SCREENING OF AEROBIC ENDOSPORE-FORMING BACTERIAL ISOLATES AS CANDIDATE BIOCONTROL AGENTS AGAINST *RHIZOCTONIA SOLANI*

by

CHARLES H. HUNTER

Submitted in fulfilment of the academic requirements for the degree of

Doctor of Philosophy

in Microbiology

School of Life Sciences
College of Agriculture, Engineering and Science
University of KwaZulu-Natal
Pietermaritzburg
South Africa

January 2016

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The research was financially supported by the National Research Foundation (NRF).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

_____

Signed: Prof. S. Schmidt (Supervisor)

Date: January 2016

_____

Signed: Prof. M.D. Laing (Co-supervisor)

Date: January 2016

_____

Signed: Prof. F.M. Wallis (Co-supervisor)

Date: January 2016

DECLARATION

I, Charles Haig Hunter, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv) this dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

b) where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

_____

Signed: C.H.Hunter (Candidate)

Date: January 2016

ABSTRACT

Bacterial-based biocontrol of soil-borne phytopathogens has gained prominence as a promising technology for developing sustainable agricultural pest control practices. Aerobic endospore-forming bacteria are seen as potential candidates for biocontrol applications due to various ecological and physiological traits which have been shown to influence plant health and disease control. Their ability to produce endospores also provides a major commercial advantage over non spore-forming bacteria. Appropriate screening methods are central to the discovery of successful biocontrol agents and should ideally be both ecologically relevant and able to evaluate a large number of isolates. A study was therefore undertaken with the aim of establishing screening methods that facilitate the selection of aerobic endospore-forming bacteria as candidate biocontrol agents against *Rhizoctonia solani*, an economically important fungal pathogen exhibiting a wide host range.

Aerobic endospore-forming bacteria were isolated from rhizosphere material of five crop types grown in composted pine bark medium and screened for *R. solani* antagonism using traditional *in vitro* dual-culture bioassays. Isolates exhibiting antifungal activity were then evaluated *in vivo* for biocontrol activity against *R. solani* in cucumber seedling trials. Selected isolates were evaluated further using several screening approaches including: genomic fingerprinting; characterization of, and PCR-based screening for genes involved in the biosynthesis of bioactive lipopeptide compounds; and, the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a means of rapidly screening bacterial isolates.

Approximately 6% of the bacterial isolates (n=400) showed antagonism towards *R. solani in vitro*. Dual-culture bioassays against *R. solani*, *Fusarium oxysporum*, *Botrytis cinerea* and *Pythium arrhenomanes* revealed that the antagonistic activity amongst isolates varied considerably and was influenced by the duration of the assay. From these assays it was possible to rank isolates based on the extent and stability of the inhibitory response *in vitro* as well as by the spectrum of antifungal activity observed. Twenty-four isolates were selected for *in vivo* screening for biocontrol activity against *R. solani,* using susceptible cucumber plants grown under greenhouse conditions. In preliminary experiments the pathogen loading rates were shown to have a marked influence on disease severity. In experiments where *R. solani* was seeded in the form of colonized agar plugs, significant differences between

treatments and controls were recorded and several potential biocontrol candidates were identified. A general observation was that isolates that achieved high rankings *in vitro* performed better in the *in vivo* trial than those with lesser rankings; although some exceptions were noted. These findings support the notion that fungal antagonism is an important determinant of biocontrol potential that can be used in preliminary biocontrol screening programmes.

Internal-transcribed spacer region (ITS) PCR and randomly amplified polymorphic DNA (RAPD) PCR were evaluated as methods to differentiate isolates exhibiting antifungal activity *in vitro*. ITS-PCR distinguished three major groupings, but proved to be limited in its ability to detect inter- and intra-specific variation amongst closely related organisms. Based on 16S rRNA gene sequence analysis, two of the groups were identified as members of the "*Bacillus subtilis*" and "*Bacillus cereus*" clusters; while, the third group consisted of a single isolate identified as a strain of *Brevibacillus laterosporus*. RAPD-PCR revealed further levels of genetic diversity within each ITS grouping. The "*Bacillus subtilis*" cluster was distinguished further into four distinct groups, which based on *gyrA* gene fragment sequence analysis, were identified as strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* respectively. Sequence matches were consistent with the RAPD-PCR groupings, indicating that this method was appropriate for differentiating related isolates at the strain and possibly the sub-species level. Clonal similarities were evident for a number of strains isolated from different plant species suggesting that these may reflect populations of rhizosphere competent strains and/or plant adapted ecotypes. Strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* were amongst the best performers in the *in vivo* biocontrol seedling trial and generally performed better than the "*Bacillus cereus*" group of isolates. RAPD-PCR of the "*Bacillus cereus*" isolates showed that they exhibited greater levels of genetic heterogeneity and that the groupings detected were not consistent when different primer sets were evaluated. Genomic fingerprinting was found to provide an insight into the prevalence, distribution and possible rhizosphere competency of related strains.

Liquid chromatography was used in conjunction with electrospray-ionization time-of-flight (ESI-TOF) mass spectrometry (MS) to characterize bioactive lipopeptides purified from culture supernatants of selected strains that ranked highly in the *in vitro/in vivo* assays. Phylogenetically related strains produced very similar lipopeptide profiles. *Bacillus subtilis*

strains were found to produce isoforms of surfactin and fengycin. In addition to these lipopeptides, *B. amyloliquefaciens* subsp. *plantarum* strains were also found to produce isoforms of bacillomycin D or iturin A. Bacillomycin/iturin and fengycin fractions exhibited antifungal activity *in vitro*, whereas surfactin fractions did not. Isolates that ranked the highest in the *R. solani* dual-culture bioassays all produced either isoforms of bacillomycin D or iturin A. Bacillomycin D producing isolates were amongst the best performers in the *in vivo* biocontrol trials.

Gene markers targeting the biosynthetic apparatus of the detected lipopeptide classes were then assessed for screening purposes using PCR. BACC1F/1R primers targeting the bacillomycin D synthetase C (*bmyC*) gene correlated well with the ESI-TOF MS findings, whereas ITUD1F/1R primers targeting the malonyl-CoA-transacylase (*ituD*) gene linked to iturin A biosynthesis were unable to distinguish between isolates that produced iturin or bacillomycin in culture. Disparities between some of the PCR and ESI-TOF MS results suggested that primers targeting *srfA* (surfactin) and *fenD* (fengycin) biosynthetic genes showed limited specificity amongst the strains screened. Phylogenetic comparisons of *srfD* and *fenD* gene sequences from selected strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* revealed that these genes clustered according to species with marked heterogeneity between clusters being evident. Using *fenD* gene sequence data from *B. amyloliquefaciens* subsp. *plantarum* FZB42, primers (FENG1F/1R) targeting fengycin synthetase genes of strains of *B. amyloliquefaciens* subsp. *plantarum* isolated in this study were successfully established.

MALDI-TOF MS was assessed as a means of identifying isolates antagonistic to *R. solani in vitro* and determining their associated lipopeptide profiles. Mass spectra were obtained in the *m/z* range 2000 to 20000 for identification and grouping purposes and in the *m/z* 750 to 2500 range in order to profile lipopeptide production. The available Bruker BDal spectral library allowed for the identification of isolates to the genus level but proved to be limited for identifying environmental isolates to the species level. Extension of the library using "in-house" mass spectra generated from isolates identified in this study significantly improved the level of isolate identification in subsequent identification runs. Cluster analysis of mass spectra allowed for the relationships between isolates to be established and provided a means of grouping closely related isolates. Strains of *B. subtilis* and *B. amyloliquefaciens* were

clearly distinguished from one another and the potential for differentiating strains at the sub-species level was also shown. MALDI-TOF MS also provided a convenient means of detecting bioactive lipopeptides directly from whole cell preparations, cell extracts and crude culture filtrates. Lipopeptide profiles varied depending on taxonomic groupings. Results for isolates within the "*Bacillus subtilis*" group supported the earlier ESI-TOF MS findings and were found to be more reliable than PCR screening for lipopeptide synthesis genes. "*Bacillus cereus*" group isolates produced distinct spectral profiles with peaks that were consistent with biomarkers previously described in the literature as isoforms of the kurstakin class of lipoheptapeptides. *Brevibacillus laterosporus* CC-R4 yielded a unique spectral profile in the *m/z* 750-2000 range with mass fragments which were similar to antimicrobial compounds recently reported in the literature. Overall, MALDI-TOF MS was found to fulfil the requirement for a practical yet robust technique suitable for processing large numbers of aerobic endospore-forming bacteria for biocontrol screening.

This study has shown that genomic fingerprinting and MALDI-TOF MS characterization of bacterial isolates are worthwhile additions to preliminary *in vitro* screening practices. They provide a level of isolate differentiation and characterization that is beneficial for selecting candidate biocontrol agents, which is not possible with traditional screening practices. Effectively, they allow traditional biocontrol screening to move away from empirically based approaches to ones which are "knowledge" based, allowing for representative groups of bacteria with specific traits to be selected for further evaluation.

ACKNOWLEDGMENTS

The work presented in this thesis has emanated from research funded by the National Research Foundation whose financial support is gratefully acknowledged. Without the help and support of many people this thesis would not have seen the light of day.

I would especially like to thank my supervisors Prof. Stefan Schmidt, Prof. Mark Laing and Prof. Mike Wallis for their encouragement, constructive suggestions and assistance throughout the course of this study.

My thanks also go to Dr Sackey Yobo for his advice and assistance with regards data analysis and to Ms Caryl Janse Van Rensburg for her assistance with the LC/ESI-MS analysis of lipopeptide fractions.

I would like to acknowledge the technical and administrative support freely given by members of the School of Life Sciences, past and present. Special thanks go to Celeste Clark, Di Fowlds, Pat Joubert and Natalie Jones.

I am also appreciative of my colleagues within the School of Life Sciences and my fellow postgraduates for their moral support and encouragement over the course of this journey.

Very special thanks go to the Hunter family for their unfailing support, their patience and all the sacrifices they have made along the way. Thank you for being a continuous reminder of what is important in life and helping me maintain my perspective.

Last, but by no means least, I would like to thank my wife Celeste without whose love, encouragement and endless supply of coffee I would probably still be trying to finish this thesis. My love and thanks to you always.

TABLE OF CONTENTS

Page

LIST OF PLATES

Page

LIST OF FIGURES

Page

LIST OF TABLES

INTRODUCTION

Developments within the field of microbial ecology over the last 50-60 years have highlighted the important role that microorganisms play within the plant-root-soil continuum. Whilst some microbes are detrimental, namely plant pathogens, the vast majority are innocuous or even beneficial; performing crucial roles in plant nutrition and health. Improved understanding of the various interactions and mechanisms involved has prompted extensive research in the fields of biological control and plant growth promotion. A major driving force has been the need to develop sustainable agronomic practices that are less reliant on synthetic pesticides that pose a potential threat to mankind and the environment in general (Nelson, 2004; Pérez-García *et al*., 2011; Deravel *et al.*, 2014).

With an estimated $39 billion spent annually (Grube *et al*., 2011) on plant protection products, the development of commercially viable and safe biological alternatives for crop protection and growth promotion has been given a strong incentive. Focus areas have revolved around the development and establishment of microbial biofertilizers, plant growth promoters and biopesticides (Lethbridge, 1989; Deravel *et al.*, 2014). To date, microbial products associated with nitrogen fixation (e.g. *Rhizobium, Azotobacter*) and insect biocontrol (e.g. *Bacillus thuringiensis*) have been the most successful in the commercial arena (Thakore, 2006).

Phytopathogens cause major economic losses in agriculture and the methods available to control many of these pathogens are often inadequate due to the inaccessibility of pathogens in soil and root environments (Khetan, 2001). There is a paucity of chemical agents suitable for the control of root diseases and added to this; the widespread application of fungicide drenches to soil is considered impractical and costly. The banning of soil fumigants such as methyl bromide has further compounded this problem. The management of soil-borne plant diseases has traditionally relied on age old agricultural practices such as crop rotation, soil tillage, water management and soil amendment (Schroth and Hancock, 1985; Cook, 1990; Messenger and Braun, 2000). Breeding for resistance in crops against soil-borne pathogens has largely been

unsuccessful. Microbial control, therefore, has been seen as a promising alternative to the use of synthetic fungicides and some notable commercial successes have already been achieved.

Aerobic endospore-forming bacteria grouped within the phylum *Firmicutes* are ubiquitous within soil ecosystems and have been routinely isolated from bulk- and rhizosphere soil (Garbeva *et al*., 2003). Members of the genus *Bacillus* are known to produce a diverse array of biologically active compounds and various strains have been identified which effect plant growth and control pathogens (Kloepper *et al*., 2004; Ongena and Jacques, 2008, Borriss, 2011; Pérez-García *et al*., 2011; Chowdhury *et al*., 2015). Their ability to produce endospores gives them a major technological advantage over non spore-forming bacterial counterparts. This property allows for a greater degree of flexibility regarding formulation, application and storage. Not surprisingly, *Bacillus*-based biocontrol products are very well represented in the bacterial biocontrol market sector (McSpadden-Gardener and Fravel, 2002; Fravel, 2005; Borriss, 2011). However, the number of strains that have been developed to a commercial level is disproportionately small compared to the numbers of organisms that have been studied over the years. This is attributable largely to the inconsistent performance of microbial inoculants when applied under field conditions. An over-reliance on inadequate screening procedures has been cited as a major reason for the poor performance of biocontrol candidates *in situ* (Pliego *et al*., 2011).

Given the complexity of plant-microbe-soil interactions it is unlikely that a single bacterial species would be able to fulfil a universal role as biopesticide/growth promoter to a range of plant species grown under varied environmental conditions. Hence, there is a continuing need to tap into underutilized sources of microbial diversity and search for new biopesticide-producing strains that are compatible with different crop types, adaptable to various growing conditions and are safe to use (Schisler and Slininger, 1997). The initial selection criteria for screening candidate organisms are crucial and will have a direct bearing on the success of subsequent stages of biopesticide development. Rather than using empirical approaches, screening strategies that are ecologically

relevant can greatly improve the chances of unearthing promising candidate organisms (Schisler and Slininger, 1997; Whipps, 1997; Pliego *et al*., 2011).

Convention dictates that biocontrol screening programmes should cast the net as widely as possible and that numerous organisms from varied sources and locations should be included (Campbell, 1986, Knudsen *et al*., 1997). Preliminary screening of isolates often involves traditional *in vitro* assays designed to select for a specific trait. These *in vitro* assays are generally simple to perform and allow for a rapid throughput of the isolates being evaluated. However, *in vitro* screening approaches are far removed from the field situation and do not take into account plant-pathogen-antagonist interactions. The inclusion of an *in vivo/in planta* screening phase is, therefore, considered an essential component of any biocontrol screening programme (Pliego *et al.*, 2011). However, traditional *in vivo* screening practices are generally laborious, resource intensive and time consuming to perform and therefore, not ideally suited to large scale screening (Marten *et al*., 2000; Pliego *et al*., 2011). Consequently, the numbers of isolates selected for *in vivo* evaluations is often restricted and invariably, strongly influenced by the outcomes of the *in vitro* bioassays. Ultimately, decisions to select certain isolates for further evaluation are often based on limited data and/or information that are not ecologically pertinent. The choice of *in vitro* assays and the criteria used to select isolates for further *in vivo* testing is therefore extremely important. Hence, it is imperative to establish *in vitro* screening methods that provide ecologically relevant information that can be used to select promising isolates for further evaluation.

With these considerations in mind a study was initiated with the aim of establishing screening methods that facilitate the selection of aerobic endospore-forming bacteria as candidate biocontrol agents. In addition to providing information that could assist with the decision making process required for selecting strains for further development, a major aim of the study was to establish screening methods that facilitate the rapid throughput of isolates. In addition to traditional *in vitro* and *in vivo* screening approaches this study sought to explore alternative screening methods which involved: the use of molecular typing as a means to compare and group isolates for dereplication purposes;

the characterization of, and screening for, active compounds involved in fungal antagonism; and, the use of matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) as a means of rapidly screening bacterial isolates.

Molecular typing is recommended as a convenient means of differentiating between closely related phenotypes; however, there is little evidence that this approach has been widely used in the preliminary phases of biological control screening programmes (Pliego *et al*., 2011; Lin *et al*., 2014). Genomic fingerprinting potentially offers a means of establishing the levels of diversity amongst a collection of environmental isolates, allowing similar strains to be grouped together. Such information would be valuable in rationalizing and selecting candidate organisms for further evaluation and characterization.

Characterization of the active compound(s) involved in fungal antagonism is important for understanding the underlying mechanisms involved in biocontrol (Ongena and Jacques, 2008; Cawoy *et al.*, 2015). Liquid chromatography (LC) used in conjunction with electrospray ionization mass spectrometry (ESI-MS) has been used successfully to distinguish and characterize antifungal lipopeptides produced by strains of *Bacillus* spp. (Li *et al.*, 2012). Molecular cloning has advanced progress in the characterization of genes associated with antibiotic biosynthesis in *Bacillus* spp. This has paved the way for specific primers and probes to be developed which can be used for PCR-based detection of specific antibiotic-producing bacteria (Ramarathnam *et al*., 2007). This approach offers a rapid means of selecting candidate organisms based on the presence of genes encoding biosynthesis of antibiotics.

MALDI-TOF MS is a well-established technique suitable for the analysis and characterization of biologically active lipopeptide compounds associated with aerobic endospore-formers (Vater *et al*., 2002; Price *et al*., 2007). It is recommended as a method requiring minimal sample preparation, which is suited to the rapid and high throughput screening of bacterial isolates (Stets *et al*., 2013). The application of MALDI-TOF MS to screen environmental isolates for specific biocontrol traits is therefore attractive. MALDI-TOF MS is also used as a means to identify bacteria based on protein

fingerprinting (Welker and Moore, 2011). This approach to bacterial identification has been applied successfully to the identification of many clinical isolates and has been used to identify strains and endospores of *B. anthracis* and *B. cereus* (Lasch *et al.*, 2009). However, the application of MALDI-TOF MS-based bacterial identification to biocontrol screening programmes still needs to be explored.

Due to its economic significance and relevance to nursery and greenhouse operations, the soil-borne fungal pathogen *Rhizoctonia solani* was chosen as a test pathogen for the screening of isolates for biocontrol potential (Huang and Huang, 2000; Chung *et al*., 2005). This pathogen has previously been shown to be susceptible to antagonism by strains of *Bacillus* spp. and was therefore considered a suitable candidate for assessing biocontrol screening methods (Asaka and Shoda, 1996; Yu *et al*., 2002; Huang *et al.*, 2012). Seedlings and young transplants are particularly susceptible to pathogens such as *R. solani*, *Pythium* spp., and *Fusarium oxysporum* which can cause pre- and post-emergence damping-off disease, uneven growth and stunting in a wide range of host plants (Huang and Huang, 2000). Sub-lethal infection by these pathogens results in poor growth and lower yields when infected seedlings are planted into the field. The application of plant growth promoting or biocontrol microorganisms to plants grown under such conditions is therefore seen as a promising approach to controlling soil-borne pathogens and maintaining "healthy" plant growth conditions.

In KwaZulu-Natal, South Africa, composted pine bark, a by-product of the local forestry industry, is well established as a growing medium for the propagation and cultivation of plants due to its low cost, physical properties and availability (Holcroft and Laing, 1995; Mupondi *et al*., 2010). Controlled environments such as those associated with greenhouses and nursery operations have been associated with greater levels of consistency when biocontrol measures have been applied and evaluated (Schroth and Becker, 1990, Paulitz and Bélanger, 2001). On this basis, composted pine bark was selected as the main source from which aerobic endospore-forming bacteria were isolated and evaluated. A previous study has shown that *Trichoderma* strains isolated from composted pine bark growing medium exhibit biocontrol activity against *R. solani in*

vitro (Askew and Laing, 1994). However, the incidence and antagonism of aerobic endospore-forming bacteria from this bark medium towards *R. solani* has not been reported on previously.

The objectives of this study were to:

i. Isolate rhizosphere competent aerobic endospore-formers from seedlings grown in composted pine bark and screen for strains able to control *R. solani* using "traditional" *in vitro* and *in vivo* techniques. This was done to obtain a pool of candidate biocontrol agents for which further screening methods could be evaluated.
ii. Use molecular typing methods to differentiate between environmental isolates to assist in rationalizing and selecting candidate organisms for further evaluation.
iii. Characterize the active compounds produced by selected isolates exhibiting broad spectrum antifungal activity using LC/ESI-MS and evaluate PCR-based screening of antibiotic biosynthesis gene markers as a means to select potential biocontrol agents.
iv. Evaluate MALDI-TOF MS as a rapid means of screening for lipopeptide antibiotic production amongst isolates and for fingerprinting and identifying environmental isolates.

The thesis has been divided into five chapters. Chapter one is a literature review providing an overview of the role and contribution that aerobic endospore-formers, specifically *Bacillus* spp., play in biological control and plant growth promotion. Chapters' two to four cover specific aspects of the research undertaken in the study. Each of these chapters is self-contained and is presented in the format of an independent scientific paper. The final chapter, Chapter five, provides a general overview of the major findings and highlights their contribution and relevance; avenues for future research are also discussed.

References

Asaka, O. and Shoda, M. (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Applied and Environmental Microbiology, 62, 4081-4085.

Askew, D.J. and Laing, M.D. (1994) The *in vitro* screening of 118 *Trichoderma* isolates for antagonism to *Rhizoctonia solani* and an evaluation of different environmental sites of *Trichoderma* as sources of aggressive strains. Plant and Soil, 159, 277-281.

Borriss, R. (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses, Springer-Verlag, Berlin, Germany, pp. 41-76.

Campbell, R. (1986) The search for biological control agents against plant pathogens: a pragmatic approach. Biological Agriculture and Horticulture, 3, 317-327.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. Microbial Biotechnology, 8, 281-295. doi: 10.1111/1751-7915.12238.

Chowdhury, S.P., Hartmann, A., Gao, X. and Borriss, R. (2015) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42: a review. Frontiers in Microbiology, 6, 780. doi:10.3389/fmicb.2015.00780.

Chung, W.C., Huang, J.W. and Huang, H.C. (2005) Formulation of a soil biofungicide for control of damping-off of Chinese cabbage (*Brassica chinensis*) caused by *Rhizoctonia solani*. Biological Control, 32, 287-294.

Cook, R. J. (1990) Twenty-five years of progress towards biological control. In: Hornby, D. (ed.), Biological Control of Soil-Borne Plant Pathogens. CAB International, Wallingford, UK, pp. 1-14.

Deravel, J., Krier, F. and Jacques, P. (2014) Les biopesticides, compléments et alternatives aux produits phytosanitaires chimiques (synthèse bibliographique)/Biopesticides, a complementary and alternative approach to the use of agrochemicals. A review. Biotechnologie, Agronomie, Société et Environnement, 18, 220-232.

Fravel, D.R. (2005) Commercialization and implementation of biocontrol. Annual Review of Phytopathology, 43, 337-359.

Garbeva, P., Van Veen, J. A. and Van Elsas, J. D. (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microbial Ecology, 45, 302-316.

Grube, A., Donaldson, D., Kiely, T. and Wu, L. (2011) Pesticides industry sales and usage. US EPA, Washington, DC, USA, 41 p.

Holcroft, D.M. and Laing, M.D. (1995) Evaluation of pine bark as a substrate for *Anthurium* production in South Africa. Acta Horticulturae: International Symposium on Growing Media and Plant Nutrition in Horticulture, 401, 177-184.

Huang, J.W. and Huang, H.C. (2000) A formulated container medium suppressive to *Rhizoctonia* damping-off of cabbage. Botanical Bulletin of Academia Sinica, 41, 49-56.

Huang, X., Zhang, N., Yong, X., Yang, X. and Shen, Q. (2012) Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiological Research, 167, 135-143.

Khetan, S. K. (2001) Microbial pest control. In: Khetan, S.K. (ed.), Microbial Pest Control, Marcel Dekker, New York, USA, pp. 3-42.

Kloepper, J.W., Ryu, C.M. and Zhang, S. (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology, 94, 1259-1266.

Knudsen, I.M.B., Hockenhull, J., Jensen, D.F., Gerhardson, B., Hökeberg, M., Tahvonen, R., Teperi, E., Sundheim, L. and Henriksen, B. (1997) Selection of biological control agents for controlling soil and seed-borne diseases in the field. European Journal of Plant Pathology, 103, 775-784.

Lasch, P., Beyer, W., Nattermann, H., Staemmler, M. and Siegbrecht, E. (2009) Identification
of *Bacillus anthracis* by using matrix-assisted laser desorption Ionization time of flight mass spectrometry and artificial neural networks. Applied and Environmental Microbiology, 75, 7229-7242.

Lethbridge, G. (1989) An industrial view of microbial inoculants for crop plants. In: Campbell, R. and Macdonald, R.M. (eds.) Microbial Inoculation of Crop Plants, Special publication of the Society of General Microbiology, vol. 25, IRL Press, Oxford, New York, USA, pp. 11-28.

Li, X.Y., Mao, Z.C., Wang, Y.H., Wu, Y.X., He, Y.Q. and Long, C.L. (2012) ESI LC-MS and MS/MS characterization of antifungal cyclic lipopeptides produced by *Bacillus subtilis* XF-1. Journal of Molecular Microbiology and Biotechnology, 22, 83-93.

Lin, Y., Du, D., Si, C., Zhao, Q., Li, Z. and Li, P. (2014) Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. Biocontrol, 71, 7-15.

Marten, P., Smalla, K. and Berg, G. (2000) Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. Journal of Applied Microbiology, 89, 463-471.

