environments screened. Rather, strains with some level of homology were found in both the bulk and rhizosphere soils. However, a few strains were found to be unique to either the bulk or rhizosphere soils. Overall, these findings suggest that certain strains resident in the bulk soil exhibited a level of rhizosphere competence. Greenhouse and field trials

would need to be performed to evaluate the environmental stability and PGPR efficacy of these isolates.

- Template DNA extraction from colony pick-offs proved to be a fast and relatively straight forward method for the preliminary fingerprinting of unknown isolates and grouping closely related strains. However, for more precise fingerprinting, kit extracted template DNA is recommended as it is difficult to standardize template DNA from colony pick-offs.

- PCR screening for zwittermicin A indicated that seven isolates harboured gene markers for this compound. Subsequent attempts to extract and confirm the presence of zwittermicin A compounds were unsuccessful. This was attributed to low concentrations of active compound being produced; or due to antifungal compound(s) binding to the filter membrane used in sterilizing the supernatant. Nonetheless, PCR based detection of antibiotic markers shows potential as a useful method for screening large numbers of isolates for the ability to produce targeted antibiotic compounds.

Large scale screening of microorganisms with PGPR potential is considered to be laborious and resource intensive. A major issue is the management and handling of large numbers of test organisms. Genomic fingerprinting of strains offers a way of determining the levels of diversity within a sample set and can assist in rationalizing and selecting candidate organisms for further evaluation. Used in conjunction, ITS-PCR and RAPD-PCR were found to be useful, rapid and cost-effective methods for establishing the levels of diversity between aerobic endospore-formers exhibiting antifungal activity. Furthermore, fingerprinting of strains can also provide useful information regarding the ecological fitness, rhizosphere competence and distribution of organisms selected for future studies.

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