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

Messenger B. and Braun A. (2000) Alternatives to methyl bromide for the control of soil-borne diseases and pests in California. Pest Management Analysis and Planning Program. California Department of Pesticide Regulation; Sacramento, CA, USA. [(accessed on 24 August 2013)]. Available online: http://www.cdpr.ca.gov/docs/emon/methbrom/alt-anal/sept2000.pdf.

Mupondi, L.T., Mnkeni, P.N.S. and Muchaonyerwa, P. (2010) Effect of pine bark goat manure medium on seedling growth and N, P, K concentration of various vegetables. South African Journal Plant and Soil, 27, 305-311.

Nelson, L.M. (2004) Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. Plant Management Network. doi:10.1094/CM-2004-0301-05-RV.

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology, 16, 115-125.

Paulitz, T.C. and Bélanger, R.R. (2001) Biological control in greenhouse systems. Annual Review of Phytopathology, 39, 103-133.

Pérez-García, A., Romero, D. and de Vicente, A. (2011) Plant protection and growth stimulation by microorganisms: biotechnological applications of bacilli in agriculture. Current Opinion in Biotechnology, 22, 187-93.

Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F. M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil, 340, 505-520.

Price, N.P.J., Rooney, A.P., Swezey, J.L., Perry, E. and Cohan, F.M. (2007) Mass spectrometric analysis of lipopeptides from *Bacillus strains* isolated from diverse geographical locations. FEMS Microbiological Letters, 271, 83-89.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W.G.D., Xuewen, G. and de Kievit, T. (2007) Molecular and biochemical detection of fengycin- and bacillomycin D- producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Canadian Journal of Microbiology, 53, 901-911.

Schisler, D. A. and Slininger, P. J. (1997) Microbial selection strategies that enhance the likelihood of developing commercial biological control products. Journal of Industrial Microbiology and Biotechnology, 19, 172-179.

Schroth, M.N. and Becker, J.O. (1990) Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In: Hornby, D. (ed.) Biological Control of Soilborne Plant Pathogens, CAB International, Wallingford, UK, pp. 389-414.

Schroth, M. N. and Hancock, J.G. (1985) Soil antagonists in IPM systems, In: Hoy, M.A. and Herzog, D.C. (eds.), Biological Control in Agricultural IPM Systems, Academic Press, Orlando, USA, pp. 425-31.

Stets, M.I., Pinto, A.S., Huergo, L.F., de Souza, E.M., Guimarães, V.F., Alves, A.C., Steffens, M.B., Monteiro, R.A., Pedrosa, F.O. and Cruz, L.M. (2013) Rapid identification of bacterial isolates from wheat roots by high resolution whole cell MALDI-TOF MS analysis. Journal of Biotechnology, 165, 167-74.

Thakore, Y. (2006) The biopesticide market for global agricultural use. Industrial Biotechnology, 2, 194-208.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. (2002) Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Applied and Environmental Microbiology, 68, 6210-6219.

Welker, M. and Moore, E.R.B. (2011) Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. Systematic and Applied Microbiology, 34, 2-11.

Whipps, J.M. (1997) Developments in the biological control of soil-borne plant pathogens. Advances in Botanical Research, 26, 1-134.

Yu, G.Y., Sinclair, J. B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry, 34, 955-963.

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Since the early 1980's extensive research has been undertaken to screen and select for microbial candidates for plant growth promotion and/or biological control of soil-borne pathogens (Cook and Baker, 1983). This has been prompted by concerns regarding the extensive use of chemical pesticides that have dominated the agricultural sector for the past 60-70 years. Issues relating to resistance development, environmental pollution, and risks to human health are increasingly being raised (Russell, 1995; Cawoy *et al*., 2011; Pérez-García *et al*., 2011; Deravel *et al*., 2014). Microbial biopesticides potentially offer a sustainable, "environmentally friendly" alternative which can be incorporated into integrated pest management (IPM) and organic farming practices (Jacobsen *et al*., 2004; Chandler *et al*., 2011). Added benefits include their specificity, ideally low toxicity to non-target organisms, low environmental risks and their relative cost effectiveness in development, production and application (Glass and Lindemann, 1992). In addition to this, soil-borne plant pathogens are inherently difficult to manage and cultural and chemical control practices are often impractical and limited in their effectiveness (Khetan, 2001; Huang *et al.*, 2012). Biocontrol, therefore, is seen as a promising alternative to the use of synthetic fungicides.

Various focus areas have emerged in the field of plant growth promotion and biological control with promising microorganisms such as *Trichoderma, Streptomyces*, *Pseudomonas* and *Bacillus* being extensively studied (Emmert and Handelsman, 1999; Harman *et al*., 2004; Kloepper *et al*., 2004; Haas and Dèfago, 2005). Interest in *Bacillus* spp. as potential plant growth promoters has only really developed in the past 15-20 years. Increasingly, a body of evidence has emerged demonstrating that strains within the genus possess a number of traits that are valuable in plant growth promotion and/or disease suppression (Emmert and Handelsman, 1999; Kloepper *et al.,* 2004; McSpadden-Gardner, 2004; Nagórska *et al.,* 2007; Francis *et al*., 2010; Borriss, 2011; Cawoy *et al*., 2011, Kumar *et al*., 2011; Pérez-García *et al*., 2011; Borriss, 2015; Chowdhury *et al*.,

2015). A major driving force has been the need to identify candidate organisms that are amenable to commercial development. In this regard, the ability of *Bacillus* spp. to form endospores is of particular significance since this trait predisposes bacilli to formulation processes that give rise to stable products with extended shelf lives. This attribute give *Bacillus* strains a distinct advantage over the less robust Gram negative pseudomonads.

In this review the role and contribution of *Bacillus* spp. to plant growth promotion and biological control within the context of the rhizosphere environment is discussed.

1.2 Rhizosphere

The root zone of plants provides a favourable environment for microorganisms, which is chemically and biologically distinct to that of the surrounding bulk soil (McCulley, 1999). The term rhizosphere refers to a narrow region of biologically active soil that is adjacent to, and directly influenced by the plant root (Bolton *et al.*, 1993). Characteristically, this region exhibits greater levels of microbiological activity than the surrounding bulk-soil. This "rhizosphere effect" is attributable to the deposition of plant derived nutrients arising primarily from root exudates (Uren, 2007; Cheng and Gershenson, 2011; Doornbos *et al.*, 2012). The rhizosphere constitutes a very large surface area where plants and microbes interact; the exchanges taking place within this environment can be expected to have a profound impact on plant health and productivity (Sørensen, 1997). Such interactions may be beneficial or harmful to the plant, and in some instances the outcome is neutral or variable (Kennedy, 1999; Singh *et al*., 2004). Examples of possible plant-microbe interactions influencing plant growth are presented in Figure 1.1. Not surprisingly, man has attempted to harness the activities of beneficial rhizobacteria for his own purposes.

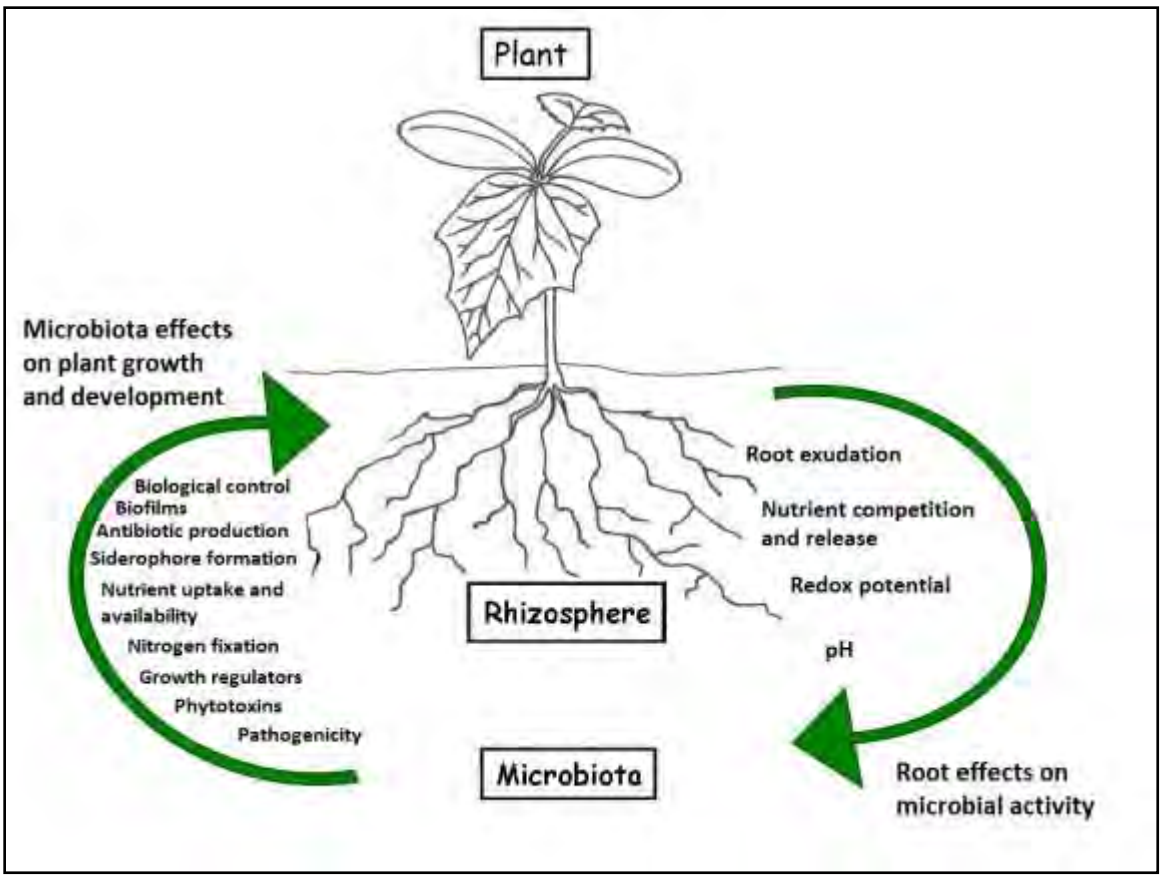


Figure 1.1 Possible plant-microbe interactions affecting plant growth

1.2.1 Plant growth promoting "rhizobacteria" (PGPR)

Root colonizing bacteria that positively influence plant development are commonly referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978; Kloepper, 1993) or more recently, as plant growth-promoting bacteria (PGPB) (Compant *et al.*, 2010). The terms "microbial inoculant" and "yield increasing bacteria" (YIB) have also sometimes been used; referring to microbial products that are marketed to promote plant and/or soil health (Shen, 1997). The promotion of plant growth by rhizosphere associated bacteria has been linked to various mechanisms, which include biological nitrogen fixation, the synthesis of plant growth regulators, enhancing nutrient availability and uptake, and, the antagonism of phytopathogens (Kloepper, 1993; Choudhary and Johri, 2009; Kumar *et al*., 2011). The so called PGPR/PGPBs act as "biological buffers" with their benefits usually being most evident at the early stages of

plant development or under sub-optimal growth conditions when the plant is stressed (Arkhipova *et al*., 2007). Improved mineral and water uptake by roots, coupled with the suppression of minor pathogens can lead to quicker germination rates, improved seedling emergence, healthier root systems and improved tolerance to plant stress (Lugtenberg and Kamilova, 2009). In nursery settings, such PGPR/PGPB can also offer some protection to seedlings once they are transplanted into a field environment.

1.2.2 Biological control agents (BCA)

Microorganisms can also contribute towards the control of plant diseases within the soil environment where they function as naturally occurring antagonists. Root associated bacteria that are antagonistic towards fungal phytopathogens are also referred to as biological control agents (BCA) or "biofungicides" (McSpadden-Gardener and Fravel, 2002). They act by reducing the activity, efficiency and/or population density of a plant pathogen through mechanisms such as antibiosis, siderophore production, competitive exclusion, parasitism, predation and induced systemic resistance (Cook and Baker, 1983; Borriss, 2011; Cawoy *et al*., 2011). The overall aim of biological control is to suppress a disease sufficiently so as to minimize yield losses and maintain crop quality (Khetan, 2001). Our understanding of the mechanisms involved has evolved from the recognition that the incidence of pathogens in soil is markedly influenced by soil type, the prevailing environmental conditions and agricultural practices used (Mathre *et al*. 1999; Berg and Smalla, 2009). Practices such as amending soil with compost and manure have long been known to stimulate microbial activity and reduce the incidence of pathogens, thereby providing an early stimulus for biocontrol research (Huber and Schneider, 1982; Hoitink and Fahy, 1986). The phenomenon of naturally occurring disease suppressing soils also provided an impetus for studying the nature of the underlying mechanisms involved (Cook, 1990). In an early study, Broadbent and Baker (1975) reported that soils suppressive of *Phytophthora cinnamomi* lost their suppressive nature when steamed at 100^{o} C but retained it after 30 minutes at 60^{o} C. This finding led to the speculation that endospore forming bacteria were somehow involved. Subsequent studies have confirmed the importance of aerobic endospore-forming bacteria in suppressing plant diseases.

1.3 Aerobic endospore-forming bacteria as plant growth promoters and antagonists of phytopathogens

Numerous reports in the literature have indicated that many strains of *Bacillus* spp. exhibit varying degrees of biocontrol activity against a range of plant diseases in various plant hosts (Table 1.1). Although members of the *B. subtilis* species complex, including *B. amyloliquefaciens,* have received much attention in this regard, other species including *B. cereus*, *B. firmus*, *B. licheniformis*, *B. megaterium*, *B. mycoides*, and *B. pumilus* have also been associated with plant growth stimulation and phytopathogen antagonism (Choudhary and Johri, 2009; Kumar *et al.,* 2011). Strains of *Paenibacillus polymyxa* have also been linked to plant growth promotion via mechanisms such as nitrogen fixation, plant hormone production and the synthesis of bioactive secondary metabolites (Borriss, 2015).

Several strains of *Bacillus* species have been commercialized and successfully marketed as plant growth promoters or biocontrol agents (Table 1.2). Insights into their modes of action will be useful for choosing and implementing screening strategies when searching for future candidate organisms. A common feature of commercial strains such as *B. subtilis* GBO3 (Kodiak^{TM}), *B. subtilis* QST713 (Serenade®), and *B. amyloliquefaciens* FZB24 (Rhizo-Plus®) is their ability to directly antagonize fungal pathogens through antibiosis (Brannen and Kenney, 1997; McSpadden-Gardener and Fravel, 2002; Fravel, 2005; Chen *et al.,* 2009b). These strains show a broad spectrum of activity towards a range of fungal pathogens and in some cases it has been shown that multiple antibiotic compounds are produced (Chen *et al.,* 2009a). Other traits such as rhizosphere competence and the indirect stimulation of plant growth have also been observed (Fan *et al.,* 2012). These observations support the early assertion of Weller (1988) that an efficient biological control agent should exhibit more than one mechanism by which it can suppress a pathogen.

Table 1.1 Examples of Bacillus-based biocontrol of fungal and oomycete plant diseases

Crop	Pathogen	*Bacills* spp.	Reference
Alfalfa	*Fusarium graminearum*	*B. subtilis*	Chan *et al.* (2003)
Apple	*Botrytis cinerea*	*B. subtilis*	Touré *et al.* (2004)
Avocado	*Colletotrichum gloeosporioides*	*B. subtilis*	Demoz & Korsten (2006)
	Phomopsis perseae		Demoz & Korsten (2006)
	Pseudocercospora purpura	*B. subtilis*	Korsten *et al.* (1997)
Banana	*Pseudocercospora musae*	*B. subtilis*	Fu *et al.* (2010)
	Colletotrichum musae		Fu *et al.* (2010)
Beet	*Cercospora beticola*	*B. subtilis*	Collins & Jacobsen (2003)
	Pythium spp.		Collins & Jacobsen (2003)
Carrot	*Alternaria dauci*	*B. subtilis*	Hernandez-Castillo *et al.* (2006)
Cauliflower	*Pythium ultimum*	*B. subtilis*	Abdelzaher (2003)
Corn	*Fusarium moniliforme*	*B. subtilis*	Bacon *et al.* (2001)
	Fusarium verticillioides	*B. subtilis*	Cavaglieri *et al.* (2005)
	Aspergillus flavus	*B. subtilis*	Nesci *et al.* (2005)
	Pythium spp.	*B. subtilis*	Cavaglieri *et al.* (2005)
	Fusarium verticillioides		Cavaglieri *et al.* (2005)
Cotton	*Fusarium oxysporum*	*B. subtilis*	Gajbhiye *et al.* (2010)
Cucumber	*Pythium aphanidermatum*	*B. subtilis*	Grosch *et al.* (1999)
	Phytophthora nicotianae	*B. subtilis*	Grosch *et al.* (1999)
	Phomopsis spp.	*B. subtilis*	Kita *et al.* (2005)
	Colletotrichum lagenarium	*B. subtilis*	Ongena *et al.* (2005)
	Sphaerotheca fuligiena	*B. subtilis*	Bettiol *et al.* (1997)
Lettuce	*P. aphanidermatum*	*B. subtilis*	Corrêa *et al.* (2010)
Oilseed rape	*Sclerotinia sclerotiorum*	*B. subtilis*	Yang *et al.* (2009a)
Potato	*R. solani*	*B. subtilis*	Brewer & Larkin (2005)
Pepper	*Phytophthora capsici*	*B. megatarium*	Ahmed *et al.* (2003)
	P. capsici	*B. subtilis*	Lee *et al.* (2008)
	P. aphanidermatum	*B. subtilis*	Nakkeeran *et al.* (2006)
Rice	*Aspergillus flavus*	*B. subtilis*	Reddy *et al.* (2009)
	R. solani	*B. subtilis*	Yang *et al.* (2009a)
Soybean	*Septoria glycines*	*B. subtilis*	Mantecon (2008)
	F. oxysporum	*B. subtilis*	Zhang *et al.* (2009)
	F. graminearum	*B. subtilis*	Zhang *et al.* (2009)
	Sclerotinia sclerotiorum	*B. subtilis*	Zhang *et al.* (2009)
	R.solani	*B. amyloliquefaciens*	Corrêa *et al* . (2009)
Sorghum	*P. ultimum*	*B. subtilis, B. cereus, B. pumilus, B. mycoides*	Idris *et al.* (2008)
Tomato	*F. oxysporum*	*B. subtilis*	Chebotar *et al.* (2009)
	Fusarium semitectum	*B. subtilis*	Nihorimbere *et al.* (2010)
	F. oxysporum f.sp. lycopersici	*B. subtilis*	Abd-Allah *et al.* (2007)
	P. aphanidermatum	*B. subtilis*	Baysal *et al.* (2008)
	R. solani	*B. subtilis*	Kondoh *et al.* (2001)
	R. solani	*B. subtilis, B. lentimorbus*	Montealegre *et al.* (2003)
Wheat	*Gaeumannomyces graminis var. tritici*	*B. subtilis*	Liu *et al.* (2009)

Table 1.2. Examples of commercially available biocontrol products incorporating *Bacillus* spp. strains (Source: Fravel, 2005; Borriss, 2011, Pérez-García *et al*., 2011)

Commercial Product	*Bacillus* strain	Target Pathogen/Disease	Crop	Manufacturer
Avogreen	*B.subtilis* B246	*Cercospora* spot	Avocado	Stimuplant, South Africa
Companion	*B. subtilis* GB03, *B.licheniformis, B.megaterium*	*Rhizoctonia, Pythium, Fusarium, Phytophthora, Sclerotinia*	Greenhouse, nursery and ornamental crops	Growth Products, USA
Kodiak™	*B. subtilis* GB03	*Rhizoctonia, Fusarium, Alternaria and Aspergillus*	Cotton, legumes and vegetables	Bayer Crop Science, USA
RhizoPlus®	*B. .amyloliquefaciens* FZB24	*R.solani, Fusarium, Alternaria, Sclerotinia, Verticillium, Streptomyces scabies*	field (potatoes, corn), vegetables, and ornamental plants	ABiTEP GmbH, Germany
Serenade®	*B. subtilis* QST716	Powdery mildew, downy mildew, *Cercospora* leaf spot, early blight, late blight, brown rot, fire blight	Cucurbits, grapes, hops, vegetables, peanuts, pome and stone fruits	AgraQuest, Inc., USA
Subtilex®	*B. subtilis* MB1600	*R. solani, Fusarium spp. and Aspergillus spp.*	Cotton, vegetables, peanuts, soybeans, forage and turf grasses, cereals	Becker Underwood Inc., Canada
YiedShield®	*B. pumillus* GB34	Soil-borne fungal pathogens causing root diseases	Soybean	Gustafson, USA

1.3.1 Genus *Bacillus*

The genus *Bacillus* comprises an important group of low G+C Gram positive endospore-forming bacteria found within the phylum *Firmicutes* (Class *Bacilli*; Order *Bacillales*; Family *Bacillaceae*) (Priest, 1993, Logan, and De Vos, 2009; Maughan and Van der Auwera, 2011). Members of this genus are aerobic or facultatively anaerobic, rod-shaped bacteria that can differentiate into endospores. Historically, strains were identified and distinguished using morphological and phenotypic characteristics which resulted in high levels of physiological and genetic diversity being evident within the genus. The G+C content of genomic DNA among species was found to vary considerably, ranging from 33 to 67 mol% indicating that the level of heterogeneity within the genus was greater than expected (Priest, 1993). The advent of phylogenetic methods based on 16S rRNA gene sequence analysis confirmed the need for members of the genus to undergo further taxonomic separation (Ash *et al*., 1991; Garrity *et al*., 2003). This has resulted in the genus undergoing a major taxonomic revision in recent years with various members

being reassigned into several new families and genera. Currently, 88 species and five sub-species are recognised within the genus (Fritze, 2004; Logan, and De Vos, 2009; Borriss *et al.*, 2011; Yi *et al*., 2014). Overall, aerobic endospore-forming bacteria found within the order *Bacillales* account for some 25 different genera incorporating over 200 species.

Bacillus subtilis Cohn 1872 is the type species of the genus and it has been used widely as a model Gram-positive bacterium to study cellular processes such as cell differentiation and regulation of gene expression (Sonenshein, 1993; Grauman, 2007). Taxonomic studies have shown that this species can be further divided into three subspecies namely *B. subtilis* subsp. *subtilis, B. subtilis* subsp. *spizizenii* and *B. subtilis* subsp. *inaquosorum* (Nakamura *et al*., 1999; Rooney *et al*., 2009; Yi *et al*., 2014). In addition to these, several closely related species have also been described which are very difficult to differentiate from *B. subtilis* based on phenotypic and phylogenetic analysis of 16S rRNA gene sequences (Fritze, 2004; Reva *et al*, 2004; Rooney *et al*., 2009). These species include *B. amyloliquefaciens, B. atrophaeus, B. axarquiensis, B. malacitensis, B. mojavensis, B. sonorensis, B. tequilensis, B. vallimortis* and *B. velezensis*. Even though DNA-DNA hybridization distinguishes these organisms as distinct species, they all exhibit very high levels of 16S rRNA gene sequence similarity (Rooney *et al*., 2009). Collectively, these bacteria have been referred to as the '*subtilis*- group' or the *B. subtilis* species complex. Phylogenetic analysis of multiple protein-coding loci has been recommended as a means of distinguishing between members of this group (Rooney *et al*., 2009; Borriss *et al*., 2011).

From a biocontrol perspective many strains associated with the *subtilis*-group are found in association with plants and possess various traits that may directly or indirectly benefit plant growth (Reva *et al*, 2004). Similarly, members of the *B. cereus* group comprising strains of *B. cereus*, *B. mycoides* and *B. thuringiensis* are common soil residents that have also been associated with PGPR/BCA attributes (Emmert and Handelsman, 1999; Fritze, 2004). Members of this group are also difficult to distinguish from each other and significantly, include the human pathogen *Bacillus anthracis* within their ranks.

The capacity to form endospores is an important survival strategy which allows vegetative *Bacillus* cells to differentiate into resting structures when nutrient depletion occurs, critical cell density thresholds are reached or other stress factors are encountered (Stragier and Losick, 1996; Grauman, 2007). Dormant endospores are extremely resistant structures which can tolerate a wide range of environmental stresses for extended periods. This adaptation has enabled bacilli to become widely dispersed, to the extent that they are considered to be ubiquitous. Soil environments are important habitats for many bacilli; here they function as saprophytes and are able to utilize a wide range of organic materials derived from plants and animals (Garbeva *et a*l., 2003). In this regard *Bacillus* spp. plays a central role in the cycling of carbon and nitrogen within the environment. Their prevalence and diversity within soil is dependent on the nutrient status of the soil as well as on the prevailing physicochemical conditions (Garbeva *et al*., 2003; McSpadden-Gardener, 2004). The extent to which strains are localized within specific niches is not well established. In some instances *Bacillus* spp. may be present within a habitat as endospores and not contribute to the functioning of that ecosystem. Increasingly it has become apparent that certain bacilli live in close proximity to plants with strains being isolated from the rhizosphere, rhizoplane and phyllosphere. Reva *et al*. (2004) found that certain strains of *B. amyloliquefaciens* were able to colonize the rhizosphere more effectively than other members of the *B. subtilis* group and proposed that they be considered a distinct ecotype. The concept of ecotypes was first put forward by Cohan (2002) and is defined as "a set of strains using the same or similar ecological resources".

The isolation of aerobic endospore-formers from environmental samples is a relatively straight forward procedure and can be achieved by implementing a heat pre-treatment step (80^{o}C for 10-30 min) which eliminates vegetative cells (Priest and Grigorova, 1990). By plating samples onto complex media or subjecting them to further enrichment steps, diverse physiological groups of bacilli can be isolated. In many instances *Bacillus* spp. strains produce extracellular hydrolytic enzymes which are involved in the breakdown of polymeric substances such as cellulose, starch, glycans, pectins and proteins (Priest and Grigorova, 1990; Priest, 1993). Consequently, the genus is viewed as an important source

of commercially useful enzymes. Various species produce interesting metabolites such as antibiotics, fine biochemicals, surfactants and bioinsecticides which have found biotechnological applications (Katz and Demain, 1977; Lancini and Lorenzetti, 1993; Zuber *et al*., 1993; Jacques, 2011). In addition to this, their amenability to large-scale production using existing fermentation technology makes them attractive candidates for commercial development (Priest, 1993).

Many saprophytic bacilli are regarded as being mostly benign and *B. subtilis* specifically, has been accorded GRAS (generally regarded as safe) status by the USA Federal Drug Administration (FDA) (Harwood and Cutting, 1990). However, members of the *B. cereus* group are known to produce enterotoxins and have been linked to frequent food-poisoning events (Granum and Lund, 1997). The use of potentially toxigenic *Bacillus* species in applications that could harm animals or humans is prohibited by the European Food Saftey Authorities (EFSA FEEDAP Panel, 2011). As a matter of course, *Bacillus* isolates selected for commercial development purposes need to be screened for toxicity and pathogenicity *in vitro* and *in vivo*.

1.3.2 *Bacillus* spp. antagonism of fungal pathogens

The antagonism of fungal pathogens is widely associated with the production of antibiotic compounds (Raaijmakers *et al*., 2002; Ongena and Jacques, 2008; Kinsella *et al*., 2009; Raaijmakers *et al*., 2010, Kumar and Johri, 2012; Cawoy *et al*., 2015). Although antibiotic production is widespread within the genus *Bacillus,* with the first commercial antibiotic bacitracin being discovered in 1945 (Johnson *et al*., 1945), the ability to produce antibiotics which are specific towards fungal and oomycete pathogens appears to be less prevalent (Stein, 2005). Cyclic lipopeptides belonging to the iturin, fengycin and surfactin families are amongst the most commonly reported antifungal compounds produced by *Bacillus* spp. (Ongena and Jacques, 2008; Cawoy *et al*., 2015). The aminopolyol zwittermycin A has also been reported to be effective in controlling soil borne fungal plant pathogens such as *Phytophthora medicaginis* and *Pythium* sp. on alfalfa and soybean plants (Stabb *et al*., 1994; Silo-suh *et al*., 1998). Kanosamine, an

aminoglycoside antibiotic produced by *B. cereus* UW85 also exhibits antagonism towards *P. medicaginis* but is regarded as being less potent than zwittermycin A (Milner *et al.* 1996). Kurstakins, a class of lipopeptides produced by strains of *B. thuringiensis* and *B. cereus* have also shown a limited degree of fungal antagonism towards fungi such as *Stachybotrys charatum* (Béchet *et al*., 2012).

To a lesser extent, antagonism of plant pathogens has also been associated with the production of volatile compounds (Chaurasia *et al*., 2005; Arrebola *et al*., 2010), inhibitory catabolic enzymes such as chitinase and glucanase (Podile and Prakash, 1996, Leelasuphakul *et al*., 2008), and siderophore production (Beneduzi *et al*., 2008; Yu *et al.*, 2011). Volatile organic compounds produced by *B. amyloliquefaciens* PPCB004 have shown potential in inhibiting or delaying the onset of postharvest citrus decay caused by *Penicillium crustosum* (Arrebola *et al*., 2010). Extracellular enzymes such as chitinase, β-1,4-N-acetyl glucosaminidase and β-1,3-glucanase play important roles in the hydrolysis of complex organic polymers that accumulate in soil environments. These enzymes can also contribute towards biocontrol through their ability to degrade fungal cell wall components (Manjula and Podile, 2005; Leelasuphakul *et al*., 2006). The siderophore 2,3-dihydroxybenzoylglycine is an iron chelating compound secreted by *Bacillus* spp. which facilitates the chelation and uptake of low concentrations of ferric ion (Fe^{3+}) present in surrounding environments (Yu *et al*., 2011). This scavenging action competitively excludes Fe^{3+} from many plant pathogens which require it for growth thereby affording some protection to the plant host.

1.3.3 *Bacillus* spp. promotion of host nutrition and growth

Plant growth promotion mediated by *Bacillus* spp. has been linked to the production of the plant growth regulators such as cytokinin, gibberellin and indole acetic acid (Selvadurai *et al*., 1991; Lebuhn *et al*., 1997; Araujo *et al*., 2005; Idris *et al*., 2007). The ability of certain strains to solubilize organic and inorganic sources of phosphorous has also been shown to increase phosphorous availability to plants (Alagawadi and Gaur, 1992; Rojas *et al.,* 2001; Idris *et al*., 2002). In soil, organic phosphate accumulates as

poorly soluble myo-inositol hexaphosphate, otherwise referred to as phytate. Microorganisms that produce extracellular phytase are able to convert phytate to a soluble form which is available for plant uptake. Solubilisation of inorganic polyphosphate is linked to organic acid production (Vazquez *et al*., 2000).

The ability to fix atmospheric nitrogen is another trait associated with plant growth promotion and has been linked to close relatives of the genus *Bacillus* namely *Paenibacillus polymyxa* and *Paenibacillus azotofixans* (Beneduzi *et al*., 2008). Plant growth promotion linked to the stimulation of mutualistic symbionts has also been reported. Several studies suggest that co-inoculation of *Bacillus* or *Paenibacillus* strains with rhizobia has a synergistic effect on plant growth in beans and pigeon-peas (Figueiredo *et al*., 2008; Rajendran *et al*., 2008). For example, Petersen *et al*. (1996) demonstrated that the presence of *P. polymyxa* within the rhizosphere of *Phaseolus vulgaris* actively promoted nodulation by *Rhizobium etli*. Enhanced uptake and accumulation of phosphorous in plants hosting mycorrhizal symbionts has also been reported when the neighbouring soil was inoculated with phosphate solubilizing *Bacillus* strains (Toro *et al*., 1997).

1.3.4 *Bacillus* spp. induced Systemic resistance (ISR)

The ability of root associated *Bacillus* spp. to elicit plant defences against a range of fungal, bacterial and viral diseases is increasingly being recognised (Ryu *et al.*, 2004; Kloepper *et al*., 2004; Choudhary and Johri, 2009; Cawoy *et al*., 2014). This is attributed to an induced systemic resistance (ISR) phenomenon which is associated with signal molecules derived from rhizosphere associated bacteria which trigger a systemic expression of disease resistance mechanisms within the host plant (Pieterse *et al*., 2001; Pieterse *et al*., 2014). The signal transduction pathways typically involve a sequential sensitization of the plant hormones jasmonic acid and/or ethylene from the site of initiator interaction (Park and Kloepper, 2000; Yan *et al*., 2002; Zhang *et al*., 2002; Ongena and Jacques, 2008). The ISR response can manifest as changes in cell wall composition, the production of pathogenesis-related-proteins, the generation of reactive

oxygen species, or in the synthesis of phytoalexins. The underlying signalling pathways involved in *Bacillus* mediated ISR have not been fully elucidated but are known to bring about the activation of a positive regulator protein NPR1 which brings about a priming effect within plants which readies them to initiate a defence response once a pathogen is encountered. Ongena *et al*. (2007) reported that the plant ISR response to elicitor compounds was not universal and appeared to be plant specific.

Several metabolites produced by *Bacillus* strains have been identified as ISR elicitors, these include 2,3–butanediol, acetoin, 2-aminobenzoic acid and the lipopeptides surfactin and fengycin (Ryu *et al*., 2004; Ongena *et al*., 2007; Jourdan *et al*., 2009; Cawoy *et al*., 2014). Ongena *et al.* (2005) for example, demonstrated that the lipopeptides surfactin and fengycin both brought about an increase in lipoxygenase activity in inoculated tomato plants and this was associated with a reduction in the incidence of anthracnose. Surfactin production by strains of *B. subtilis* and *B. amyloliquefaciens* has also been positively correlated to the generation of reactive oxygen species (Cawoy *et al*., 2014).

Although ISR does not bring about complete protection against pathogen infection it holds promise since it confers a long-lasting defence response and is not conducive to the development of pathogen resistance (Ongena and Jacques, 2008). Comprehensive reviews on this topic are provided by Kloepper *et al*. (2004), Choudhary and Johri, (2009) and Pieterse *et al*., (2014).

1.3.5 *Bacillus* antibiotics associated with biological control of phytopathogens

Members of the genus *Bacillus* are well known for their ability to produce a variety of biologically active compounds and several comprehensive reviews are available covering this topic (e.g. Katz and Demain, 1977; Zuber *et al*., 1993; Stein, 2005 and Ongena and Jacques, 2008; Kumar and Johri, 2012). Peptide antibiotics appear to be the predominant class of antimicrobial compounds found within the genus (Stein, 2005). To a lesser extent, non-peptide antibiotics such as polyketides, aminopolyols and aminoglycosides

have also been isolated. Most of these compounds are characteristically produced as secondary metabolites and exhibit a range of activities which includes antibacterial, antifungal, antiviral, antitumour and insecticidal modes of action. From a biological control perspective, bacilli that produce antibiotic compounds antagonistic towards fungal and oomycete phytopathogens are of particular interest.

Antibiotic activity is relatively easy to screen for *in vitro* using dual-culture bioassays. However, establishing the causal relationship between antibiosis and biocontrol activity has been more challenging to prove (Haas and Dèfago, 2005). Through the use and testing of antibiotic deficient mutants it has been shown that antibiotics play a key role in the suppression of various soil-borne plant pathogens (Raaijmakers *et al*., 2002). Asaka and Shoda (1996) for example showed that iturin A producing *B. subtilis* RB14 was able to suppress damping-off in tomatoes whereas antibiotic deficient mutant strains were unable to. Further to this, it has been shown that *B. subtilis* 168, a domesticated laboratory strain that lost its ability to produce lipopeptides due to a frameshift mutation, was able to regain this trait when a native *sfp* gene coding for 4`-phosphopantetheine transferase was introduced thereby enabling it to exhibit biocontrol activity (Tsuge *et al*., 1999). Additionally, the development of sensitive analytical methods such as matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass-spectrometry and electrospray ionization mass-spectrometry have also permitted the quantitative and qualitative detection of antibiotic compounds *in vivo* (Asaka and Shoda, 1996; Kinsella *et al*., 2009).

1.4 Lipopeptide antibiotics

The ability to produce lipopeptide antibiotics is widespread within the genus *Bacillus*. Examples include strains of *B. amyloliquefaciens* (Yu *et al*., 2002; Arrebola *et al*., 2009; Chen *et al*., 2009a), *B. cereus* (Sadfi *et al*., 2002; Ramarathnam *et al*., 2007), *B. licheniformis* (Patel *et al*., 2004; Tendulkar *et al*., 2007), *B. pumilis* (Leifert *et al*., 1995; Munimbazi and Bullerman, 1998), *B. subtilis* (Vater *et al*., 2002; Mizumoto *et al*., 2007; Romero *et al.*, 2007; Hsieh *et al*., 2008) and *B. thuringiensis* (Kim *et al*., 2004). The

capacity for a single strain to produce multiple antimicrobial compounds appears to be less prevalent. A shared feature of commercialized biocontrol strains (viz. *B. subtilis* GB03, *B. subtilis* QST716, and *B. amyloliquefaciens* FZB42) is their wide spectrum of antimicrobial activity which most likely is linked to their ability to synthesize multiple antimicrobial compounds (Brannen and Kenny, 1997; Emmert and Handelsman, 1999; Chen *et al*., 2009b). *Bacillus amyloliquefaciens* FZB42 for example, produces four distinct cyclic peptides (viz. surfactin, bacillomycin, fengycin, and an unidentified peptide) and three antibacterial polyketides (viz. macrolactin, bacillaene, and difficidin) (Chen *et al*., 2009b).

Structurally, lipopeptides are cyclic in nature comprising a hydrophobic β-amino or β-hydroxy fatty acid component incorporated into a hydrophilic peptide moiety (Magnet-Dana and Peypoux, 1994; Romero *et al.*, 2007). Differentiation of the lipopeptide families is mainly based on the composition of the amino acid components as well as the level of fatty acid branching (Vater *et al*., 2002). The amino acid constituents can include L- and D-isomers of the same amino acid and a possible consequence of this is that lipopeptides are often resistant to protease/peptidase catalysed hydrolysis (Kleinkauf and Döhren, 1990). Another feature of their structures is that some lipopeptides show remarkable stability and retain activity even after exposure to elevated temperatures and/or wide pH ranges (Souto *et al*., 2004). Within each lipopeptide family closely related isoforms can be distinguished where the length and composition of the fatty acid side chain varies (Stein, 2005; Ongena and Jacques, 2008). Amino acid substitution within the peptide ring structure also occurs. Several reports indicate that the length of the fatty acid side chain affects the efficacy of these compounds with longer hydrocarbon side chains being more bioactive against target membranes (Toure *et al*., 2004; Leclere *et al*., 2005). Three main families of antifungal lipopeptides are recognized, the surfactins, iturins and fengycins (or plipstatins). Representative structures and examples of each of these lipopeptides are presented in Figure 1.2.

The iturin family which comprises iturin A, B, C, D, E, bacillomycin D, F,L and mycosubtilin are heptapeptides with a β-amino fatty acid side chain (C14 to C17)

(Magnet-Dana and Peypoux, 1994). Compounds within the surfactin family (viz. surfactin, lichenysin, pumilacidin) also comprise a heptapeptide moiety but differ from the iturins in that it is interlinked to the carboxyl and hydroxyl groups of a β-hydroxy fatty acid (C13 to C15) to form a cyclic lactone ring structure (Peypoux *et al.*, 1999). Members of the fengycin family, including plipastatin are decapeptides with an internal lactone ring in the peptide moiety and incorporating a β-hydroxy fatty acid side chain (C14 to C18) (Vanittanakom *et al.,* 1986).

The amphiphilic nature of lipopeptides enables them to interact with biological membranes and interfere with membrane function (Magnet-Dana and Peypoux, 1994). Their disruptive effect is thought to be concentration dependant and susceptibility to them is linked to membrane composition and sterol content (Carillo *et al*., 2003). Iturins are antagonistic towards a range of yeast and fungi but show virtually no antibacterial activity. The hydrophobic moiety of iturin penetrates into the fungal cytoplasmic membrane resulting in an aggregation of molecules which leads to pore formation and efflux of essential ions which can lead to osmotic disruption of the lipid bilayer and cell cytoplasm (Stein, 2005). Surfactins exhibit haemolytic, antiviral, and antibacterial activities whereas their action against fungi is less pronounced (Jacques, 2011). As surfactant compounds they display powerful emulsifying and foaming properties which facilitates membrane solubilisation and disruption (Peypoux *et al*., 1999). Fengycins also exhibit strong activity against filamentous fungi and it is thought to involve changes in membrane structure and permeability (Deleu *et al*., 2005). There is some evidence to suggest that combinations of these compounds may act in a synergistic manner thereby enhancing their antagonistic effect towards fungal phytopathogens (Magnet-Dana *et al*., 1992; Romero *et al*., 2007).

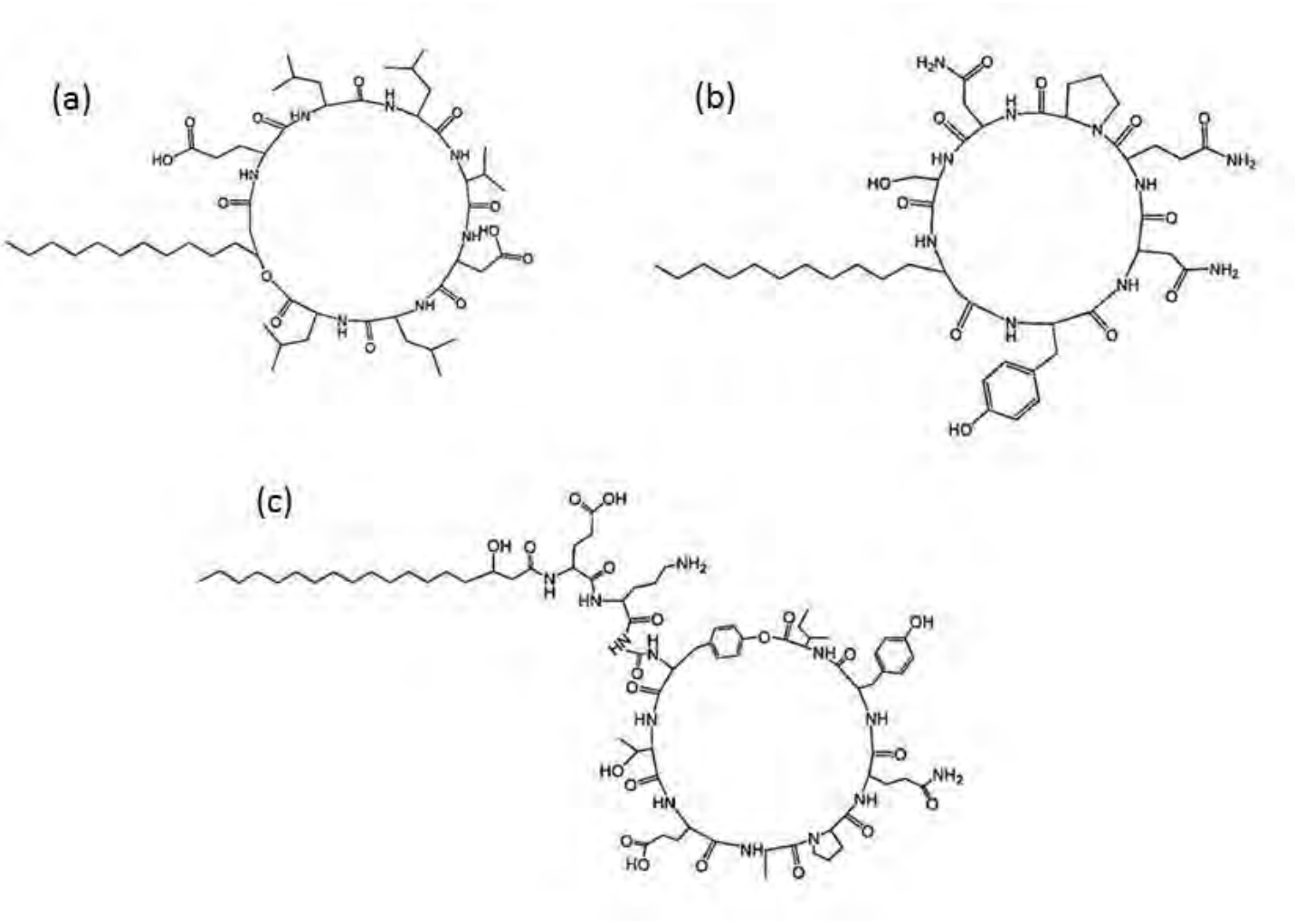


Figure 1.2 Representative members of antifungal lipopeptide families synthesized by *Bacillus* spp., (a) surfactin, (b) iturin A, (c) fengycin (Adapted from Jacques, 2011)

In general, the biosynthesis of these lipopeptide compounds is accomplished nonribosomally by large multienzyme systems referred to as non-ribosomal peptide synthetases (NRPS) (Finking and Marahiel, 2004; Stein, 2005). NRPS require post-translational modification to become functional and are mediated by 4`-phosphopantetheinyl transferase encoded by the *sfp* gene. These NRPS are comprised of modularly arranged catalytic domains which are responsible for the assembly of the lipopeptide components. The structure and activity of each peptide product is determined by the arrangement of the NRPS modules and the functional domains associated with different peptide synthetases. The process involves three core domains namely, an adenylation domain which is responsible for targeting specific amino acids and generating an enzymatically stabilized aminoacyl adenylate; a thiolation or peptidyl carrier domain to which the adenylated aminoacid substrate is transferred and thioesterified; and, a condensation domain responsible for catalyzing th formation of new peptide bonds (Stein *et al*., 1996). NRPS systems can readily undergo natural rearrangements which accounts for the broad heterogeneity and numerous isoforms associated with lipopeptides generated from these systems (Stein, 2005).

1.4.1 Why produce antibiotics?

It is estimated that strains of *B. subtilis* and *B. amyloliquefaciens* dedicate between 4.5-8.5% of their genome to the synthesis of antibiotic compounds (Stein, 2005; Chen *et al*., 2007). Over the years the exact purpose of these compounds has been debated widely. Besides their seemingly obvious role in suppressing and inhibiting competitor organisms various other functions have been proposed. Lipopeptide compounds are secondary metabolites and their production is linked to factors such as high cell densities, nutrient limitation, general stress responses and a transition from a planktonic to a sessile growth phase (Cosby *et al*., 1998; Toure *et al*., 2004; Mizumoto *et al*., 2007).

The regulatory mechanisms governing lipopeptide production have not been fully established and appear to differ between lipopeptide compounds (Duitman *et al*., 2007). Surfactin production is initiated during late exponential phase and has been found to be linked to competence development (Roggiani and Dubnau, 1993; Hamoen *et al*., 2003). A ComA/ComP signal transduction pathway triggered by quorum sensing pheromones ComA and PhrC oversees the transcriptional regulation of the surfactin synthetase operon *srf*A.

Interestingly, expression of the *srf*A locus is essential for competence development, which enables bacteria to take up exogenous DNA from the environment. In the case of mycosubtilin Duitman *et al*. (2007) found that the *myc* operon is regulated by the transition-state regulator AbrB. The production of bacillomycin D is regulated at multiple levels and expression of the *bmy* operon is dependent on the σ^A–dependant promoter P_{bmy} (Koumoutsi *et al*., 2007). The regulators DegU and ComA were also required for full transcriptional activity of *bmy*. The pleiotropic regulator DegQ has been linked to fengycin production; specifically, by regulating expression of the fengycin synthetase gene (Tsuge *et al*., 1999; Wang *et al*., 2015).

The investment in secondary metabolite production is thought to play an important role in determining the ecological fitness of an organism and provide means for bacteria to adapt to nutrient limited conditions (Hamoen *et al.*, 2003; Farjardo and Martinez, 2008; Raaijmakers *et al*., 2010; Raaijmakers and Mazzola, 2012). In addition to their antimicrobial action lipopeptides such as surfactin and fengycin are now known to play important roles in root colonization and biofilm development (Danhorn and Fuqua, 2007). These compounds can also stimulate host defense mechanisms by eliciting induced systemic resistance in plants (Ongena *et al*., 2007). Surfactin production has also been linked to the swarming action associated with wild type bacilli (Kearns *et al*., 2004). Farjado and Martinez (2008) suggest that certain antibiotic compounds, including nonribosomal peptides, may act as signalling molecules that play important roles in intercellular and interspecies communication. For example, surfactin has been shown to act as a signal molecule which triggers biofilm formation in strains of *B. subtilis* (Lopez et al., 2009a). Additionally, bacillaene, a peptide/polyketide antibiotic produced by *B. subtilis* has been linked to interspecies interactions which suppress the defensive mechanisms associated with *Streptomyces coelicolor* (Yang *et al*., 2009b). Bacillaene has also been shown to protect *B. subtilis* from predation by *Myxococcus xanthus*; thereby allowing *B. subtilis* to form endospores which are inherently resistant to predation (Müller *et al*., 2014).

1.5 Ecological considerations for plant growth promotion and biological control

Ultimately, the success of a microbial inoculant depends on its ability to disseminate and establish itself within the rhizosphere of a targeted host plant and then execute its desired trait (Whipps, 2001; Berg, 2009). The root zone is a dynamic environment and introduced

microorganisms have to contend with a range of complex interactions involving the host plant, the resident microflora as well as the surrounding soil. In order to be effective, introduced bacteria must be able to establish themselves at sufficiently high numbers before root infection by the plant pathogen takes place. Inconsistency in the efficacy of microbial inoculants is often attributed, in part, to factors such as inefficient root colonization, and cell densities that are low or short-lived. Hence, an understanding of gene expression under environmental conditions is warranted. Several authoritive reviews pertaining to the microbial ecology of the rhizosphere are available (e.g. Rovira, 1965; Curl and Truelove, 1986; Lynch, 1990; Sørensen, 1997; Pinton *et al.*, 2001; Cardon and Whitbeck, 2011; Mendes *et al*., 2013; Philippot *et al*., 2013).

Plant and soil type both have a profound influence on the structure of the rhizosphere community structure (Marschner *et al*., 2001; Berg and Smalla, 2009). Root structure varies considerably between plant types and therefore can be expected to influence microbial colonization of the rhizosphere region. Dicotyledonous plants for example generally produce primary roots which bear many lateral branches; whereas, monocotyledon plants often develop shallow fibrous root systems which spread horizontally through the soil profile (Curl and Truelove, 1986). These rooting systems represent a major route for the flow of organic carbon into soil and factors influencing rhizodeposition will also have a direct bearing on root colonization (Brimecombe *et al.*, 2001). Plant derived nutrients originate from sources such as exudates, lysates and mucilagenous material. Estimates suggest that up to 40% of photosynthetically derived carbon can be released in to the rhizosphere in the form of root exudates (Whipps, 2001; Doornbos *et al*., 2012). These exudates vary in composition and quantity depending on plant type, cultivar, age and physiological state (Morgan and Whipps, 2001; Pinton *et al.*, 2001). Physical and chemical properties of soil also have a marked influence on rhizodeposition as well as the activity and distribution of rhizosphere populations. Factors such as soil structure, texture, porosity and consistency can influence rooting depth, water movement and aeration within the root region (Baker, 1987; Uren, 2007). Chemical properties such as soil organic matter, cation exchange capacity, soil mineralogy and pH also contribute to biological activity and redox conditions within the rhizosphere microhabitat (Garbeva *et al*., 2004). Biotic factors such as the presence bacterial grazing protozoa, bacterial phages or even bacterial predators such as *Myxococcus xanthus*

within the rhizosphere can also be expected to impact microbial community structure and function (Sørensen, 1997; Müller *et al*., 2014).

The rhizosphere is not a uniform environment and is subject to concentration gradients of both organic and inorganic compounds which are influenced by rates of root exudation and the degree to which the rhizodeposits are utilized (Campbell and Greaves, 1990). Microorganisms congregate at localized sites within the rhizosphere that are associated with elevated levels of rhizodeposition; these include apical regions directly behind root tips, cell junctions and zones of root elongation (e.g. root hairs). Microbes within the rhizosphere can also stimulate root exudation by altering the permeability of root cells through the production of various metabolites such as plant growth regulators, extracellular enzymes and phytotoxins (Lynch, 1990).

Colonization of the rhizosphere of a host plant by introduced bacteria is considered a key determinant of the organism's ability to positively influence plant growth (Compant *et al*, 2010). This process typically involves several steps which include recognition, adherence, colonization and growth. A basic understanding of mechanisms involved in rhizosphere colonization and factors which influence the distribution and functioning of rhizobacteria are important in order to predict how bacterial inoculants interact with plants (Compant *et al*., 2010). Parameters such as growth, chemotaxis, motility, and biofilm formation all play essential roles in this regard (Benizri *et al*., 2001; Ramey *et al*., 2004; Zhang *et al*., 2014). A wide range of biotic and abiotic parameters can affect bacterial distribution and survival and influence root colonization (Figure 1.3). Much of our current understanding of rhizosphere competence traits and the underlying genetic determinants is based on studies that have looked at the pseudomonads which are found ubiquitously within the rhizosphere (Dekkers *et al*., 1999). Rhizosphere competence studies involving bacilli are relatively scarce in comparison (Raaijmakers *et al*., 2010).

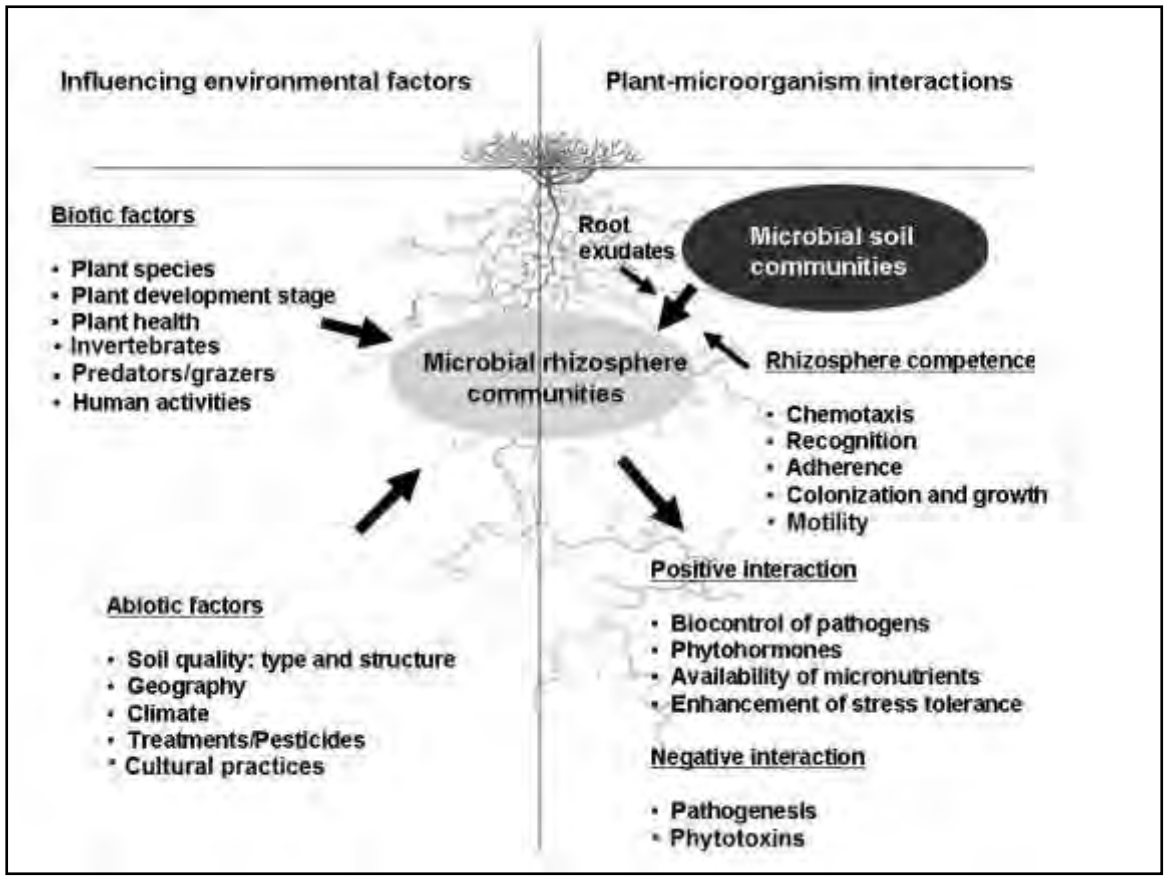


Figure 1.3 Factors influencing rhizosphere competence (Modified from: Berg and Smalla, 2009)

A range of application methods have been used to introduce microbial inoculants into their targeted niche; these include seed treatment, soil drenches and root dipping at transplant (Bashan, 1998). In instances where selected biocontrol bacteria can establish themselves and function in the same environmental niche as the plant pathogen they are to control, a single application may suffice (Weller *et al*., 1995). This classical biocontrol approach relies on the inoculants ability to maintain a permanent presence. More commonly, an augmentative biocontrol approach is used, whereby microbial inoculants impart their influence for a limited period of time and need be re-applied at frequent intervals (Cook, 1993). In such cases the organisms may be applied inundatively to temporarily raise the population to a very high level, or augmentatively to bolster or supplement existing populations of the strains that have already been introduced.

Dispersion of rhizobacteria within a growing medium and migration to the roots appears to be dependent on two main factors namely; bacterial motility and passive mechanisms such as water percolation, mass flow and root elongation (Parke, 1991). The relative significance of each of these with regard to bacterial colonization of the rhizosphere is dependent on the

prevailing conditions (e.g. pore size and available pore water) as well as the method of microbial inoculation (Benizri *et al*., 2001). Abiotic factors such as soil type, soil texture and structure, pH and temperature have all been found to influence viability, root colonization, leaching and adsorption of inoculated microbes to organic matter, root surfaces or soil particles. In the case of a *B. licheniformis* strain used as PGPR on alder seedlings, soil type was found to have a marked influence on the persistence of the inoculated strain; this was attributed to differences in soil structure and nutrient availability (Ramos *et al*. 2003). Maplestone and Campbell (1989) demonstrated that a motile strain of *B. pumilus* colonized the roots of wheat plants grown under gnotobiotic conditions in sand and population densities of 10^3 to 10^6 colony forming units (cfu) g^{-1} dry root were achieved. Colonization occurred even in the absence of watering but was hampered when the soil growing medium had an elevated clay composition.

Reva *et al*. (2004) noted that the biocontrol activity of selected *Bacillus* strains was directly related to their ability to colonize and persist on the roots of seedlings after seed inoculation. Strains of *B. amyloliquefaciens* were generally found to be more proficient in colonizing the rhizosphere than other members of the *B. subtilis* group. Studies using non-motile mutants of *B. megaterium* B153-2-2 have shown that there is a strong correlation between flagellation and the ability to colonize roots (Zheng and Sinclair, 1996; Zheng and Sinclair, 2000). Chemotaxis in response to root or seed exudates is also an important determinant (Zhang *et al*., 2014). Motile bacteria that are able to respond to chemo-attractant gradients are thought to gain a competitive advantage early within a root's development. Early colonization of the seed spermosphere is particularly important for microbial inoculants in order to establish themselves in significant numbers and have a positive influence on seed germination and root growth. Interestingly, Yaryura *et al*. (2008) noted that seed exudates derived from soybeans induced a chemotactic response in *B. amyloliquefaciens*, whereas root exudates did not. Not surprisingly, the composition, quality and quantity of plant exudates, as well as other rhizodeposits, changes over time and is influenced by parameters such as plant age, nutritional status, soil type, water stress and disease incidence (Berg and Smalla, 2009). The chemo-attractants released by plants may also be plant specific and in some instances even cultivar specific.

Once rhizobacteria have reached the root zone of a young seedling, their continued presence or further distribution is determined largely by their ability to adhere to root surfaces (Benizri *et al*., 2001). In bacilli, this has been linked to extracellular polysaccharides secretion and an ability to form biofilms (Bais *et al*., 2004; Liu *et al*., 2010). The ability of *B. amyloliquefaciens* C06 to colonize apple surfaces and form mucoid colonies on semi-solid surfaces is associated with the secretion a high molecular weight compound comprised primarily of the capsule polymer γ-polyglutamic acid (γ-PGA) (Liu *et al*., 2010). γ-PGA deficient mutants demonstrated reduced efficiency in swarming, surface adhesion and biofilm formation. Similarly, Bais *et al*. (2004) found that the biocontrol efficacy of *B. subtilis* 6051 against *Pseudomonas syringae* pv *tomato* on *Arabidopsis* roots was positively linked to its ability to colonize roots via biofilm formation, which in turn is linked to the secretion of surfactin.

Bacterial inoculants have to be able to compete with existing microbial inhabitants for available nutrients. High growth rates are also considered to be a desirable trait for successful establishment within the rhizosphere (Benizri *et al*., 2001). In this regard *Bacillus* spp. are not as prolific or widespread as the pseudomonads and Seddon *et al*. (1995) have cautioned that *Bacillus* based inoculants may be slow to actively colonize seeds and the emerging root sufficiently to deal with fast-growing plant pathogens. To overcome this they propose that biocontrol agents be used in conjunction with seed dressing fungicides which would provide an initial level of protection that allows the bacilli to establish within the rhizosphere. However, this approach contradicts the whole purpose of using biocontrol agents in the first place.

Root colonization has also been positively correlated to high inoculum densities (Pierson *et al*., 1998; Fray *et al*., 1999; Jjemba and Alexander, 1999). The formulation and application of microbial inoculants is therefore a crucial consideration when applying these products. Various methods of inoculation such as seed treatments and soil drenches have been advocated (Bashan, 1998). Szczech and Shoda (2006) reported that the ability of *B.subtilis* RB14-C to colonize plant roots and protect tomato seedlings against *Rhizoctonia solani* damping-off varied depending on the application method. Seed coating was found to be less effective than direct application of the strain to potting soil several days prior to planting. These results differ from the findings of other authors who have reported that seed

bacterization with strains of *Bacillus* spp. improved plant growth and decreased pathogen infection (Liu and Sinclair, 1993; Zheng and Sinclair, 2000).

The distribution of rhizobacteria along a root surface is not uniform and it was shown that they congregate at sites associated with elevated or enhanced root exudation, such as root hair zone, lateral root emergence sites, or at junctures between epidermal cells (Dandurand *et al*., 1997). The distribution and population size of rhizobacteria in the rhizosphere also varies greatly over time and may be related to plant age, nutritional status or prevailing conditions. A common observation is that high population densities of bacilli introduced into the rhizosphere are short lived and are usually followed by a rapid and steady decline in population density over several days or weeks (Reddy and Rahe, 1989; Liu and Sinclair, 1993; Zheng and Sinclair, 2000; Szcech and Shoda, 2006). With seed inoculation the greatest densities of rhizobacteria appear to be associated with the point of inoculation and diminished population sizes occur the further one moves from this point. This type of information becomes very useful when selecting application methods and deciding on appropriate rates and frequency of applying microbial inoculants. In many instances the early stages of seed germination and seedling development are critical periods for effective biological control to take place and hence rhizosphere colonization should be ensured during this period (Khetan, 2001).

One of the main constraints in the field of microbial ecology is the limited availability of suitable methods to track or follow the fate of specific microbes within complex environments (Fan *et al.,* 2011). Early population dynamic studies with *Bacillus* strains made use of antibiotic resistant mutants to study rhizosphere and soil colonizing ability (Thomashow *et al*., 1996; Mahaffee *et al*., 1997). Concerns regarding the ecological fitness of such mutants as well as the incidence of naturally occurring antibiotic resistant populations have detracted from the value of these methods (Mahaffee *et al*., 1997). Detection of wild-type microorganisms by monitoring specific bacterial genotypes can be done by using culture-dependent or culture-independent methods or combinations thereof [e.g most probable number PCR (MPN-PCR), colony hybridisation and fluorescent *in situ* hybridisation (FISH)] (van Elsas *et al*., 1998; Gamalero *et al*., 2003; Lynch *et al*., 2004). Felici *et al*. (2008) for example, developed a strain-specific molecular marker for tracking *B.subtilis* 101 in the rhizosphere of tomato using RAPD-PCR to obtain and develop a

sequence characterized amplified region (SCAR) marker. Using qualitative PCR and Southern blotting a detection limit of ca. 10^2 cfu g^{-1} dry wt soil was achieved.

Various molecular markers such as chromogenic (xylE, *gus*A and *lac*Z), luminescent (*lux*AB and *luc*) and green fluorescent protein (GFP) markers are available to study root colonization (Gamalero *et al*., 2003). Few of these methods have been successfully applied to *Bacillus* strains due to instability and poor expression within natural environments. Recently, Fan *et al.* (2011) were able to introduce a stable GFP marker to *B. amyloliquefaciens* FZB42 by integrating a *gfp* gene copy into the chromosome using a homologous recombination approach. Colonization studies using the gfp^+ tagged strain of *B. amyloliquefaciens* FZB42 revealed that root colonization varied with plant type and appeared to be linked to differences in the sites of exudate release. To establish which genes are involved in root colonization, Dietel *et al.* (2013) evaluated the fate of selected GFP-labelled *B. amyloliquefaciens* FZB42 mutants applied to *Arabidopsis* seedlings. An ability to colonize roots was found to be directly linked to biofilm formation and swarming motility. Mutants exhibiting impaired growth promotion abilities (*abrB-, nrfA-*) as well as those that which were deficient in the ability to form biofilms (*yusV-*) or synthesise alternative sigma factors (*sigH-, sigD-*) all exhibited diminished root colonization compared to a wild-type control.

1.5.1 Biofilm formation

Microbial colonization of the rhizosphere appears to be synonymous with biofilm formation (Ramey *et al*., 2004; Rudrappa *et al*., 2008; Bogino *et al*., 2013). Within the root zone biofilms occur as multicellular assemblies of bacteria embedded in a network of extracellular matrix material. These assemblages occur at localized regions on the rhizoplane and can vary in size from small microcolonies to large cell aggregates forming extensive biofilms. Increasingly it is recognized that this biofilm development occurs in response to various extracellular signals and involves “cross talking” or communication between plants and rhizobacteria (Dunn and Handelsman, 2002; Bais *et al*., 2006; Rudrappa *et al*., 2008; Beauregard *et al*., 2013). Root exudates are thought to be key drivers in triggering root colonization and biofilm associations but relatively little is known about how plants and rhizobacteria regulate this interaction. Various physiological processes, including the production of secondary metabolites, are known to be dependent on population density (Johnson *et al*., 2005; Barnard *et al*., 2007). Cell density-dependant quorum-sensing is

therefore thought to play an important role in regulating genes necessary for root colonization; these include nutrient recognition and uptake, stress response and the secretion of exoenzymes and/or antibiotic production. Much of the research in this area has focused on *Pseudomonas* spp. and further research focusing on *Bacillus* spp. is needed (Vlamakis *et al*., 2013).

Biofilm formation by *B. subtilis* was first described by Cohn in 1877 (Cohn, 1877). *Bacillus subtilis* has been used as a model organism to examine the molecular mechanisms involved in biofilm formation, which has been shown to be controlled by a number of integrated regulatory pathways (Lemon *et al.*, 2008; Vlamakis *et al*., 2013; Cairns *et al*., 2014). Biofilm formation by *B. subtilis* involves a switch from a unicellular motile mode to a non-motile multicellular one. When bacterial cells attach to a surface they lose their motility and begin to form long chains that adhere to each other by secreting an extracellular matrix (Branda *et al*., 2001; Branda *et al.*, 2006). The expression of genes involved in matrix secretion appears to be triggered by external signal molecules, such as surfactin, nystatin and acetic acid (Lopez *et al*., 2009a; Chen *et al*., 2015). Biofilms do not form uniform structures; as they develop and mature, genetically identical cells undergo differentiation giving rise to functionally distinct cell types (*viz*., matrix producing cells, motile cells and sporulating cells) that co-exist (Lopez *et al.*, 2009b). The spatial-temporal organization of cells is dynamic and appears to follow an ordered sequence of differentiation in response to changing conditions (Lopez and Kolter, 2010). Eventually, biofilms reach a stage of maturation whereby matrix-producing cells begin to sporulate and the biofilm disassembles. At this point biofilm cells secrete molecules such as D-amino acids and polyamines which break down the extracellular matrix allowing cells to disperse.

Extracellular matrix is primarily composed of exopolysaccharide (EPS), proteins and DNA (Branda *et al*., 2005). These extracellular molecules are shared within biofilm matrix for the common good of the biofilm community. TasA and BslA proteins are the two main structural components associated with biofilms and pellicles of *B. subtilis* strains (Branda *et al*., 2005; Kobayashi and Iwano, 2012). TasA binds thioflavin and forms amyloid-like fibres between cells in the matrix (Romero *et al*., 2010). The TasA fibres are anchored to cell walls by the protein TapA, which is also involved in the assembly of the TasA fibres (Romero *et al*.,

2009). BslA is an amphiphilic protein that confers hydrophobicity to biofilms by forming a layer on the surface of biofilm matrix (Kobayashi and Iwano, 2012).

Biofilm formation is an energetically expensive process and the genes involved in the synthesis of matrix components are under strict transcriptional control. For *B. subtilis,* a network of several regulatory pathways that initiate and control biofilm formation have been identified (Lopez and Kolter, 2010; Vlamakis *et al*., 2013; Cairns *et al*., 2014). Activation of the master transcriptional regulator Spo0A is central to biofilm initiation in response to various extracellular signals (Branda *et al*., 2001; Hamon and Lazazzera, 2001; Kearns *et al*., 2005). For example surfactin has been identified as an important signal molecule that triggers the phosphorylation of Spo0A resulting in increased expression of extracellular matrix genes (Lopez *et al*., 2009a).

Matrix production has been shown to be critical for bacterial colonization on plant root surfaces for wild-type strains of *B. subtilis* (Chen *et al*., 2013). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates was positively correlated to the presence and expression of conserved genes mediating biofilm formation. The capacity to form biofilms can therefore be considered an important requirement for the colonization of plant roots by wild-type *Bacillus* spp. and potential biocontrol agents.

1.6 Screening strategies for selecting microbial inoculants

Biocontrol screening programmes typically involve several tiers of screening and represent a major undertaking, which impacts heavily on time, space, labour and resources (Marten *et al*., 2000; Pliego *et al.,* 2011). Each level of screening involves a level of decision making that allows for the shortlisting of promising candidates that can be taken forward for further evaluation. Screening processes are inherently selective; consequently, the selection criteria used will have a major impact on the outcomes of the screening process. Rather than using purely empirical approaches, screening strategies that incorporate ecologically relevant selection criteria can greatly improve the chances of unearthing promising candidate organisms (Schisler and Slininger, 1997; Whipps *et al*., 1997). In this regard, Cook and Baker (1983) recommended that antagonists of soil-borne root pathogens should be isolated from the rhizosphere of targeted crops grown under conditions reflective of their intended use.

Specific baiting or enrichment techniques can also be used whereby potential candidate organisms are isolated from the roots of plant exposed to and/or grown in pathogen infested soil (Schisler and Slininger, 1997). An understanding of the epidemiology and biology of the targeted pathogen is also recommended for designing appropriate screening strategies (Pliego *et al*., 2011).

The amenability of an organism to bulk production and formulation has also been proposed as important selection criterion for candidate biocontrol agents (Pérez-García *et al*., 2011). Aerobic endospore-formers are particularly well suited in this regard and members of the genus *Bacillus* are considered to be good candidates for biocontrol screening. Bacilli can be isolated from soil and rhizosphere environments with relative ease and are readily cultured within a laboratory setting. Their ability to form endospores is an important feature which is advantageous for formulation and commercialization purposes. The mechanisms associated with plant growth promotion and plant disease antagonism are fairly well characterized and provide a basis from which to develop screening methods that target specific traits or markers.

The initial phase of biocontrol screening should ideally involve the assessment of large numbers of organisms isolated from varied sources and locations (Campbell, 1989, Knudsen *et al*., 1997). A hierarchical approach to screening is commonly used whereby screening methods progress from simple, high throughput procedures to those which are more complex but which yield ecologically relevant information. Preliminary screening tests are performed in order to identify isolates exhibiting a specific trait based on a particular mode of action. Traditional laboratory-based *in vitro* screening methods are often chosen for their ease of use (Pliego *et al*., 2011). However, such approaches are often far removed from the field situation and should not be relied on exclusively. Further rounds of testing and screening, which take into account important ecological considerations such as rhizosphere competence and plant-pathogen-antagonist interactions, should also be considered (Pliego *et al*., 2011). *In vivo/in planta* screening approaches play an important role in this regard.

Various *in vitro* screening approaches have been established to select for antagonistic microorganisms; these include dual-culture bioassays to detect antibiosis or hyperparasitism and assays that select for siderophore and lytic enzyme production (Chaiharn *et al*., 2009;

Pliego *et al.,* 2011; Pandya and Saraf, 2014). Screens for plant growth promotion including assays for phytohormone production, phosphorous solubilisation and nitrogen fixation have also been established (Ahmad *et al*., 2008; Lin *et al.,* 2014). *In vitro* assays that screen for chemotaxis and biofilm formation in response to plant exudates have also been developed to select for isolates exhibiting rhizosphere competence (Kamilova *et al.,* 2005; Zhang *et al*., 2014).

In planta screening approaches have been developed to select for biocontrol or plant growth promoting bacterial candidates based on *in vivo* plant growth promotion, induced systemic resistance or disease suppression assessments (Knudsen *et al*., 1997; Kloepper *et al*., 2004; Compant *et al*., 2005; Validov *et al*., 2007). *In vivo* assays are important since they can address factors such as rhizosphere colonization and niche competition, and incorporate variables that are relevant to the field situation. Bacterial isolates that possess multiple biocontrol or plant growth promotion modes of action may also be selected for, or promoted under these conditions. The incorporation of non-sterile growing media in these types of screening procedures is also recommended since isolates that effectively compete with naturally occurring microflora are selected for (Lumsden and Lewis, 1989). *In vivo* screening, therefore, provides a basis for evaluating isolates obtained from preliminary stages of screening and a means of rationalizing the selection of isolates for further evaluation. The adoption of a particular screening approach is influenced by its practicality when used at a large scale. Many of the more complex *in vivo* screening methods described in the literature are not suited to high throughput screening approaches. The selection of isolates for *in vivo* testing is therefore heavily reliant on the results of preliminary screening procedures using selection criteria which often appear to be applied arbitrarily.

A major issue associated with preliminary screening procedures is the management and handling of large numbers of test organisms. To avoid unnecessary replication the grouping of isolates with similar phenotypic or genomic characteristics is recommended so that representatives can selected for further testing; thereby saving time, money and resources (Dieckmann *et al*., 2005; Ghyselinck *et al*., 2011). This process is referred to as dereplication and several approaches involving genomic fingerprinting and/or MALDI-TOF MS typing of bacterial strains have been used previously (Marten *et al*., 2000; Ghyselinck *et al*., 2011).

Various molecular methods have been developed as tools to screen for potential biological control agents. For example, Benitez and McSpadden-Gardener (2009) developed a microbial community profiling approach involving terminal restriction fragment length polymorphism (T-RFLP) analyses to identify microbial populations associated with disease suppressive environments. This approach allows for the comparison of bacterial populations from different environments thereby facilitating the identification of novel bacterial candidates for biocontrol screening. Joshi and McSpadden-Gardener (2006) used PCR-based suppressive-subtractive hybridization (SSH) to identify genetic markers associated with commercialized biocontrol strains of *B. subtilis*, namely, GB03 and QST713. The genomes of these strains were compared with *B. subtilis* 168, a strain which exhibits no defined biocontrol ability. DNA fragments unique to the biocontrol strains were sequenced in order to identify genetic markers that could be used to establish the diversity, ecology and biocontrol potential of different *B. subtilis* strains. The characterization of genomes of *Bacillus* spp. strains exhibiting biocontrol and/or growth promotion potential have been useful for identifying genes associated with important biocontrol traits such as root colonization and antibiotic production (Chen *et al*., 2007; Chen *et al*., 2009a; Fan *et al*., 2011; Rückert *et al*., 2011); such information can be used to identify functionally important genes for biocontrol screening purposes.

PCR-based screening for genetic markers associated with antibiotic production is attractive for screening *Bacillus* spp. since it potentially offers a means of bypassing *in vitro* and *in vivo* screening methods (Giacomodonato, *et al*., 2001; Ramarathnam *et al.,* 2007; Mora *et al*., 2015). It provides information regarding the type(s) of antibiotic compound potentially produced without having to resort to biochemical characterization and has been recommended as a means of speeding up the rate of discovery of new biocontrol candidates (Hsieh *et al*., 2004; Joshi and McSpadden-Gardener, 2006; Hsieh *et al*., 2008). Genes associated with the biosynthesis of *Bacillus* antibiotics such as iturin A, bacillomycin D, fengycin, surfactin and zwittermicin A have been used (Abushady *et al*., 2005; Ramarathnam *et al.,* 2007; Hsieh *et al*., 2008; Athukorala *et al*., 2009). However, the detection of biosynthetic genes does not guarantee their expression; confirmation of antibiotic production using analytical techniques such as MALDI-TOF MS is therefore recommended.

1.7 Future prospects

Legitimate concerns have been raised regarding the efficacy and reliability of microbial biocontrol products over a range of environmental conditions (Pal and McSpadden Gardener, 2006; Pliego *et al*., 2011). Many studies have given rise to organisms showing promising biocontrol or plant growth promotion attributes in laboratory bioassays or greenhouse trials however, subsequent field trials have often failed to show consistent results and fall far below expectations; consequently, the widespread commercialization of such technology has been comparatively slow. This is attributable partly to inadequate selection criteria for microbial agents as well as a lack of understanding of gene expression under "real life" conditions. As a result, recommendations have been made suggesting that bacterial inoculants be used where a degree of environmental control or consistency can be achieved. Examples include applications on protected crops in green houses and in post-harvest infestation control (Paulitz and Bélanger, 2001; Arrebola *et al*., 2010).

Microbial inoculants do not always compare favourably to chemical products in areas such as speed of kill, range of targets, storage and ease of use. The primary benefits of biological agents over chemicals are that they are capable of reproducing, spreading, and having a lasting impact within agro-ecosystems. Rather than competing directly with markets in which effective chemical products exist, it has been suggested that alternative opportunities for biocontrol agents be sought (Lethbridge, 1989; Pérez-García *et al*., 2011). Suggestions include their application in situations where no effective chemical control is available or where resistance to chemicals is prevalent. Prohibitive chemical costs and the banning of certain chemicals have also created market opportunities for biological control products. Legislative pressure, lobbying from environmental groups and public awareness are also drivers needed to create an environment conducive for biological products to become acceptable and attractive for farmers to use. The successful implementation of biocontrol products will require farmers to change their perceptions and expectations as well as their current agronomic practices (Lethbridge, 1989). A shift from a chemical paradigm to a biological one is necessitated and the successful application of microbial inoculants will require more knowledge-intensive management.

1.7.1 Integrated Pest Management (IPM)

Jacobsen *et al*. (2004) highlighted the potential for using *Bacillus*-based biocontrol in intergrated pest management (IPM) systems to control plant disease and indicated that it is an area requiring further attention. IPM has been defined as "A sustainable approach to managing pests by combining biological, cultural, physical and chemical tools in a way that minimizes economic, health and environmental risks". Since the level of disease control meted out by biocontrol agents targeting plant pathogenic fungi is rarely equal to corresponding fungicides, integration of BCAs with other disease management tools potentially offers a means of achieving more consistent levels of disease control. Various approaches have been reviewed by Jacobsen *et al.* (2004) and include:

i. Linking biocontrol agent application with resistant plant cultivars to achieve greater and more consistent disease control;
ii. Integrating biocontrol agents with fungicides to lower fungicide rates and the number of spray applications required;
iii. Combining biocontrol agents with different modes of action to enhance efficacy, reduce variability or produce synergistic effects in disease control;
iv. Combining biocontrol agents with cultural control practices to achieve greater levels of disease control.

Microbial inoculants are usually most effective before pathogen infection has been initiated and hence combinations of biocontrol agents with fungicides have been recommended as a means of affording BCA organisms the opportunity to establish themselves within the rhizosphere (Korsten *et al*., 1997). The application of BCA mixtures with isolates exhibiting different modes of action, or isolates adapted to different environmental conditions has also been suggested as a way of improving the reliability of disease control. Examples include the combined application of a yeast, *Pichia guilermondii,* with *Bacillus mycoides* B16 used to produce a more constant control of *Botrytis cinerea* on strawberry leaves (Guetsky *et al*., 2001). Additionally, Jetiyanon *et al*. (2003) found that combinations of *B. amyloliquefaciens* IN937A with strains of *B. pumilus* yielded greater growth promotion and anthracnose disease control on cayenne pepper than with individual applications.

Disease management using cultural practices such as crop rotation, tillage, sanitation, improved drainage, plant nutrition management and the selection of favourable environments for planting have long been practiced in traditional farming systems (Cook, 1990). The integration of *Bacillus*-based biocontrol or plant growth promotion practices into such systems has not been widely reported in the literature and needs to be explored (Jacobsen *et al.,* 2004; Pérez-García *et al*., 2011). It has also been proposed that biocontrol agents can contribute to fungicide resistance management programmes. Since the mode of biocontrol action is usually different to synthesized chemicals, it has been proposed that they could be used in alternation with fungicides in order to reduce fungicide resistance developing within crops (Jacobsen *et al*., 2004).

1.8 Conclusions

Members of the genus *Bacillus* can be considered to be promising candidates for biocontrol screening. A growing body of evidence points to the positive role that *Bacillus* strains play in plant growth promotion and antagonism of plant disease. The mechanisms associated with these attributes are reasonably well characterized and, therefore, provide a basis from which to establish methods of screening bacteria for specific markers or traits. The key to uncovering promising candidates lies in implementing screening strategies that are underpinned by sound knowledge of the interactions of the biocontrol microbes with their biotic and abiotic environment. The successful screening of candidates for biocontrol applications remains a major challenge.

Candidates for biocontrol applications should, ideally, be able to antagonize or competitively exclude a targeted pathogen in a consistent and reproducible manner. In doing so, candidate isolates need to be compatible with the host plant and the environment they are introduced, demonstrate rhizosphere competence and compete with the extant microflora. A major constraint to the successful implementation of *Bacillus*-based biocontrol is the inherent variability of biotic and abiotic factors experienced in the field. Various characteristics of both the plant and the inoculated bacteria determine rhizosphere competence and the extent of root colonization. Plant-microbe interactions in turn are intricately linked to the prevailing conditions and can be influenced by a wide range of biotic and abiotic factors. Many questions still remain with regards to the ecology, biochemistry and regulation of plant-microbe interactions. For instance, the specificity of plant root colonization and the

mechanisms involved needs to be resolved. The expression of biocontrol traits within the rhizosphere also needs further investigation, as does the role and influence of plant and/or microbe signal molecules in the regulation of these traits. Further characterization of ISR pathways and the elicitors produced by biocontrol organisms is also warranted. A better understanding of the structural and functional diversity of microbial populations within the rhizosphere in relation to biocontrol/plant growth promotion activity is also required.

1.9 References

Abd-Allah, E.F., Ezzat, S.M. and Tohamy, M.R. (2007) *Bacillus subtilis* as an alternative biologically based strategy for controlling *Fusarium* wilt disease in tomato: A histological study. Phytoparasitica, 35, 474-478.

Abdelzaher, H.M.A. (2003) Biological control of root rot of cauliflower (caused by *Pythium ultimum* var. *ultimum*) using selected antagonistic rhizospheric strains of *Bacillus subtilis.* New Zealand Journal of Crop and Horticultural Science, 31, 209-220.

Abushady, H.M., Bashandy, A.S., Aziz, N.H. and Ibrahim, H.M.M. (2005) Molecular characterization of *Bacillus subtilis* surfactin producing strain and the factors affecting its production. International Journal of Agriculture and Biology, 3, 337-344.

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

Ahmed, A.S., Ezziyyani, M., Sanchez, C.P. and Candela, M.E. (2003) Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. European Journal of Plant Pathology, 109, 633-637.

Alagawadi, A. R. and Gaur, A. C. (1992) Inoculation of *Azospirillum brasiliense* and phosphate solubilizing bacteria on yield of sorghum (*Sorghum bicolor* L Moench) in dryland. Tropical Agriculture, 69, 347-350.

Araujo, F.F., Henning, A.A. and Hungria, M. (2005) Phytohormones and antibiotics produced by *Bacillus subtilis* and their effects on seed pathogenic fungi and on soybean root development. World Journal of Microbiology Biotechnology, 21, 1639-1645.

Arkhipova, T.N., Prinsen, E.A., Veselov, S.U., Martinenko, E.V., Melentiev, A.I. and Kudoyarova, G.R. (2007) Cytokinin producing bacteria enhance plant growth in drying soil. Plant and Soil, 292, 305-315.

Arrebola, E., Jacobs, R. and Korsten, L. (2009) Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. Journal of Applied Microbiology, 108, 386-395.

Arrebola, E., Sivakumar, D. and Korsten, L. (2010) Effect of volatile compounds produced by *Bacillus strains* on postharvest decay in citrus. Biological Control, 53, 122-128.

Asaka, O. and Shoda, M. (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Applied and Environmental Microbiology, 62, 4081-4085.

Ash, C., Farrow, J. A. E., Wallbanks, S. and Collins, M. D. (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. Letters in Applied Microbiology, 13, 202-206.

Athukorala, S.N.P., Fernando, W.G.D. and Rashid, K.Y. (2009) Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Canadian Journal of Microbiology, 55, 1021-1032.

Bacon, C.W., Yates, I.E., Hinton, D.M. and Meredith, F. (2001) Biological control of *Fusarium moniliforme* in maize. Environmental Health Perspectives, 109, 325-332.

Bais, H.P., Fall, R. and Vivanco, J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology, 134, 307-319.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S. and Vivanco, J.M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology, 57, 233-266.

Baker, K.F. (1987). Evolving concepts of biological control of plant pathogens. Annual Review of Phytopathology, 25, 67-85.

Barnard, A.M., Bowden, S.D., Burr, T., Coulthurst, S.J., Monson, R.E. and Salmond, G.P. (2007) Quorum sensing, virulence and secondary metabolite production in plant soft-rotting bacteria. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 362, 1165-1183.

Bashan, Y. (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnology Advances, 16, 729-770.

Baysal, O., Caliskan, M. and Yesilova, O. (2008) An inhibitory effect of a new *Bacillus subtilis* strain (EU07) against *Fusarium oxysporum* f. sp *radicis-lycopersici*. Physiological and Molecular Plant Pathology, 73, 25-32.

Beauregard, P.B., Chai, Y., Vlamakis, H., Losick, R. and Kolter, R. (2013) *Bacillus subtilis* biofilm induction by plant polysaccharides. Proceedings National Academy of Science, USA, 110, E1621-E1630.

Béchet, M., Caradeca, T., Husseina, W., Abderrahmanib, A., Cholleta, M., Leclèreb, M., Dubois, T., Lereclusc, D., Pupind, M. and Jacques, P. (2012) Structure, biosynthesis and properties of Kurstakins, nonribosomal lipopeptides from *Bacillus* spp. Applied Microbiology and Biotechnology, 95, 593-600.

Beneduzi, A., Peres, D., Vargas, L.K., Bodanese–Zanettini, M.H. and Passaglia, L.M.P. (2008) Evaluation of genetic diversity and plant growth promoting activities of nitrogen–fixing bacilli isolated from rice fields in South Brazil. Applied Soil Ecology, 4, 311-320.

Benitez, M. S. and Gardener, B. B. M. (2009) Linking sequence to function in soil bacteria: sequence-directed isolation of novel bacteria contributing to soilborne plant disease suppression. Applied and Environmental Microbiology, 75, 915-924.

Benizri, E., Baudoin, E. and Guckert, A. (2001) Root colonization by inoculated plant growth-promoting rhizobacteria. Biocontrol Science and Technology, 11, 557-574.

Berg, G. (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology, 84, 11-18.

Berg, G. and Smalla, K. (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiology Ecology, 68, 1-13.

Bettiol, W., Garibaldi, A. and Migheli, Q. (1997) *Bacillus subtilis* for the control of powdery mildew on cucumber and zucchini squash. Bragantia, 56, 281-287.

Bogino, P.C., Oliva, M.D.L.M., Sorroche, F.G. and Giordano, W. (2013) The role of bacterial biofilms and surface components in plant-bacterial associations. International Journal of Molecular Sciences, 14, 15838-15859.

Bolton, H., Frederickson, J.K. and Elliott, L.F. (1993) Microbial ecology of the rhizosphere. Soil Microbial Ecology: Applications. In: Metting, F.B. (ed.), Agricultural and Environmental Management. Marcel Dekker, New York, USA, pp. 27-63.

Borriss, R. (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents, In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses. Springer, Heidelberg, Germany, pp. 41-76.

Borriss, R. (2015) *Bacillus*, a plant beneficial bacterium, In: Lugtenberg, B (ed.), Principles of Plant-Microbe Interactions. Microbes for Sustainable Agriculture, Springer, Berlin, Germany, pp. 379-391.

Branda, S.S., Chu, F., Kearns, D.B., Losick, R. and Kolter, R. (2006) A major protein component of the *Bacillus subtilis* biofilm matrix. Molecular Microbiology, 59, 1229-1238.

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. (2001) Fruiting body formation by *Bacillus subtilis*. Proceedings of the National Academy of Sciences, 98, 11621-11626.

Branda, S.S., Vik, S., Friedman, L. and Kolter, R. (2005) Biofilms: the matrix revisited. Trends in Microbiology, 13, 20-26.

Brannen, P.M. and Kenney, D.S. (1997) Kodiak® – a successful biological-control product for suppression of soil-borne plant-pathogens of cotton. Journal of Industrial Microbiology and Biotechnology, 19, 169-171.

Brewer, M.T. and Larkin, R.P. (2005) Efficacy of several potential biocontrol organisms against *Rhizoctonia solani* on potato. Crop Protection, 24, 939-950.

Brimecombe, M.J., De Lelj, F.A. and Lynch, J.M. (2001) The Rhizosphere: the effect of root exudates on rhizosphere microbial populations. *In:* Pinton, R., Varanini, Z. and Nannipieri, P. (eds).The Rhizosphere. Biochemistry and Organic Substances at the Soil-plant Interface. Marcel Dekker, New York, USA, pp. 95-140.

Broadbent, P. and Baker, K.F. (1975) Soils suppressive to *Phytophthora cinnamomi* in eastern Australia. In: Bruehl, G.W., (ed.), Biology and Control of Soilborne Plant Pathogens. American Phytopathological Society Press, St Paul, USA , pp.152-157.

Cairns, L.S., Hobley, L. and Stanley-Wall, N.R. (2014) Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. Molecular Microbiology, 93, 587-598.

Campbell, R. (1989) Biological Control of Microbial Plant Pathogens. Cambridge University Press, New York, USA, 212 p.

Campbell, R. and Greaves, M.P. (1990) Anatomy and community structure of the rhizosphere. In: Lynch, J.M (ed.), The Rhizosphere. Wiley, Chichester, UK, pp. 11-34.

Cardon, Z.G. and Whitbeck, J.L. (eds) (2011) The Rhizosphere: an Ecological Perspective. Elsevier Academic Press, Burlington, USA, 214 p.

Carrillo, C., Teruel, J.A., Aranda, F.J. and Ortiz, A. (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochimica et Biophysica Acta, 1611, 91-97.

Cavaglieri, L., Orlando, J., Rodriguez, M.I., Chulze, S. and Etcheverry, M. (2005) Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and at the maize root level. Research in Microbiology, 156, 748-754.

Cawoy, H., Bettiol, W., Fickers, P. and Ongena, M. (2011) *Bacillus*-based biological control of plant diseases. In: Stoytcheva, M. (ed.) Pesticides in the Modern World - Pesticides Use and Management, InTech, Rijeka, Croatia, pp. 273-302.

Cawoy, H., Mariutto, M., Henry, G., Fisher, C., Vasilyeva, N., Thonart, P., Dommes, J. and Ongena, M. (2014) Plant defence stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. Molecular Plant-Microbe Interactions, 27, 87-100.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens.* Microbial Biotechnology, 8, 281–295. doi: 10.1111/1751-7915.12238.

Chaiharn, M., Chunhaleuchanon, S. and Lumyong, S. (2009) Screening siderophore producing bacteria as potential biological control agent for fungal rice pathogens in Thailand. World Journal of Microbiology and Biotechnology, 25, 1919-1928.

Chan, Y.K., McCormick, W.A. and Seifert, K.A. (2003) Characterization of an antifungal soil bacterium and its antagonistic activities against *Fusarium* species. Canadian Journal of Microbiology, 49, 253-262.

Chandler, D., Bailey, A.S., Tatchell, G.M., Davidson, G., Greaves, J. and Grant, W.P. (2011) The development, regulation and use of biopesticides for integrated pest management. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 366, 1987-1998.

Chaurasia, B., Pandey, A., Palni, L.S., Trivedi, P., Kumar, B. and Colvin, N. (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi *in vitro*. Microbiological Research 160, 75-81.

Chebotar, V.K., Makarova, N.M., Shaposhnikov, A.I. and Kravchenko, L.V. (2009) Antifungal and phytostimulating characteristics of *Bacillus subtilis* Ch-13 rhizospheric strain, producer of bioprepations. Applied Biochemistry and Microbiology, 45, 419-423.

Chen, Y., Gozzi, K., Yan, F. and Chai, Y. (2015) Acetic acid acts as a volatile signal to stimulate bacterial biofilm formation. MBio, 6, e00392-15. doi:10.1128/mBio.00392-15.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O. and Junge, H. (2007) Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nature Biotechnology, 25, 1007-1014.

Chen, X.H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Sussmuth, R., Piel, J. and Borriss,R. (2009a) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. Journal of Biotechnology, 140, 27-37.

Chen, X.H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S. and Borriss, R. (2009b) Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. Journal of Biotechnology, 140, 38-44.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R. and Guo, J.H. (2013) Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environmental Microbiology, 15, 848–864.

Cheng, W. and Gershenson, A. (2011) Carbon fluxes in the rhizosphere. In: Cardon, Z. G. and Whitbeck, J. L. (eds). The Rhizosphere: an Ecological Perspective. Academic Press, Burlington, USA, pp. 31-52.

Choudhary, D.K. and Johri, B.N. (2009). Interactions of *Bacillus* spp. and plants – with special reference to induced systemic resistance (ISR). Microbiological Research, 164, 493-513.

Chowdhury, S.P., Hartmann, A., Gao, X. and Borriss, R. (2015) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42: a review. Frontiers in Microbiology, 6, 780. doi:10.3389/fmicb.2015.00780.

Cohan, F.M. (2002) What are bacterial species? Annual Review Microbiology, 56, 457-87.

Cohn, F. (1877) Untersuchungen über bacterien. IV. Beiträge zur biologie der Bacillen. Beiträge zur Biologie der Pflanzen, 7, 249–276.

Collins, D.P. and Jacobsen, B.J. (2003) Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet *Cercospora* leaf spot. Biological Control, 26, 153-161.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, C. and Barka, E. A. (2005) Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Applied and Environmental Microbiology, 71, 1685-1693.

Compant, S., Clément, C. and Sessitsch, A. (2010) Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, 42, 669-678.

Cook, R. J. (1990) Twenty-five years of progress towards biological control. In: Hornby, D. (ed), Biological Control of Soil-Borne Plant Pathogens. CAB International, Wallingford, UK, pp. 1-14.

Cook, R. J. and Baker, K. F. (1983). The nature and practice of biological control of plant pathogens. American Phytopathological Society Press, St Paul, USA, 539 p.

Correa, O.S., Montecchina, M.S., Berti, M.F., Fernandez Ferrari, M.C., Pucheu, N.L., Kerber, N.L. and Garcia, A.F. (2009) *Bacillus amyloliquefaciens* BNM122 a potential microbial biocontrol agent applied on soybean seeds, cause a minor impact on rhizosphere and soil microbial communities. Applied Soil Ecology, 41, 185-194.

Corrêa, E.B., Bettiol, W. and Sutton, J.C. (2010) Controle biológico da podridão radicular (*Pythium aphanidermatum*) e promoção de crescimento por *Pseudomonas chlororaphis* 63-28 e *Bacillus subtilis* GB03 em alface hidropônica. Summa Phytopathologica, 36, 275-281.

Cosby, W.M., Vollenbroich, D., Lee, O.H. and Zuber, P. (1998) Altered *srf* expression in *Bacillus subtilis* resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. Journal of Bacteriology. 180, 1438-1445.

Curl, E. A. and Truelove, B. (1986) The Rhizosphere. Springer-Verlag. Berlin, Germany, 288 p.

Dandurand, L.M., Schotzko, D.J. and Knudsen, G.R. (1997) Spatial patterns of rhizoplane populations of *Pseudomonas fluorescens*. Applied and Environmental Microbiology. 63, 3211-3217.

Danhorn, T. and Fuqua, C. (2007) Biofilm formation by plant-associated bacteria. Annual Reviews Microbiology, 61, 401-422.

Dekkers, L.C., Phoelich, C.C. and Lugtenberg, B.J.J. (1999) Bacterial traits and genes involved in rhizosphere colonization. In: Bell, C.R., Brylinskym, M. and Johnson-Green, P. (eds) Proceedings of the 8th International Symposium on Microbial Ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Deleu, M., Paquot, M. and Nylander, T. (2005) Fengycin interaction with lipid monolayers at the air–aqueous interface—implications for the effect of fengycin on biological membranes. Journal of Colloid and Interface Science, 283, 358-365.

Demoz, B.T. and Korsten, L. (2006) *Bacillus subtilis* attachment, colonization, and survival on avocado flowers and its mode of action on stem-end rot pathogens. Biological Control, 37, 68-74.

Deravel, J., Krier, F. and Jacques, P. (2014) Les biopesticides, compléments et alternatives aux produits phytosanitaires chimiques (synthèse bibliographique)/ Biopesticides, a complementary and alternative approach to the use of agrochemicals. A review. Biotechnologie, Agronomie, Société et Environnement, 18, 220-232.

Dieckmann, R., Graeber, I., Kaesler, I., Szewzyk, U. and Döhren, H. (2005) Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by Intact-Cell-MALDI-TOF mass spectrometry (ICM-MS). Applied Microbiology and Biotechnology 67, 539-548.

Dietel, K., Budiharjo, A. and Borriss, R. (2013) Bacterial traits involved in colonization of *Arabidopsis thaliana* roots by *Bacillus amyloliquefaciens* FZB42. The Plant Pathology Journal, 29, 59-66.

Doornbos, R. F., van Loon, L. C. and Bakker, P. A. (2012) Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agronomy for Sustainable Development, 32, 227-243.

Duitman, E.H., Wyczawski, D., Boven, L.G., Venema, G., Kuipers, O.P. and Hamoen, L.W. (2007) Novel methods for genetic transformation of natural *Bacillus subtilis* isolates used to study the regulation of the mycosubtilin and surfactin synthetases. Applied and Environmental Microbiology, 73, 3490-3496.

Dunn, A.K. and Handelsman, J. (2002) Toward an understanding of microbial communities through analysis of communication networks. Antonie van Leewenhoek, 81, 565-574.

Emmert, E.A.B. and Handelsman, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiology Letters, 171, 1-9.

European Food Safety Authority (EFSA) (2011) Technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA Journal, 9, 2445-2458.

Fajardo, A. and Martínez, J. L. (2008) Antibiotics as signals that trigger specific bacterial responses. Current Opinion in Microbiology, 11, 161-167.

Fan, B., Chen, X.H., Budiharjo, A., Bleiss, W., Vater, J. and Borriss, R. (2011) Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. Journal of Biotechnology, 151, 303-311.

Fan, B., Borriss, R., Bleiss, W. and Wu, X. (2012) Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. The Journal of Microbiology, 50, 38-44.

Felici, C., Vettori, L., Toffanin, A. and Nuti, M. (2008) Development of a strain-specific genomic marker for monitoring a *Bacillus subtilis* biocontrol strain in the rhizosphere of tomato. FEMS Microbiology Ecology, 65, 289-298.

Figueiredo, M.V.B., Martinez, C.R., Burity, H.A. and Chanway, C.P. (2008) Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). World Journal of Microbiology and Biotechnology, 24, 1187-1193.

Finking, R. and Marahiel, M.A. (2004) Biosynthesis of non-ribosomal peptides. Annual Review of Microbiology, 58, 453-488.

Francis, I., Holsters, M. and Vereecke, D. (2010) The Gram-positive side of plant-microbe interactions. Environmental Microbiology, 12, 1-12.

Fravel, D. R. (2005) Commercialization and implementation of biocontrol. Annual Review of Phytopathology, 43, 337-359.

Fray, R.G., Throup, J.P., Daykin, M., Wallace, A., Williams, P., Stewart, G.S. and Grierson, D. (1999) Plants genetically modified to produce N-acylhomoserine lactones communicate with bacteria. Nature Biotechnology, 17, 1017-1020.

Fritze, D. (2004) Taxonomy of the genus *Bacillus* and related genera: The aerobic endospore-forming bacteria. Phytopathology, 94, 1245-1248.

Fu, G., Huang, S.L., Ye, Y.F., Wu, Y.G., Cen, Z.L. and Lin, S.H. (2010) Characterization of a bacterial biocontrol strain B106 and its efficacy in controlling banana leaf spot and post-harvest anthracnose diseases. Biological Control, 55, 1-10.

Gajbhiye, A., Rai, A.R., Meshram, S.U. and Dongre, A.B. (2010) Isolation, evaluation and characterization of *Bacillus subtilis* from cotton rhizospheric soil with biocontrol activity against *Fusarium oxysporum*. World Journal of Microbiology and Biotechnology, 26, 1187-1194.

Gamalero, E., Lingua, G., Berta, G. and Lemanceau, P. (2003) Methods for studying root colonization by introduced beneficial bacteria. Agronomie-Sciences des Productions Vegetales et de l'Environnement, 23, 407-418.

Garbeva, P., Van Veen, J. A. and Van Elsas, J. D. (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microbial Ecology, 45, 302-316.

Garbeva, P., Van Veen, J.A. and Van Elsas, J.D. (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review Phytopathology, 42, 243-270.

Garrity, G. M., Bell, J. A. and Lilburn, T. G. (2003) Taxonomic Outline of the Prokaryotes: Bergey's Manual of Systematic Bacteriology. doi No. http://dx.doi.org/ 10.1007/ bergeysoutline. Published online by Springer-Verlag, New York.

Ghyselinck, J., Van Hoorde, K., B., H., Heylen, K. and De Vos, P. (2011) Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. Journal of Microbiological Methods 86, 327–336.

Giacomodonato, M.N., Pettinari, M.J., Souto, G.I., Méndez, B.S. and López, N.I. (2001) A PCR-based method for the screening of bacterial strains with antifungal activity in suppressive soybean rhizosphere. World Journal of Microbiology Biotechnology, 17, 51-55.

Glass, D.J. and J. Lindemann. (1992) Biotechnology in Agriculture: The Next Decade. Decision Resources Inc., Burlington, USA, 176 p.

Granum, P.E. and Lund, T. (1997) *Bacillus cereus* enterotoxins. FEMS Microbiology Letters, 157, 223-228.

Graumann, P. (ed.) (2007) *Bacillus*: Cellular and Molecular Biology. Caister Academic Press, Hethersett, UK, 398 p.

Grosch, R., Junge, H., Krebs, B. and Bochow, H. (1999) Use of *Bacillus subtilis* as a biocontrol agent. III. Influence of *Bacillus subtilis* on fungal root diseases and on yield in soil-less culture. Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection, 106, 568-580.

Guetsky, R., Shtienberg, D., Elad, Y. and Dinoor, A. (2001) Combining biocontrol agents to reduce the variability of biological control. Phytopathology, 91, 621-627.

Haas, D. and Défago, G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology, 3, 307-319.

Hamoen, L.W., Venema, G. and Kuipers, O.P. (2003) Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology, 149, 9-17.

Hamon, M.A. and Lazazzera, B.A. (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. Molecular Microbiology, 42, 1199-1209.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. and Lorito, M. (2004) *Trichoderma* species opportunistic, avirulent plant symbionts. Nature Reviews Microbiology, 2, 43-56.

Harwood, C. R. and Cutting, S. M. (1990) Molecular Biological Methods for *Bacillus*. Wiley, New York, USA, 618 p.

Hernandez-Castillo, F., Aquirre-Aguirre, A., Lira-Saldivar, R., Guerrero-Rodriguez, E. and Gallegos-Morales, G. (2006) Biological efficiency of organic biological and chemical products against *Alternaria dauci* Kuhn and its effects on carrot crop. Phyton-International Journal of Experimental Botany, 75, 91-101.

Hoitink, H.A.J. and Fahy, P.C. (1986) Basis for the control of soil-borne plant pathogens with composts. Annual Review of Phytopathology, 24, 93–114.

Hsieh, F.C., Li, M.C., Lin, T.C. and Kao, S.S. (2004) Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Current Microbiology, 49, 186-191.

Hsieh, F.C., Lin, T.C., Meng, M. and Kao, S.S. (2008) Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide Iturin A. Current Microbiology, 56, 1-5.

Huang, X., Zhang, N., Yong, X., Yang, X. and Shen, Q. (2012) Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiological Research, 167, 135-143.

Huber, D. M. and Schneider, R. W. (1982) Description and occurrence of suppressive soils. In: Schneider, R.W. (ed.) Suppressive Soils and Plant Disease. APS Press, St. Paul, USA, pp. 1-8.

Idris, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R. and Bochow, H. (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth promoting effect, Microbiology, 148, 2097-109.

Idris, E.E.S., Iglesias, D.J., Talon, M. and Borriss, R. (2007) Tryptophan-dependent production of indole-3-actic acid (IAA) affects level of plant growth-promotion by *Bacillus amyloliquefaciens* FZB42. Molecular Plant-Microbe Interactions, 20, 619-26.

Idris, H.A., Labuschagne, N. and Korsten, L. (2008) Suppression of *Pythium ultimum* root rot of sorghum by rhizobacterial isolates from Ethiopia and South Africa. Biological Control, 45, 72-84.

Jacobsen, B. J., Zidack, N. K. and Larson, B. J. (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: Plant diseases. Phytopathology, 94, 1272-1275.

Jacques, P. (2011) Surfactin and other lipopeptides from *Bacillus* spp. In: Soberσn-Chàvez, G. (ed.) Biosurfactants. Springer-Verlag, Berlin Heidelberg, Germany, pp. 57-91.

Jetiyanon, K., Fowler, W.D. and Kloepper, J.W. (2003) Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Disease, 87, 1390-1394.

Jjemba, P. K. and Alexander, M. (1999) Possible determinants of rhizosphere competence of bacteria. Soil Biology and Biochemistry, 31, 623-632.

Johnson, B., Anker, H. and Meleney, F. (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science, 102, 376-377.

Johnson, M.R., Montero, C.I., Conners, S.B., Shockley, K.R., Bridger, S.L. and Kelly, R.M. (2005) Population density-dependent regulation of exopolysaccharide formation in the hyperthermophilic bacterium *Thermotoga maritime*. Molecular Microbiology, 55, 664-674.

Joshi, R. and McSpadden-Gardener, B.B. (2006) Identification of genes associated with pathogen inhibition in different strains of *B. subtilis*. Phytopathology, 95,145-154.

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthelemy, J.P., Thonart, P. and Ongena, M. (2009) Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Molecular Plant Microbe Interactions, 22, 456-468.

Kamilova, F., Validov, S., Azarova, T., Mulders, I. and Lugtenberg, B.J.J. (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environmental Microbiology, 7, 1809-1817.

Katz, E. and Demain, A.L. (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. Bacteriology Reviews, 41, 449-474.

Kearns, D.B., Chu, F., Rudner, R. and Losick, R. (2004) Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Molecular Microbiology, 52, 357-369.

Kearns, D.B., Chu, F., Branda, S.S., Kolter, R. and Losick, R. (2005) A master regulator for biofilm formation by *Bacillus subtilis*. Molecular Microbiology, 55, 739-749.

Kennedy, A.C., (1999) The rhizosphere and spermosphere. In: Sylvia, D.M., Fuhrmann, J.J., Hartel, P.G. and Zuberer, D.A. (eds), Principles and Applications of Soil Microbiology. Prentice Hall, Upper Saddle River, USA, pp. 389-407.

Khetan, S. K. (2001). Microbial pest control. In: Khetan, S.K. (ed), Microbial Pest Control, Marcel Dekker, New York, USA, pp. 3-42.

Kim, P.I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. and Chi, Y-T (2004) Isolation and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. Journal of Applied Microbiology, 97, 942-949.

Kinsella, K., Schulthess, C.P., Morris, T.F. and Stuart, J.D. (2009) Rapid quantification of *Bacillus subtilis* antibiotics in the rhizosphere. Soil Biology and Biochemistry, 41, 374-379.

Kita, N., Ohya, T., Uekusa, H., Nomura, K., Manago, M. and Shoda, M. (2005) Biological control of damping-off of tomato seedlings and cucumber *Phomopsis* root rot by *Bacillus subtilis* RB14-C. JARQ-Japan Agricultural Research Quarterly, 39, 109-114.

Kleinkauf, H. and von Döhren, H. (1990) Non-ribosomal biosynthesis of peptide antibiotics. European Journal of Biochemistry, 192, 1-15.

Kloepper, J.W. (1993) Plant growth-promoting rhizobacteria as biological control agents. In: Metting B (ed) Soil Microbial Technologies. Marcel Dekker, New York, USA, pp. 255-274.

Kloepper, J.W., Ryu, C.M. and Zhang, S.A. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp.. Phytopathology, 94, 1259-1266.

Kloepper, J.W. and Schroth, M.N. (1978) Plant growth promoting rhizobacteria on radishes, In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Angers, France, pp. 879-882.

Knudsen, I.M.B., Hockenhull, J., Funck-Jensen, D., Gerhardson, B., Hökeberg, M., Tahvonen, R., Teperi, E., Sundheim, L. and Henriksen, B. (1997) Selection of biological control agents for controlling soil and seed-borne diseases in the field. European Journal of Plant Pathology, 103, 775-784.

Kobayashi, K. and Iwano, M. (2012) BslA (YuaB) forms a hydrophobic layer on the surface of Bacillus subtilis biofilms. Molecular Microbiology, 85, 51-66.

Kondoh, M., Hirai, M. and Shoda, M. (2001) Integrated biological and chemical control of damping-off caused by *Rhizoctonia solani* using *Bacillus subtilis* RB14-C and Flutolanil. Journal of Bioscience and Bioengineering, 91, 173-177.

Korsten, L., de Villiers, E.E., Wehner, F.C. and Kotze, J.M. (1997) Field sprays of *Bacillus subtilis* and fungicides for control of preharvest fruit diseases of avocado in South Africa. Plant Disease, 81, 455-459.

Koumoutsi, A, Chen, X-H, Vater, J. and Borriss, R. (2007) DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillus amyloliquefaciens* Strain FZB42, Applied and Environmental Microbiology, 73, 6953-6964.

Kumar, A. and Johri, B.N. (2012) Antimicrobial lipopeptides of *Bacillus*: natural weapons for biocontrol of plant pathogens. In: Satyanarayana, T., Johri, B. N., and Prakash, A. (eds), Microorganisms in Sustainable Agriculture and Biotechnology, Springer, Netherlands, pp. 91-111.

Kumar, A., Prakash, A. and Johri, B.N. (2011) *Bacillus* as PGPR in crop ecosystem, In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses, Springer-Verlag, Berlin, Germany, pp. 37-56.

Lancini, G. and Lorenzetti, R. (1993) Biotechnology of Antibiotics and Other Bioactive Microbial Metabolites. Plenum Press, New York, 244 p.

Lebuhn, M., Heulin, T. and Hartmann, A. (1997) Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. FEMS Microbiology Ecology, 22, 325-334.

Leclere, V., Bechet, M., Adam, A., Guez, J.S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M. and Jacques, P. (2005) Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. Applied and Environmental Microbiology, 71, 4577-4584.

Lee, K.J., Kamala-Kannan, S., Sub, H.S., Seong, C.K. and Lee, G.W. (2008) Biological control of *Phytophthora* blight in red pepper (*Capsicum annuum* L.) using *Bacillus subtilis*. World Journal of Microbiology and Biotechnology, 24, 1139-1145.

Leelasuphakul, W., Sivanunsakul, P. and Phongpaichit, S. (2006) Purification, characterization and synergistic activity of ß 1-3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. Enzyme and Microbial Technology, 28, 990-997.

Leelasuphakul, W., Hemmanee, P. and Chuenchitt, S. (2008) Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biology and Technology, 48, 113-121.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D. and Harbour, A. (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology, 78, 97-108.

Lemon, K.P., Earl, A.M., Vlamakis, H.C., Aguilar, C. and Kolter, R. (2008) Biofilm development with an emphasis on *Bacillus subtilis*. Current Topics in Microbiology and Immunology, 322, 1-16.

Lethbridge, G. (1989) An industrial view of microbial inoculants for crop plants. In: Campbell, R. and Macdonald, R.M. (eds.) Microbial Inoculation of Crop Plants, Special publication of the Society of General Microbiology, vol. 25, IRL Press, Oxford, New York, USA, pp. 11-28.

Lin, Y., Du, D., Si, C., Zhao, Q., Li, Z. and Li, P. (2014) Potential biocontrol *Bacillus* sp. strains isolated by an improved method from vinegar waste compost exhibit antibiosis against fungal pathogens and promote growth of cucumbers. Biological Control, 71, 7-15.

Liu, J., He, D., Li, X.Z., Gao, S., Wu, H., Liu, W. and Zhou, T. (2010) γ-Polyglutamic acid (γ-PGA) produced by *Bacillus amyloliquefaciens* C06 promoting its colonization on fruit surface. International Journal of Food Microbiology, 142, 190-197.

Liu, B., Qiao, H.P., Huang, L.L., Buchenauer, H., Han, Q.M., Kang, Z.S. and Gong, Y.F. (2009) Biological control of take-all in wheat by endophytic *Bacillus subtilis* E1R-j and potential mode of action. Biological Control, 49, 277-285.

Liu, Z.L. and Sinclair, J.B. (1993) Colonization of soybean roots by *Bacillus megaterium* B 153-2-2. Soil Biology and Biochemistry, 25, 849-855.

Logan, N.A. and De Vos, P. (2009) Genus *Bacillus* Cohn 1872. In: De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B (eds). Bergey's Manual of Systematic Bacteriology, 2nd Edition, Volume 3. Springer, New York, USA, pp. 21-127.

Lopez, D., Fischbach, M.A., Chu, F., Losick, R. and Kolter, R. (2009a) Structurally diverse natural products that cause potassium leakage trigger multicellularity in *Bacillus subtilis*. Proceedings National Academy of Sciences, USA, 106, 280-285.

Lopez, D. and Kolter, R. (2010) Extracellular signals that define distinct and coexisting cell fates in Bacillus subtilis. FEMS Microbiology Reviews, 34, 134-149.

Lopez, D., Vlamakis, H., and Kolter, R. (2009b) Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiology Reviews, 33, 152-163.

Lugtenberg, B. and Kamilova, F. (2009) Plant growth-promoting rhizobacteria. Annual Review of Microbiology, 63, 541-556.

Lumsden, R.D. and Lewis, J.A., (1989) Selection, production, formulation and commercial use of plant disease biocontrol fungi: problems and progress. In: Whipps, J.M. and Lumsden, R.D. (eds), Biotechnology of Fungi for Improving Plant Growth, Cambridge University Press, Cambridge, UK, pp.171-190.

Lynch J.M. (1990) The Rhizosphere, John Wiley and Sons Ltd, Chichester, UK, 458 p.

Lynch, J.M., Benedetti, A., Insam, H., Nuti, M.P., Smalla, K., Torsvik, V. and Nannipieri, P. (2004) Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. Biology and Fertility of Soils, 40, 363-385.

Magnet-Dana, R. and Peypoux, F. (1994) Iturins, a special class of pore forming lipopeptides: biological and physicochemical properties. Toxicology, 87, 151-174.

Magnet-Dana, R., Thimon, L., Peypoux, F. and Ptak, M. (1992) Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochemie, 74, 1047-1051.

Mahaffee, W.F., Bauske, E.M., van Vuurde, J.W., Van der Wolf, J.M., Van Den Brink, M. and Kloepper, J.W. (1997) Comparative analysis of antibiotic resistance, immunofluorescent colony staining, and a transgenic marker (bioluminescence) for monitoring the environmental fate of rhizobacterium. Applied and Environmental Microbiology, 63, 1617-1622.

Manjula, K. and Podile, A.R. (2005) Production of fungal cell wall degrading enzymes by a biocontrol strain of *Bacillus subtilis* AF 1. Indian Journal of Experimental Biology, 43, 892-896.

Mantecon, J.D. (2008) Efficacy of chemical and biological strategies for controlling the soybean brown spot (*Septoria glycines*). Ciencia E Investigacion Agraria, 35, 211-214.

Maplestone, P.A. and Campbell, R. (1989) Colonization of roots of wheat seedlings by bacilli proposed as biocontrol agents against take-all. Soil Biology and Biochemistry, 21, 543-550.

Marschner, P., Yang, C. H., Lieberei, R. and Crowley, D. E. (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biology and Biochemistry, 33, 1437-1445.

Marten, P., Smalla, K. and Berg, G. (2000) Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. Journal of Applied Microbiology 2000, 89, 463-471.

Mathre, D.E., Cook, R.J. and Callan, N.W. (1999) From discovery to use. Traversing the world of commercializing biocontrol agents for plant disease control. Plant Disease, 83, 972-983.

Maughan, H. and Van der Auwera, G. (2011) *Bacillus* taxonomy in the genomic era finds phenotypes to be essential though often misleading. Infection, Genetics and Evolution, 11, 789-797.

McCully, M.E. (1999) Roots in soil: unearthing the complexities of roots and their rhizospheres. Annual Review of Plant Biology, 50, 695-718.

McSpadden-Gardener, B.B. (2004) Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathology, 94, 1252-1258.

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

Mendes, R., Garbeva, P. and Raaijmakers, J.M. (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiology Reviews, 37, 634-663.

Milner, J.L., Silo-Suh, L., Lee, J.C., He, H., Clardy, J. and Handelsman J (1996) Production of kanosamine by *Bacillus cereus* UW85. Applied and Environmental Microbiology, 62, 3061-3065.

Mizumoto, S., Hirai, M. and Shoda, M. (2007) Enhanced iturin A production by *Bacillus subtilis* and its effect on suppression of the plant pathogen *Rhizoctonia solani*. Applied Microbiology and Biotechnology, 75, 1267-1274.

Montealegre, J.R., Reyes, R., Perez, L.M., Herrera, R., Silva, P. and Besoain, X. (2003) Selection of bioantagonistic bacteria to be used in biological control of *Rhizoctonia solani* in tomato. Electronic Journal of Biotechnology, 6, 115-127.

Mora, I., Cabrefiga, J. and Montesinos, E. (2015) Cyclic lipopeptide biosynthetic genes and products, and inhibitory activity of plant-associated Bacillus against phytopathogenic bacteria. PloS one, 10, e0127738. doi:10.1371/journal.pone.0127738.

Morgan, J.A.W. and Whipps, J.M. (2001) Methodological approaches to the study of rhizosphere carbon flow and microbial population dynamics. In: Pinton, R., Varanini, Z. and Nannipieri, P. (eds) The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface. Marcel Dekker, New York, USA. pp. 373-409.

Müller, S., Strack, S.N., Hoefler, B.C., Straight, P.D., Kearns, D.B. and Kirby, J.R. (2014) Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. Applied and Environmental Microbiology, 80, 5603-5610.

Munimbazi, C. and Bullerman, L.B. (1998) Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. Journal of Applied Microbiology, 84, 959-968.

Nagorska, K., Bikowski, M. and Obuchowki, M. (2007) Multicellular behaviour and production of a wide vaiety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochimica Polonica, 54, 495-508.

Nakamura, L. K., Roberts, M. S. and Cohan, F. M. (1999) Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. *nov*. and *Bacillus subtilis* subsp. *spizizenii* subsp. *nov*. International Journal of Systematic Bacteriology, 49, 1211-1215.

Nakkeeran, S., Kavitha, K., Chandrasekar, G., Renukadevi, P. and Fernando, W.G.D. (2006) Induction of plant defence compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping-off of hot pepper caused by *Pythium aphanidermatum.* Biocontrol Science and Technology, 16, 403-416.

Nesci, A., Bluma, R. and Etcheverry, M. (2005) *In vitro* selection of maize rhizobacteria to study potential biological control of *Aspergillus* section *Flavi* and aflatoxin production. European Journal of Plant Pathology, 113, 159-171.

Nihorimbere, V., Ongena, M., Cawoy, H., Brostaux, Y., Kakana, P., Jourdan, E. and Thonart, P. (2010) Beneficial effects of *Bacillus subtilis* on field-grown tomato in Burundi: Reduction of local *Fusarium* disease and growth promotion. African Journal of Microbiology Research, 4, 1135-1142.

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonart, P. (2005) *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Applied Microbiology and Biotechnology, 67, 692-698.

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology, 16, 115-125.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B. and Thonart, P. (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environmental Microbiology, 9, 1084-1090.

Pal, K.K. and McSpadden Gardener, B. (2006) Biological control of plant pathogens. The Plant Health Instructor, doi: 10.1094/PHI-A-2006-1117-02.

Pandya, U. and Saraf, M. (2014) *In Vitro* evaluation of PGPR strains for their biocontrol potential against fungal pathogens. In: Kharwar, R.N., Upadhyay, R.S., Dubey, N.K. and Raghuwanshi, R. (eds) Microbial Diversity and Biotechnology in Food Security, Springer India. pp. 293-305.

Park, K.S. and Kloepper, J.W. (2000) Activation of PR-1 a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. Biological Control, 18, 2-9.

Parke, J.L. (1991) Root colonization by indigenous and introduced microorganisms. In: Keister, D.L. and Cregan, P.B. (eds). The Rhizosphere and Plant Growth. Springer, Netherlands. pp 33-42.

Patel, V. J., Tendulkar, S. R. and Chattoo, B. B. (2004) Bioprocess development for the production of an antifungal molecule by *Bacillus licheniformis* BC98. Journal of Bioscience and Bioengineering, 98, 231-235.

Paulitz, T.C. and Bélanger, R.R. (2001) Biological control in greenhouse systems. Annual Review Phytopathology, 39,103-133.

Pérez-García, C., Romero, D., Zerioub, H. and de Vicente, S. (2011) Biological control of phytopathogenic fungi by aerobic endospore-formers. In: Logan, N.A. and De Vos, P. (eds) Endospore-forming Soil Bacteria, Soil Biology Volume 27, Springer-Verlag, Berlin Heidelberg, Germany, pp.157-180.

Petersen, D. J., Srinivasan, M. and Chanway, C. P. (1996) *Bacillus polymyxa* stimulates increased *Rhizobium etli* populations and nodulation when co-resident in the rhizosphere of *Phaseolus vulgaris*. FEMS Microbiology letters, 142, 271-276.

Peypoux, F., Bonmatin, J.M. and Wallach, J. (1999) Recent trends in the biochemistry of surfactin. Applied Microbiology and Biotechnology, 51, 553-563.

Philippot, L., Raaijmakers, J. M., Lemanceau, P. and van der Putten, W. H. (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 11, 789-799.

Pierson, E.A., Wood, D.W., Cannon, J.A., Blachere, F.M. and Pierson, L.S. (1998) Interpopulation signaling via N-acyl-homoserine lactones among bacteria in the wheat rhizosphere. Molecular Plant-Microbe Interactions, 11, 1078-1084.

Pieterse, C.M.J., van Pelt, J.A., van Wees, S.C.M., Ton, J., Léon-Kloosterziel, K., Keurentjes, J.J.B., Verhagen, B.W.M., Knoester, M., van der Sluis, I., Bakker, P.A.H.M. and van Loon L.C. (2001) Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. European Journal of Plant Pathology, 107, 51-61.

Pieterse, C.M., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C. and Bakker, P.A. (2014) Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology, 52, 347-375.

Pinton, R., Varanini, Z. and Nannipieri, P. (2001) The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface. Marcel Dekker, New York, USA, 424 p.

Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F.M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil, 340, 505-520.

Podile, A.R. and Prakash, A.P. (1996) Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. Canadian Journal of Microbiology, 42, 533-538.

Priest F (1993) Systematics and Ecology of *Bacillus: Bacillus subtilis* and other Gram-positive Bacteria, Biochemistry, Physiology, and Molecular Genetics. American Society for Microbiology, Washington DC, USA, pp. 3-16.

Priest, F.G. and Grigorova, R. (1990) 18 methods for studying the ecology of endospore-forming bacteria. Methods in Microbiology, 22, 565-591.

Raaijmakers, J.M., De Bruijn, I., Nybroe, O. and Ongena, M. (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiology Reviews, 34, 1037-1062.

Raaijmakers, J.M. and Mazzola, M. (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annual Review of Phytopathology, 50, 403-424.

Raaijmakers, J.M., Vlami, M. and de Souza, J.T. (2002) Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek, 81, 537-547.

Rajendran, G., Sing, F., Desai, A.J. and Archana, G. (2008) Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. Bioresource Technology, 99, 4544-4550.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W.G.D., Xuewen, G. and de Kievit, T. (2007) Molecular and biochemical detection of fengycin- and bacillomycin D- producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Canadian Journal of Microbiology, 53, 901-911.

Ramey, B.E., Koutsoudis, M., von Bodman, S.B. and Fuqua, C. (2004) Biofilm formation in plant–microbe associations. Current Opinion in Microbiology, 7, 602-609.

Ramos, B., García, J.A.L., Probanza, A., Barrientos, M.L. and Mañero, F.J.G. (2003) Alterations in the rhizobacterial community associated with European alder growth when inoculated with PGPR strain *Bacillus licheniformis*. Environmental and Experimental Botany, 49, 61-68.

Reddy, M.S. and Rahe, J.E. (1989) Growth effects associated with seed bacterization not correlated with populations of *Bacillus subtilis* inoculant in onion seedling rhizospheres. Soil Biology and Biochemistry, 21, 373-378.

Reddy, K.R.N., Reddy, C.S. and Muralidharan, K. (2009) Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. Food Control, 20, 173-178.

Reva, O.N., Dixelius, C., Meijer, J. and Priest, F.G. (2004) Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. FEMS Microbiology Ecology, 48, 249-259.

Roggiani, M. and Dubnau, D. (1993) ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*. Journal of Bacteriology, 175, 3182-3187.

Rojas, A., Holguin, G., Glick, B.R. and Bashan, Y. (2001) Synergism between *Phyllobacterium* sp. (N_2-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere. FEMS Microbiology Ecology, 35, 181-187.

Romero, D., Aguilar, C., Losick, R. and Kolter, R. (2010) Amyloid fibres provide structural integrity to *Bacillus subtilis* biofilms. Proc Natl Acad Sci U S A, 107, 2230-2234.

Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.W., Arrebola, E.,Cazorla, F.M., Kuipers, O.P., Paquot, M. and Perez-Garcia, A. (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Molecular Plant-Microbe Interactions, 20, 430-440.

Romero, D., Vlamakis, H., Losick, R. and Kolter, R. (2009) An accessory protein required for anchoring and assembly of amyloid fibres in *B. subtilis* biofilms. Molecular Microbiology, 80, 1155-1168.

Rooney, A.P., Price, N.P., Ehrhardt, C., Swezey, J.L. and Bannan, J.D.(2009) Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. *nov*. International Journal of Systematic and Evolutionary Microbiology, 59, 2429-2436.

Rovira, A.D. (1965) Interactions between plant roots and soil microorganisms. Annual Reviews in Microbiology, 19, 241-266.

Rückert, C., Blom, J., Chen, X., Reva, O. and Borriss, R. (2011) Genome sequence of *B. amyloliquefaciens* type strain DSM7(T) reveals differences to plant-associated *B. amyloliquefaciens* FZB42. Journal of Biotechnology, 155, 78-85.

Rudrappa, T., Biedrzycki, M.L. and Bais, H.P. (2008) Causes and consequences of plant-associated biofilms. FEMS Microbiology Ecology, 64, 153-166.

Russell, P.E. (1995) Fungicide resistance: occurrence and management. Journal of Agricultural Science, 124, 317-323.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X. and Kloepper, J.W. (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology, 134, 1017-26.

Sadfi, N., Cherif, M., Hajlaoui, M. R., Boudabbous, A. and Belanger, R. (2002) Isolation and partial purification of antifungal metabolites produced by *Bacillus cereus*. Annals of Microbiology, 52, 323-338.

Schisler, D.A. and Slininger, P.J. (1997) Microbial selection strategies that enhance the likelihood of developing commercial biological control products. Journal of Industrial Microbiology and Biotechnology, 19, 172-179.

Seddon, B., Edwards, S.G. and Rutland, L. (1995) Development of *Bacillus* species as antifungal agents in crop protection. In: Lyr, H., Russell, P.E. and Sisler, H.D. (eds), Modern Fungicides and Antifungal Compounds. Proceedings of the 11th International Reinhardsbrunn Symposium, Thuringia, Germany, pp. 14-20.

Selvadurai, E.L., Brown, A.E. and Hamilton, J.T.G. (1991) Production of indole-3-acetic acid analogues by strains of *Bacillus cereus* in relation to their influence on seedling development. Soil Biology and Biochemistry 23, 401-403.

Shen, D. (1997) Microbial diversity and application of microbial products for agricultural purposes in China. Agriculture, Ecosystems and Environment, 62, 237-245.

Singh, B. K., Millard, P., Whiteley, A. S. and Murrell, J. C. (2004). Unravelling rhizosphere–microbial interactions: opportunities and limitations. Trends in Microbiology, 12, 386-393.

Silo-Suh, L.A., Stabb, E.V., Raffel, S.J. and Handelsman, J. (1998) Target range of Zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Current Microbiology, 37, 6-11.

Sonenshein A. L. (1993) Introduction to metabolic pathways. In: Sonenshein A. L., Hoch J. A., Losick R. (eds) *Bacillus subtilis* and other Gram-positive Bacteria: Physiology, Biochemistry, and Molecular Biology. American Society for Microbiology, Washington, USA, pp.127-132.

Sørensen, J. (1997) The rhizosphere as a habitat for soil microorganisms, In: Van Elsas, J.D. Trevors, J.T. and Wellington, EM.H. (eds), Modern Soil Microbiology. Marcel Dekker Inc., New York, USA, pp. 21-45.

Souto, G.I., Correa, O.S., Montecchia, M.S., Kerber, N.L., Pucheu, N.L., Bachur, M. and Garcia, A.F. (2004) Genetic and functional characterization of a *Bacillus* sp. strain excreting surfactin and antifungal metabolites partially identified as iturin-like compounds. Journal of Applied Microbiology, 97, 1247-1256.

Stabb, E.V., Jacobson, L.M. and Handelsman, J. (1994) Zwittermicin A producing strains of *Bacillus cereus* from diverse soils. Applied and Environmental Microbiology, 60, 4404-4412.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology, 56, 845-857.

Stein, T., Vater, J., Kruft, V., Otto, A., Wittmann-Liebold, B., Franke, P., Panico, M., McDowell, R. and Morris, H.R. (1996) The multiple carrier model of non-ribosomal peptide biosynthesis at modular multienzymatic templates. Journal of Biological Chemistry, 271, 15428-15435.

Stragier, P. and Losick, R. (1996) Molecular Genetics of Sporulation in *Bacillus subtilis*. Annual Reviews, 30, 297-341.

Szczech, M. and Shoda, M. (2006) The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*. Journal of Phytopathology, 154, 370-377.

Tendulkar, S.R., Saikumari, Y.K., Patel, V., Raghotama, S., Munshi, T.K., Balaram, P. and Chattoo, B.B. (2007) Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*. Journal of Applied Microbiology, 103, 2331-2339.

Thomashow, L.S. (1996) Biological control of plant root pathogens. Current Opinion in Biotechnology, 7, 343-347.

Toro, M., Azcón, R. and Barea, J.M. (1997) Improvement of arbuscular mycorrhizal development by inoculation with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (32P) and nutrient cycling. Applied and Environmental Microbiology, 63, 4408-4412.

Touré, Y., Ongena, M., Jacques, P., Guiro, A. and Thonart, P. (2004) Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. Journal of Applied Microbiology, 96, 1151-1160.

Tsuge, K., Ano, T., Hirai, M., Nakamura, Y. and Shoda, M. (1999) The genes *degQ*, *pps*, and *lpa-8* (sfp) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrobial Agents Chemotherapy, 43, 2183-2192.

Uren, N.C. (2007) Types, amounts and possible functions of compounds released in the rhizosphere by soil-grown plants. In: Pinton, R., Varanini, Z. and Nannipieri, P. (eds.). The Rhizosphere: Biochemistry and Organic Substances at the Soil-plant Interface. CRC press, Boca Raton, USA, pp.1-23.

Validov, S., Kamilova, F., Qi, S., Stephan, D., Wang, J. J., Makarova, N. and Lugtenberg, B. (2007) Selection of bacteria able to control *Fusarium oxysporum* f. sp. *radicis-lycopersici* in stonewool substrate. Journal of Applied Microbiology, 102, 461-471.

Van Elsas, J.D., Duarte, G.F., Rosado, A.S. and Smalla, K. (1998) Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. Journal of Microbiological Methods, 32, 133-154.

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. (1986) Fengycin—a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. The Journal of Antibiotics, 39, 888-901.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. (2002) Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Applied and Environmental Microbiology, 68, 6210-6219.

Vazquez, P., Holguin, G., Puente, M. E., Lopez-Cortes, A. and Bashan, Y. (2000) Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. Biology and Fertility of Soils, 30, 460-468.

Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. and Kolter, R. (2013) Sticking together: building a biofilm the *Bacillus subtilis* way. Nature Reviews Microbiology, 11, 157-168.

Wang, P., Guo, Q., Ma, Y., Li, S., Lu, X., Zhang, X. and Ma, P. (2015) DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NCD-2. Microbiological Research, 178, 42-50.

Weller, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Reviews Phytopathology, 26, 379-407.

Weller, D.M., Thomashow, L.S. and Cook, R.J. (1995) Biological control of soil-borne pathogens of wheat: benefits, risks and current challenges. In: Hokkanen, H. and Lynch, J. (eds) Biological Control: Benefits and Risks. Plant and Microbial Biotechnology Research Series. Cambridge University Press, Cambridge, UK, pp. 149-160.

Whipps, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany, 52, 487-511.

Whipps, J.M., van Elsas, J.D., Trevors, J.T. and Wellington, E.M.H. (1997) Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. Modern Soil Microbiology, 525-546.

Yan, Z., Reddy, M.S., Ryu, C-M., McInroy, J.A., Wilson, M. and Kloepper, J.W. (2002) Induced systemic resistance against tomato late blight elicited by plant growth-promoting rhizobacteria. Phytopathology, 92, 1329-33.

Yang, D.J., Wang, B., Wang, J.X., Chen, Y. and Zhou, M.G. (2009a) Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and *Sclerotinia* stem rot of rape. Biological Control, 51, 61-65.

Yang, Y.L., Xu, Y., Straight, P. and Dorrestein, P.C. (2009b) Translating metabolic exchange with imaging mass spectrometry. Nature Chemical Biology. 5, 885-887.

Yaryura, P.M., León, M., Correa, O.S., Kerber, N.L., Pucheu, N.L. and García, A.F. (2008) Assessment of the role of chemotaxis and biofilm formation as requirements for colonization of roots and seeds of soybean plants by *Bacillus amyloliquefaciens* BNM339. Current Microbiology, 56, 625-632.

Yi, H., Chun, J. and Cha, C. J. (2014) Genomic insights into the taxonomic status of the three subspecies of *Bacillus subtilis*. Systematic and Applied Microbiology, 37, 95-99.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry, 34, 955-963.

Yu, X., Ai, C., Xin, L. and Zhou, G. (2011) The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium* wilt and promotes the growth of pepper, European Journal of Soil Biology, 47, 138-145.

Zhang, S., Moyne, A-L., Reddy, M.S. and Kloepper, J.W. (2002) The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco, BiolControl, 25, 288-296.

Zhang, J.X., Xue, A.G. and Tambong, J.T. (2009) Evaluation of seed and soil treatments with novel *Bacillus subtilis* strains for control of soybean root rot caused by *Fusarium oxysporum* and *F. graminearums*. Plant Disease, 93, 1317-1323.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q. and Zhang, R. (2014) Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. Plant and Soil, 374, 689-700.

Zheng, X.Y. and Sinclair, J.B. (1996) Screening and isolation of mutants of *Bacillus megaterium* B153–2–2 for motility, chemotaxis, antagonism, and sporulation. Physiological and Molecular Plant Pathology, 84, 233-244.

Zheng, X.Y. and Sinclair, J.B. (2000) The effects of traits of *Bacillus megaterium* on seed and root colonization and their correlation with the suppression of *Rhizoctonia* root rot of soybean. Biocontrol, 45, 223-243.

Zuber, P., Nakano, M.M. and Marahiel, M.A. (1993) Peptide antibiotics. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (eds) *Bacillus subtilis* and Other Gram-positive Bacteria. ASM, Washington, USA, pp. 897-916.

CHAPTER TWO

SCREENING FOR BIOLOGICAL CONTROL OF *RHIZOCTONIA SOLANI* USING *IN VITRO* AND *IN VIVO* APPROACHES IN CONJUNCTION WITH PCR-BASED MOLECULAR TYPING METHODS

2.1 Introduction

Biological control of plant diseases is regarded as a viable and environment-friendly alternative to the use of agricultural pesticides, which pose a potential pollutant threat to man and the environment (Nelson, 2004; Cawoy *et al*., 2011). Interest in aerobic endospore forming bacteria, such as members of the genus *Bacillus* as candidates for biological control and plant growth promotion applications, has increased substantially in recent years (Emmert and Handelsman, 1999; Kloepper *et al*., 2004; McSpadden-Gardner**,** 2004; Francis *et al.*, 2010; Borriss, 2011; Cawoy *et al*., 2011; Kumar *et al*., 2011; Pérez-García *et al*., 2011; Chowdhury *et al.*, 2015). Increasingly, a body of evidence has emerged indicating that strains within the genus *Bacillus* possess a number of traits that are valuable in promoting plant growth and/or disease suppression. Mechanisms frequently associated with potential biocontrol *Bacillus* spp. include antagonism of pathogens, promotion of host nutrition and growth, and stimulation of plant host defences (Bais *et al*., 2004; Stein, 2005; Nagorska *et al*., 2007; Ongena *et al*., 2007; Ongena and Jacques, 2008; Choudhary and Johri, 2009).

The ability of *Bacillus* spp. strains to antagonize soil borne phytopathogens is generally ascribed to antibiotic production (Raaijmakers *et al*., 2002; Ongena and Jacques, 2008; Kinsella *et al*., 2009; Ongena *et al*., 2010). This trait can be screened for *in vitro* using plate assays and has been used widely as a starting point for selecting isolates for further testing (Kloepper and Schroth, 1981; Anith *et al*., 2003; Pliego *et al*., 2011). These traditional assays are simple and cost effective to perform, allowing large numbers of isolates to be screened with relative ease. However, laboratory-based *in vitro* screening is inherently artificial and far removed from the more complex field situation. Consequently, additional *in vivo/in planta* screening is considered a crucial component of any screening strategy targeting potential biocontrol agents. However, the traditional large scale *in vivo* screening of microorganisms is considered to be resource intensive, time consuming and in many cases subjective (Marten *et al*., 2000; Pliego *et al*., 2011). Accordingly, the number of isolates selected for further *in vivo* testing is often restricted and invariably, strongly influenced by the outcomes of the *in vitro* bioassays. Therefore, the selection of isolates for further evaluation is based on data and/or

information that are not ecologically pertinent. The inclusion of additional ecologically relevant information in screening protocols is therefore warranted.

Growing evidence suggests that certain *Bacillus* spp. strains found in close association to plants can be considered distinct ecotypes (Reva *et al*., 2004; Borriss *et al*., 2011). For example, Reva *et al*. (2004) found that strains of *B. amyloliquefaciens* were better adapted to colonization of the rhizosphere than other members of the *B. subtilis* group. Molecular typing using polymerase chain reaction (PCR)-based fingerprinting methods, potentially offers a simple, reliable and rapid means of differentiating environmental isolates that cannot be distinguished phenotypically (Rademaker *et al*., 2005). This approach facilitates the grouping of phenotypically related strains for dereplication purposes and could be used to rationalise and select potential biocontrol agents for further evaluation and characterization. The specific targeting and identification of plant specific ecotypes using molecular fingerprinting techniques is therefore desirable since they represent a valuable pool of plant adapted strains from which biocontrol traits can be screened for.

Various molecular approaches have been applied to describe the diversity of *Bacillus* species and differentiate closely related strains. These include 16S rRNA sequence analysis (Garbeva *et al*, 2003), repetitive extragenomic palindromic PCR (REP-PCR) (de Bruijn *et al*., 1996; Freitas *et al.,* 2008), macrorestriction of genomic DNA coupled with pulse field gel electrophoresis (PFGE) (Marten *et al*., 2000), amplified ribosomal DNA restriction analysis (ARDRA) (Wu *et al*., 2006), restriction fragment length polymorphism of rRNA operons (RFLP) (Shaver *et al.,* 2002; Mohanty *et al.,* 2011), random amplified polymorphic DNA fingerprinting (RAPD) (Nilsson *et al*., 1998; Sorokulova *et al*., 2003; Katara *et al.,* 2013) and ribosomal intergenic transcribed spacer region analysis (ITS-PCR) (Xu and Côté, 2003; Martínez and Siňeriz, 2004). Each of these methods varies in complexity, ease of use, reproducibility and in the level of phylogenetic and taxonomic resolution provided (Rademaker *et al*., 2005). ITS-PCR and RAPD-PCR have both been used and recommended as fast and reproducible methods to differentiate and group environmental isolates belonging to the genus *Bacillus* based on different banding patterns (Martínez and Siňeriz, 2004; Logan *et al*., 2009). In this study, both these methods were evaluated as a means to differentiate *Bacillus* spp. isolates that collectively exhibited antifungal activity *in vitro*.

ITS-PCR involves amplification of the internal transcribed spacer (ITS) intergenic region adjacent to the 16S and 23S rRNA genes (Jensen *et al*., 1993). Since it is assumed to not be subjected to the same selective pressure as rRNA structural genes, this region is less conserved and, hence, exhibits greater variability in terms of sequence composition at the genus and species level (Nagpal *et al*., 1998). Using universal primers to target conserved regions of the 16S and 23S rRNA genes, ITS polymorphisms can be analysed. Variation in the ITS region is also attributable to the presence of multiple rRNA operons within certain bacterial species as well as the presence of different types of tRNA genes present in variable numbers (Daffonchio *et al*., 1998). Daffonchio *et al*. (2000 and 2003) found that ITS-PCR analysis allowed for differentiation between bacterial strains at inter- and intra-species levels. Polymorphism in the ITS region, which is attributable to the presence of tRNA genes, has been used in discriminating between closely related strains and species in the *Bacillus cereus* group (Daffonchio *et al*., 2000).

Random amplification of polymorphic DNA (RAPD) involves the PCR amplification of random fragments of genomic DNA using short arbitrary primers (Welsh and McCleland, 1990). Due to the simplicity of this method a rapid throughput of large numbers of isolates is possible. Logan *et al*. (2009) recommended the use of RAPD to differentiate *Bacillus* strains into more manageable groups prior to further characterization. Although this method has been criticized for its poor reproducibility across different laboratories, it is well suited for in-house screening of isolates. RAPD has found application in differentiating strains of *B. thuringiensis* (Brousseau *et al*., 1993; Katara *et al.,* 2013), determining the genetic diversity of *Bacillus* spp. strains involved in bread spoilage (Sorokulova *et al*., 2003), screening the genetic diversity of amicoumacin producing *Bacillus* spp. from different habitats (Pinchuk *et al*., 2002), genotyping strains of *B. subtilis* and *B. pumilus* in cured sausages (Matarante *et al*., 2004), and in the large-scale typing of *B. cereus* strains associated with food contamination (Nilsson *et al*., 1998).

An initial aim of the study was to screen *Bacillus* spp. isolates for biological control activity against *Rhizoctonia solani* using "traditional" *in vitro* and *in vivo* techniques. This was done to obtain a pool of candidate biocontrol isolates from which further molecular typing methods could be applied to determine diversity levels amongst isolates and identify closely related groups. *Rhizoctonia solani* is an economically important pathogen, which exhibits a wide

host range (Huang and Huang, 2000; Chung *et al*., 2005). This pathogen has previously been found to be susceptible to *Bacillus* spp. antagonism and was therefore chosen as a relevant test organism suited for evaluating biocontrol screening methods (Asaka and Shoda, 1996; Yu *et al*., 2002; Huang *et al.,* 2012).

In this study *Bacillus* spp. were isolated from the rhizospheres of five vegetable crop types grown in composted pine bark media and then tested for *in vitro* antagonism of *R. solani* using dual-culture bioassays. Isolates exhibiting antifungal activity were selected for further *in vitro* testing against *Fusarium oxysporum*, *Botrytis cinerea* and *Pythium arrhenomanes* to determine the spectrum and extent of antagonistic activity amongst isolates. These isolates were then evaluated *in vivo* for their biocontrol potential against *R. solani* using susceptible cucumber plants grown under greenhouse conditions. ITS-PCR and RAPD-PCR fingerprinting methods were then evaluated as means to differentiate between the selected isolates. *Bacillus* spp. were first grouped according to profiles obtained using ITS-PCR. The diversity within each of these groups was then established using RAPD-PCR fingerprinting. The identity of representatives from each of the fingerprint groupings distinguished was then determined using 16S rRNA gene sequence analysis. Further differentiation of closely related strains within the *B. subtilis* group of related taxa was investigated by comparative sequence analysis of *gyrA* gene fragments that code for DNA gyrase subunit A.

2.2 Materials and methods

2.2.1 *In vitro* and *in vivo* screening *of Bacillus* spp. for biocontrol of *Rhizoctonia solani*

2.2.1.1 Plant growth conditions

Endospore-formers were isolated from the roots of five different crop plants, cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon lycopersicum* L.), lettuce (*Lactuca sativa* L.), capsicum pepper (*Capsicum annuum* L.) and cabbage (*Brassica oleracea* L. var. *capitata*) growing in composted pine bark medium (Gromor Seedling Mix, National Plant Foods, Cato Ridge, RSA). In addition, seedlings were also grown in bark media amended (20% $^{v}/_{v}$) with either sieved garden compost or a Mispah clay-loam soil (Krasilnikov and Arnold, 2009) from the University of KwaZulu-Natal's Plant Pathology demonstration vegetable garden (Co-ordinates: 29°, 37'S and 30°, 24'E).

Untreated seeds were sourced from McDonalds Seeds (Pty) Ltd., Pietermaritzburg, South Africa. Five seeds of each plant type were planted separately into duplicate pots (150 mm diameter) filled with approximately 0.4 kg (dry weight after 72 h at 70^{o}C) of each respective medium. Prior to planting, each pot was irrigated with potable water (Msunduzi municipality, Pietermaritzburg, South Africa) until the maximum water holding capacity had been exceeded and run-through was achieved. The pots were arranged in a randomized block design in a polycarbonate tunnel. During the four week experiment, diurnal ambient temperatures ranged between 18-28^{o}C. The pots were fertigated three times daily for 5 minutes, using microjet overhead irrigation (Inverted mini wobbler, Sennenger, USA). This irrigation regime ensured that the moisture content of the growing medium did not fall below 50% of its water holding capacity. The irrigation water (pH 6.4) contained per L 1 g NPK soluble fertilizer [3:1:3(38)] Complete (Ocean Agriculture, RSA) and was maintained at 20^{o}C (Pro Heat 2000 Plus, RSA).

2.2.1.2 Isolation of aerobic endospore-forming bacteria

At harvest, seedlings were carefully removed from each pot and shaken to remove loosely adhering particles from the roots. The excised roots were transferred into sterile containers and transported to the laboratory for processing. Approximately 1 g of bulked root material was suspended in nine times the volume of sterile de-ionized water and agitated at 500 rpm for 30 minutes at ambient temperature on a benchtop shaker (Vortemp 1550, Labnet, USA). The suspension was heated to 80°C for 15 min to eliminate vegetative microbial cells (Priest, 1989) and then serially diluted (10^{-1} to 10^{-5}), before spread plating (0.1 ml) onto duplicate Tryptic Soy agar (TSA) (Merck, RSA) plates. After incubation at 30°C for 48 h, separated colonies with colony morphologies representing *Bacillus* spp. were picked randomly from each set of plates. These colonies were aseptically transferred onto TSA plates, following a grid pattern with 16 isolates per plate. The plates were incubated as described above and then used as template cultures for preliminary dual-culture bioassays to screen for antifungal activity. In total 400 isolates were selected for *in vitro* screening.

2.2.1.3 Maintenance and storage of isolates

Selected isolates from the original TSA template were sub-cultured (streak plating) onto normal strength TSA plates and 10% ($^{w}/_{v}$) TSA (amended with 15 g per L bacteriological

agar), to obtain distinct colonies and to check for purity. Colony characteristics were noted and all isolates were Gram stained and analysed using bright field microscopy at x1000 magnification (Axiophot, Zeiss, Germany) after 24 h and 72 h of incubation to confirm the Gram reaction and determine whether endospores were present. Pure cultures were established on 10% ($^{w}/_{v}$) TSA slants and stored at 4^{o}C until required. For long term storage, pure cultures were grown in Tryptic Soy Broth (TSB) (Merck) at 30^{o}C and 150 rpm for 48 h and then stored at –80^{o}C in 1.8 ml CryoTubes (Nunc, Roskilde, Denmark) containing a final concentration of 20% ($^{v}/_{v}$) glycerol. Samples from each broth culture were Gram stained to confirm the presence of endospores before making up each glycerol stock. To estimate cell dimensions, samples taken from the TSB cultures (16 h) were examined as wet mounts at x1000 magnification using a bright field microscope fitted with a calibrated ocular micrometer (Zeiss, Germany).

Before further testing, isolates were revived from the stock cultures by streaking onto TSA to obtain distinct colonies. Revived cultures were never sub-cultured more than twice, to minimize mutations that could result in loss of wild type characteristics.

2.2.1.4 Fungal isolates used for in vitro screening

The microorganisms used to screen for antimicrobial activity in dual-culture bioassays are detailed in Table 2.1. All of these isolates were cultured and maintained on Potato Dextrose Agar (PDA) (Merck).The strain of *R. solani* used for *in vivo* testing was originally isolated from cabbage (*Brassica oleracea* L. var. *capitata*) seedlings exhibiting symptoms of damping-off. The identity of this strain was confirmed by Dr Isabel Rong, based at the Biosystematics Division of the Plant Protection Research Institute (Agricultural Research Council, Queenswood, RSA) (PPRI Accession number 03212). The fungal test pathogens specified in Table 2.1 were cultured and maintained on 10% ($^{w}/_{v}$) PDA (amended with 15 g per L bacteriological agar) and sub-culturing was undertaken on a monthly basis. Cultures were incubated at 28^{o} for 5-7 days before being stored in the dark at room temperature.

Table 2.1 Test organisms used in dual-culture bioassays

Test organism	Strain designation	Origin	
		Host/substrate	Locality
Rhizoctonia solani[a]	PPRI 03212	Cabbage	KwaZulu-Natal, RSA
Pythium arrhenomanes[a]	PPRI 8710	Maize	Western Cape, RSA
Fusarium oxysporum[a]	PPRI 10177	Maize	Limpopo, RSA
Botrytis cinerea[b]	KYBc 01	Strawberry	KwaZulu-Natal, RSA

Source: [a] - Plant Protection Research Institute (PPRI) Culture Collection, Agricultural Research Council (ARC), Private Bag X134, Pretoria, 001, RSA; [b] - Discipline of Plant Pathology Culture Collection, University of KwaZulu-Natal (UKZN), Private Bag X01, Scottsville, 3209, RSA.

2.2.1.5 Rhizoctonia solani used in in vivo testing

Before each *in vivo* pot trial was undertaken, the pathogenicity of the *R. solani* strain was first checked by growing cucumber seedlings in bark media seeded with *Rhizoctonia* colonized PDA plugs (3x3 mm). *Rhizoctonia* was then re-isolated from diseased plant material according to the method described by Guttierez *et al*. (1997). Briefly, sections of infected plant material were washed under running tap water and then blotted dry before being placed on Water Agar (20 g per L) (Biolab, RSA) plates and incubated at 28^{o}C for 2-3 days. Sub-cultures onto PDA were made from plates showing hyphal growth and then incubated, as described previously. Wet mount slides of fungal mycelium were prepared and examined using bright field microscopy (x 400) to confirm that the hyphal morphology was characteristic of *Rhizoctonia* sp. (Barnes, 1979). The sub-cultures were used for subsequent *in vivo* pot trials and inoculum was prepared either by growing *R. solani* on 10% PDA at 28^{o}C for 7-10 days or by culturing it on sterile barley grains which had been soaked in H_2O for 12 h. Visual confirmation of mycelial colonisation of the barley grains was made prior to use.

2.2.1.6 Dual-culture bioassays

a) Preliminary *in vitro* screening for antagonism of *R. solani*

The *in vitro* dual-culture plate assay used to screen bacterial isolates for antagonistic activity towards *R. solani,* was adapted from various authors (Utkhede and Rahe, 1983; Berg *et al*., 1996; Sadfi *et al*., 2002). Isolates were inoculated as pea-size spots on duplicate PDA plates (four isolates per plate). Inoculation sites were ~10mm from the periphery of the plates at the four corners of an imaginary square. Colonized PDA plugs (3 x 3 mm), taken from a 7 day-old culture of *R. solani,* were aseptically transferred to the center of each bioassay plate.

Controls were also established in which only *R. solani* was inoculated onto the PDA plate. All plates were incubated at 28°C and then rated for antifungal activity after 72 h. Isolates that showed evidence of antifungal activity, indicated by a clear zone of inhibition surrounding them, were considered to be antagonistic towards *R. solani*. These isolates were selected for further evaluation and were maintained and stored as described above.

b) *In vitro* screening for antifungal activity

To determine their levels and spectra of antifungal activity, isolates were tested for antagonism towards strains of *B. cinerea, F. oxysporum, P. arrhenomanes* in addition to *R. solani* (Table 2.1). Broth cultures were established by transferring one colony of each isolate into 5 ml of sterile TSB with incubation at 28°C and shaking at 150 rpm. Twenty microliters of overnight (18 h) broth cultures of each bacterial isolate were pipetted onto three sterile filter-discs (8 mm in diameter, Oxoid, Cambridge, UK) placed ~10 mm from the periphery's of PDA plates (standardized at 20 ml of PDA per plate). Inoculated plates were incubated at 28°C for 24 h to allow bacterial colonies to establish before the fungal test organisms were transferred to the plates as described above. Plates inoculated with *B. cinerea* and *R. solani* were incubated at 28°C and rated after 72 h, while those inoculated with *Fusarium oxysporum* and *P. arrhenomanes* were incubated at 25°C and rated after 120 h. Further ratings of *R. solani* and *F. oxysporum* were made at 168 h and 240 h respectively. All plates were sealed with parafilm to ensure that the agar did not dry out. Inhibition zone size was measured from the edge of each disc to the leading edge of mycelium growth using a Vernier caliper, and rated according to the systems used by Walker *et al*. (1998) and Berg *et al*. (2001):

- Isolates showing no inhibition of fungal growth were rated (–);
- Isolates showing weak inhibition of fungal growth (>0-4 mm wide zone of inhibition) were rated (+);
- Isolates showing moderate to good inhibition of fungal growth (5-9 mm wide zone of inhibition) were rated (++);
- Isolates exhibiting strong inhibition of fungal growth (i.e., ≥10 mm wide zone of inhibition) were rated (+++)

The strains *B. amyloliquefaciens* R16 and *B. subtilis* B81 (departmental strain collection), which showed biocontrol potential in previous studies (Ugoji and Laing, 2008; Yobo *et al*., 2009) were used as positive controls for comparative purposes. The assignment of these strains to a taxonomic rank was based on comparisons of their morphological and physiological characteristics and their 16S rRNA gene sequences to those in GenBank (http://www.ncbi.nlm.nih.gov) and SILVA databases (http://www.arb-silva.de) (similarities ≥ 99.8%, E-value 0.0) (This work). PDA plates on which filter paper discs were inoculated with sterile TSB (20 µl) served as controls. Three replicate bioassays were conducted for each bacterial isolate, and each set of experiments was performed twice.

2.2.1.7 In vivo screening of aerobic endospore-forming isolates for biocontrol of R. solani damping-off

A series of *in vivo* plant trials were set up to establish the biological control potential of those isolates selected from the dual-culture bioassays described above. Cucumber was selected as the test crop based on its susceptibility to *R. solani* damping-off (Huang *et al*., 2012). Untreated cucumber seeds (cultivar Ashley) were obtained from McDonalds Seeds (Pty) Ltd., Pietermaritzburg, South Africa. Seeds were planted in a commercial seedling-mix medium comprising composted pine bark amended with coir (pH 5.96; EC 189.3 mS/m; Air fill porosity 10.2%; Water holding capacity, 29.6% of total drained medium by volume) (Gromor Seedling Mix, National Plant Foods, Cato Ridge, KZN, RSA). The seedling-mix was not pasteurized so as to avoid diminishing any possible competitive interactions between the isolates being screened and the resident autochthonous microflora. To determine heterotrophic plate counts for microorganisms present in this medium three replicate samples (~1 g) of seedling-mix medium were each suspended in nine times volume of sterile Ringer's solution (¼ strength) and agitated at 500 rpm for 30 minutes on a benchtop shaker (Vortemp 1550) before being serially diluted up to 10^{-6} and spread plated 100 µl in duplicate onto TSA. Plates were incubated at 30^{o}C for 48 h and the average number of colony forming units (CFU) per gram of the medium (dry weight) was determined. Dry weight of the seedling-mix medium was determined by drying triplicate 1 g samples of medium at 70^{o}C for 48 h and averaging their final masses. Counts for viable endospore-forming bacteria were also determined by heating each seedling-mix suspension to 80^{o}C for 15 min prior to decimal dilution and plating onto TSA.

a) Influence of pathogen inoculum loading

Initially, a pot trial was run to determine the influence of pathogen inoculum loading on disease incidence and severity. Loading rates of 0, 1, 2 and 3 grains of *R. solani* colonized barley per 250 ml volume of growing medium were tested; with and without the addition of a microbial drench (*B. amyloliquefaciens* R16). The efficacy of applying the microbial drench at two rates (~2.0 x 10^6 and ~2.0 x 10^5 cells per cm^2) was also evaluated. A total of twelve treatments were assessed.

Bacillus amyloliquefaciens strain R16 was first cultured in 100 ml of TSB (Merck) for 16 h at 30°C with agitation at 150 rpm. Approximate cell numbers and the proportion of endospores present was determined by phase contrast microscopy using a bacterial counting chamber (Modified Thoma, Marienfield, Germany). An average cell count of 3.8 x 10^8 cells x ml^{-1} (n=3) was determined; with only motile vegetative cells and no free endospores being evident in the culture. The culture was then centrifuged at 10,976 x g, 4°C, for 30 minutes (JA-10 rotor, Avanti J-26XPI, Beckman). Pelleted cells were washed by resuspending in sterile, deionized H_2O (20 ml) and repeating the centrifugation step. Thereafter, the supernatant was discarded and the pelleted cells resuspended in deionized H_2O to achieve final concentrations of ~10^6 and ~10^7 cells x ml^{-1}, which were verified using the bacterial counting chamber.

Perforated polystyrene cups (250 cm^3) were half filled with growing medium. Pathogen inoculation was made by placing a specified number of *R. solani* colonized barley grains in the centre of each cup before filling them to a final volume of 250 cm^3 with medium. Three cucumber seeds were then planted to a depth of 5-10 mm in each cup. Depending on the treatment, the cups were drenched (10 ml) with either the bacterial suspensions described above or deionized water. Each treatment comprising five planted cups was triplicated giving a total of 45 seeds per treatment. Each treatment (5 cups) was placed in 2 l polypropylene trays to facilitate ease of handling. The trays were perforated to ensure good drainage, thereby avoiding water-logging. The inoculated cups were all moved to a polycarbonate tunnel and arranged in a randomized block design. The growing conditions have been described previously (Section 2.2.1.1). Seed germination was determined after 72 h and seedling survival was rated after three weeks. At the end of the trial, stem and leaf material

were collected from each replicate and oven dried at 70^{o}C for 72 h to determine the total dry weight of seedling biomass per replicate (Yobo *et al*., 2004). The experiment was duplicated.

b) *In vivo* screening of endospore-forming isolates for biological control of *R. solani*

In vivo greenhouse trials were established to screen endospore-forming isolates, selected from *in vitro* dual-culture bioassays, for biological control of *R. solani* using cucumber seeds planted into 24 well polystyrene Speedling® seedling trays. In the first round of screening, pathogen inoculation was achieved by adding a single grain of *R. solani* colonized barley to each cell (63 cm^3), half filled with bark seedling-mix (Gromor, Cato Ridge, RSA). The trays were then filled and seeds planted to a depth of ~5 mm. Subsequently, in a second round of trials, a single PDA plug (3 x 3 mm) colonized by *R. solani* was added to each well instead of colonized barley grains. Bacteria were applied as drenches at a rate of ~2.0 x 10^5 cells per cm^2 with 2.5 ml of bacterial suspension (~10^6 cells x ml^{-1}) being applied to each cell. Controls comprised seeds planted into trays without pathogen. Conversely, disease controls comprised *R. solani* infected seeds with no bacterial cells added. All controls were drenched with 2.5 ml of sterile water per cell. Each treatment had three replicates, each comprising a 24 well seedling tray, giving a total of 72 seeds per treatment. Trays were arranged in a randomized block design and kept in a polycarbonate tunnel for three weeks. The greenhouse conditions were maintained as described previously (Section 2.2.1.1). In addition, relative humidity was maintained at 85% with an automated fogging system (Enviromist, Johannesburg, RSA). Seed germination, seedling survival and dry weight biomass data were determined, as described previously (Section 2.2.1.7 a). Each set of screening trials was repeated and set up within three to five days of each other to ensure that similar environmental conditions were maintained for each replicated trial.

2.2.1.8 Statistical analysis

A general linear model (GLM) was used to run an analysis of variance (ANOVA) on seed germination, the number of surviving seedlings and dry weight of plant material for each treatment in each greenhouse experiment (Genstat Version 12.1). Where the ANOVA results were significantly different ($P<0.05$), the means were separated using Fishers's unprotected least significant difference test.

2.2.2 Genomic fingerprinting of isolates

2.2.2.1 Bacterial strains

Isolates showing *in vitro* antagonism of *R. solani* were selected for genomic fingerprinting. *Bacillus amyloliquefaciens* R16 and *B. subtilis* B81, which had shown biocontrol potential in earlier studies, were included for comparative purposes. Six reference strains were also included in the study, namely: *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^T; *B. licheniformis* DSM 13^T; *B. pumilus* DSM 27^T*; B. subtilis* subsp. *subtilis* DSM 10^T; *B. subtilis* subsp. *spizizenii* DSM 347 (ATCC 6633); and, *B. subtilis* DSM 3258 (DSMZ, Deutsche, Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Strains were routinely grown in Tryptic Soy broth (TSB) or on agar (TSA) and incubated at 30^{o}C. Stock cultures of each strain were maintained on 10% ($^w/_v$) TSA slants at 4^{o}C or at -80^{o}C in TSB amended with 20% ($^v/_v$) glycerol.

2.2.2.2 DNA extraction

Strains were cultured overnight in 10 ml sterile Luria Bertani (LB) broth (Tryptone 10 g x l^{-1}, Yeast extract 5 g x l $^{-1}$, NaCl 10 g x l $^{-1}$; pH 7) at 30^{o}C in a rotary shaker (MRC, Holon, Israel) at 150 rpm. One millilitre aliquots were centrifuged in 1.5 ml minifuge tubes at 12000 x g for 10 min. DNA was extracted from the resultant pellet using a NucleoSpin® DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol recommended for Gram positive bacteria. Eluted samples were then subjected to a further purification step following a standard ethanol precipitation protocol (Sambrook and Russell, 2006) and resuspension in 100 µl Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8). The presence of DNA in the final sample was verified by agarose (0.8% $^w/_v$) gel electrophoresis. Eluted samples (5 µl) were mixed with 2 µl of loading dye (Promega, Madison, USA) before loading onto gels which were then run in 1x Tris-acetate-EDTA buffer (TAE) (40 mM Tris-acetate, 1 mM EDTA, pH 8) at 80 V for 60 min. A 1 kb DNA molecular weight marker (Promega) was also included in each electrophoretic run. Gels were stained with ethidium bromide (0.5 µg x ml^{-1}) for 15 min and then examined using a UV trans-illuminator (254 nm) and documented (Versadoc Imaging Sytem 3000L, Quantity One gel detection software Version 4.4.1, Biorad, Hercules, United States). The concentration and purity of extracted DNA samples were determined spectrophotometrically by taking absorbance measurements at 260 nm, 280 nm and 230 nm (Nanodrop 1000, Thermo

Scientific, Wilmington, USA). DNA purity was assessed according to its A_{260}/A_{280} and A_{260}/A_{230} ratios and DNA quantities were determined according to the Lambert-Beer equation assuming that 50 µg pure ds DNA corresponds to an A_{260} of 1. The DNA samples were stored at -20^{o}C.

2.2.2.3 ITS-PCR and RAPD-PCR fingerprinting

All PCR reactions (25 µl) were carried out in sterile 200 µl thin-walled PCR tubes using GoTaq® PCR reagents (Promega, Madison, USA) in a Bioer thermal-cycler (XP Cycler, Bioer, Hangzhou, PRC).

ITS-PCR was adapted from the method of Martínez and Siñeriz (2004) using the forward (ISR-1494) and reverse (ISR-35) primers shown in Table 2.2. Each PCR reaction contained 1.5 mM $MgCl_2$, 5 µl of (5x) reaction buffer, 0.2 mM of each dNTP, 0.4 µM of forward and reverse primer, 0.6 U of Taq polymerase, 50-100 ng template DNA and was adjusted to a final volume of 25µl with nuclease-free water (Promega). The temperature cycling profile described by Martínez and Siñeriz (2004) was used but modified by increasing the number of reaction cycles from 30 to 35.

RAPD-PCR was performed using four arbitrary primers OPG-5, OPG-8, OPG-11 and OPG-16 (Table 2.2) according to a method adapted from Felici *et al*. (2008). RAPD-PCR reactions consisted of 1.5 mM $MgCl_2$, 5 µl of (5x) reaction buffer, 0.2 mM of each dNTP, 0.4 µM of primer, 1.25 U of *Taq* polymerase, 50-100 ng template DNA, made up to a final volume of 25 µl with nuclease-free water. The temperature cycling profile described by Felici *et al*. (2008) was used.

PCR reaction mixtures in which template DNA was omitted by using nuclease-free water (Promega) served as controls for each set of PCR experiments performed. After each set of amplification reactions PCR products were evaluated by agarose (1.5% $^{w}/_{v}$) gel electrophoresis run in 1x Tris-Borate-EDTA buffer (TBE) (89 mM Tris base, 89 mM Boric acid and 2 mM EDTA, pH 8.0) at 80V for 90 min. Gels were stained and examined as described previously (Section 2.2.2 2).

Table 2.2 Oligonucleotide primers used for ITS-PCR, RAPD-PCR, 16S rRNA-PCR and *gyrA*-PCR analysis

Primer*	Sequence (5´-3´)	Nucleotides	Reference
ITS-PCR			
ISR-1494	GTCGTAACAAGGTAGCCGTA	20	Martínez and Siñeriz (2004)
ISR-35	CAAGGCATCCACCGT	15	
RAPD-PCR			
OPG-5	CTGAGACGGA	10	Daffonchio *et al*. (1998)
OPG-8	TCACGTCCAC	10	
OPG-11	TGCCCGTCGT	10	
OPG-16	AGCGTCCTCC	10	
16S rRNA			
BacF	GGGAAACCGGGGCTAATACCGGAT	24	Garbeva *et al*. (2003)
R1378	CGGTGTGTACAAGGCCCGGGAACG	24	Heuer *et al*. (1997)
gyrA			
p-gyrA-f	CAGTCAGGAAATGCGTACGTCCTT	24	Roberts *et al*. (1994)
p-gyrA-r	CAACGTAATGCTCCAGGCATTGCT	24	

* Primers synthesized and supplied by Inqaba Biotech™ Hatfield, Pretoria, RSA

2.2.2.4 Taxonomic identification of isolates

a) 16S rRNA gene sequence analysis

The partial 16S rRNA gene (~1200 bp) from different endospore-forming isolates was amplified according to the method of Garbeva *et al*. (2003) using BacF, a *Bacillus* specific forward primer, in conjunction with R1378, a universal 16S rRNA reverse primer (Heuer *et al*., 1997) (Table 2.2). Template DNA from *B. subtilis* subsp. *spizizenii* DSM 347 (ATCC 6633) was included as a positive control in each round of amplifications. Reactions in which template DNA was omitted were used as negative controls. PCR amplification of the targeted gene fragment (~1200 bp) was confirmed by agarose gel electrophoresis as described previously (Section 2.2.2.2). The resultant amplification products were purified and sequenced by Inqaba Biotech™ (Hatfield Pretoria, RSA) using the forward and reverse primers and an ABI 3130*XL* sequence analyser (Applied Biosystems, Foster city, California, USA).

The base chromatograms for each isolate were checked (visually) for reading errors and edited using Chromas Lite software (Version 2.01), where an ambiguity threshold of < 0.5% was adhered to. Contiguous sequences for each amplicon were assembled using BioEdit software (Version 7.1.3.0) (Hall, 1999) and checked for chimeras by submitting the

sequences in FASTA format to DECIPHER, a web-based chimera detection tool (http://decipher.cee.wisc.edu) (Wright *et al.,* 2012). Sequences were then compared to 16S rRNA gene sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov) and SILVA (http://www.arb-silva.de) using the Megablast algorithm (word size 28, Expect threshold 10, Match/Mismatch score 1,-2) (Zhang *et al.,* 2000) and the SINA alignment tool (using default alignment parameters) (Pruesse *et al*., 2012) respectively. The GenBank search was limited to the NCBI-complete genomes database and type and reference strains within the 16S ribosomal RNA gene sequence database.

Phylogenetic analyses of gene sequence data were conducted using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods using Mega 6 (Version 6.06) software (Tamura *et al.,* 2013). The Jukes-Cantor model was used to calculate genetic distances for the NJ analysis and the topology of resultant phylogenetic tree was inferred using bootstrap analyses based on 1000 pseudoreplicates (Felsenstein, 1985). ML analysis was conducted using the Tamura-Nei substitution model to estimate evolutionary distances (site rate variation setting: gamma-distributed with invariant sites) and the reliability of internal branches of the resultant phylogenetic tree determined from 1000 bootstrap pseudoreplicates.

For comparative purposes 16S rRNA gene sequence data of phylogenically related taxa sourced from GenBank were also included in the analysis. The rooting of trees was done by using the 16S rRNA gene sequence of *Clostridium acetobutylicum* DSM1731 as an out-group. All sequences were aligned using the alignment algorithm MUSCLE (Edgar, 2004) and all positions containing alignment gaps or missing data were excluded from pairwise sequence comparisons. For the final dataset, aligned sequences spanning 1148 bp of the 16S rRNA gene were used. The sequences determined in this study were deposited in GenBank under accession numbers KT920446-KT920463.

b) *gyrA* sequence analysis

Comparative sequence analysis of partial *gyrA* genes, coding for DNA gyrase sub unit A, was performed in order to further differentiate and identify selected isolates that, based on 16S rRNA sequence analysis, showed close similarity to *B. subtilis* strains and related taxa. G*yrA* fragments, corresponding to *B. subtilis gyrA* numbering positions 43-1065 (Kunst *et al*. 1997)

were amplified using two oligonucleotide primers p-gyrA-f and p-gyrA-r (Roberts *et al*. 1994) (Table 2.2).

Each PCR reaction comprised 1.5 mM $MgCl_2$, 5 µl (5x) reaction buffer 0.2 mM of each dNTP, 0.4 µM of primers p-gyrA-f and p-gyrA-r, 1.25 U of Taq polymerase, 50-100 ng template DNA made up to a final volume of 25 µl with nuclease-free water. The temperature cycling profile used for *gyrA*-PCR comprised an initial denaturation at 95°C for 2 min, followed by 35 cycles each consisting of a denaturation step of 95°C for 1 min, an annealing step of 60°C for 30 s, and an elongation step of 72°C for 1 min; followed by a final single extension step at 72°C for 10 min, after which samples were cooled to 4°C. Template DNA from *B. subtilis* subsp. *spizizenii* DSM 347 (ATCC 6633) was included as a positive control. Reactions in which template DNA was omitted and replaced with nuclease free water were used as negative controls.

PCR amplification of the targeted gene fragment (~1024 bp) was confirmed by agarose gel electrophoresis as described above (Section 2.2.2.2). Sequence analysis of the *gyrA* gene fragments was carried out as described previously for the 16S rRNA gene sequence analysis. The sequences were subjected to a BLAST-N search (Megablast algorithm) of the GenBank database to identify closely related sequences. The evolutionary relationships of *gyrA* sequences from isolates and phylogenically related taxa, sourced from the GenBank database, were then determined using the NJ and ML methods as described previously (Section 2.2.3 a). The *gyrA* gene sequence of *C. acetobutylicum* DSM1731was included as an out-group for rooting purposes. Partial *gyrA* nucleotide sequences of the test strains were deposited in the Genbank database under accession numbers KT960036-KT960047.

2.3 Results

2.3.1 Screening of isolates for antifungal antagonism *in vitro*

Out of a total of 400 isolates screened, twenty-five showed *in vitro* antagonism towards *R. solani* and were chosen for further evaluation. The spectrum of fungal antagonism and the degree of antifungal activity of each isolate tested is shown in Table 2.3. Results for the dual-culture bioassays were reproducible when repeated independently. All of the isolates tested exhibited activity against *R. solani* and *F. oxysporum* at the first rating; whereas, only 59% and 67% of the isolates antagonised *P. arrhenomanes* and *B. cinerea* respectively, at the corresponding rating intervals. Representative examples of fungal antagonism exhibited by bacterial isolates from the dual-culture bioassays are shown in Plates 2.1-2.4.

Fifteen of the isolates, including the reference strains R16 and B81, showed antifungal activity against all of the four species tested (Table 2.3). The duration of the dual-culture bioassay was also found to influence inhibition levels. For example, the degree of *R. solani* antagonism decreased over time in many instances suggesting that these interactions were fungistatic in nature or that the concentration of active compound(s) decreased due instability, diffusion or a cessation of production by the test strain. Isolates which showed weak zones of inhibition at 72 h were completely overgrown after 168 h (viz., CC-R7, CC-R10, CP-R30, CL-R59). Several isolates, including R16, CP-R23, CP-R43, CP-R45, CL-R51 and CL-R53 retained prominent zones of inhibition against *R. solani* after 168 h incubation. The spectrum of antifungal activity against *F. oxysporum* mirrored these findings over a 240 h incubation period. Whereas, the levels of inhibition against *P. arrhenomanes* were generally weak; the only exception was CC-R4 which yielded a moderate inhibition zone (++) (Plate 2.3). Isolates which exhibited prominent zones of inhibition against *R. solani* and *F. oxysporum* also demonstrated high ratings against *B. cinerea* (Plate 2.4).

Table 2.3 Antifungal activity of aerobic endospore-forming bacterial isolates determined from dual-culture bioassays

Isolate	Fungal antagonism rating[a]						Source	
	Rhizoctonia solani		*Fusarium oxysporum*		*Pythium arrhenomones*	*Botrytis cinerea*	Plant type	Growing medium[b]
	72h	168h	120h	240h	120h	72h		
CC-R4	++	+	++	+	++	+	Cucumber roots	CPB + S
CC-R7	++	-	+	-	-	-	Cucumber roots	CPB
CC-R9	++	++	++	++	+	++	Cucumber roots	CPB
CC-R10	+	-	+	-	-	-	Cucumber roots	CPB
CP-R15	++	+	+	-	-	-	Capsicum pepper roots	CPB + C
CP-R23	+++	+++	+++	+++	+	+++	Capsicum pepper roots	CPB + S
CP-R25	++	+	++	+	-	-	Capsicum pepper roots	CPB
CP-R30	+	-	+	-	-	-	Capsicum pepper roots	CPB + C
CP-R41	+++	++	++	++	+	++	Capsicum pepper roots	CPB
CP-R42	++	+	+	-	-	+	Capsicum pepper roots	CPB
CP-R43	+++	++	+++	++	+	++	Capsicum pepper roots	CPB
CP-R45	+++	++	+++	++	+	++	Capsicum pepper roots	CPB
CL-R49	+++	++	+++	++	+	+++	Lettuce roots	CPB + S
CL-R51	+++	++	+++	++	+	+++	Lettuce roots	CPB + S
CL-R53	+++	+++	+++	+++	+	+++	Lettuce roots	CPB + S
CL-R59	+	-	+	+	+	-	Lettuce roots	CPB + S
CL-R64	++	++	++	+	-	+	Lettuce roots	CPB
CT-R67	++	+	++	++	+	++	Tomato roots	CPB + C
CT-R73	++	+	+	-	-	-	Tomato roots	CPB
CT-R77	++	+	+	-	-	-	Tomato roots	CPB
CT-R89	++	+	++	+	-	+	Tomato roots	CPB + S
CT-R90	++	+	++	+	+	++	Tomato roots	CPB + S
CT-R92	++	+	++	+	+	++	Tomato roots	CPB + S
CB-R105	+	+	+	-	-	-	Cabbage roots	CPB + S
CB-R106	++	++	++	++	+	++	Cabbage roots	CPB + S
R16[c]	+++	+++	+++	+++	+	+++	Lettuce roots	Soil
B81[c]	+++	++	++	+	+	++	Pumpkin roots	Soil

[a] Antagonism rating: + represents >0-4 mm wide inhibition zone; ++ represents 5-9 mm wide inhibition zone; +++ represents > 10mm wide zone of inhibition; - represents no evidence of inhibition. The data shown are based on duplicate bioassays.

[b] Medium used in initial isolation: CPB-composted pine bark; S-soil; C- garden compost.

[c] Isolates R16 and B81 are included as reference strains exhibiting broad-spectrum antifungal activity.

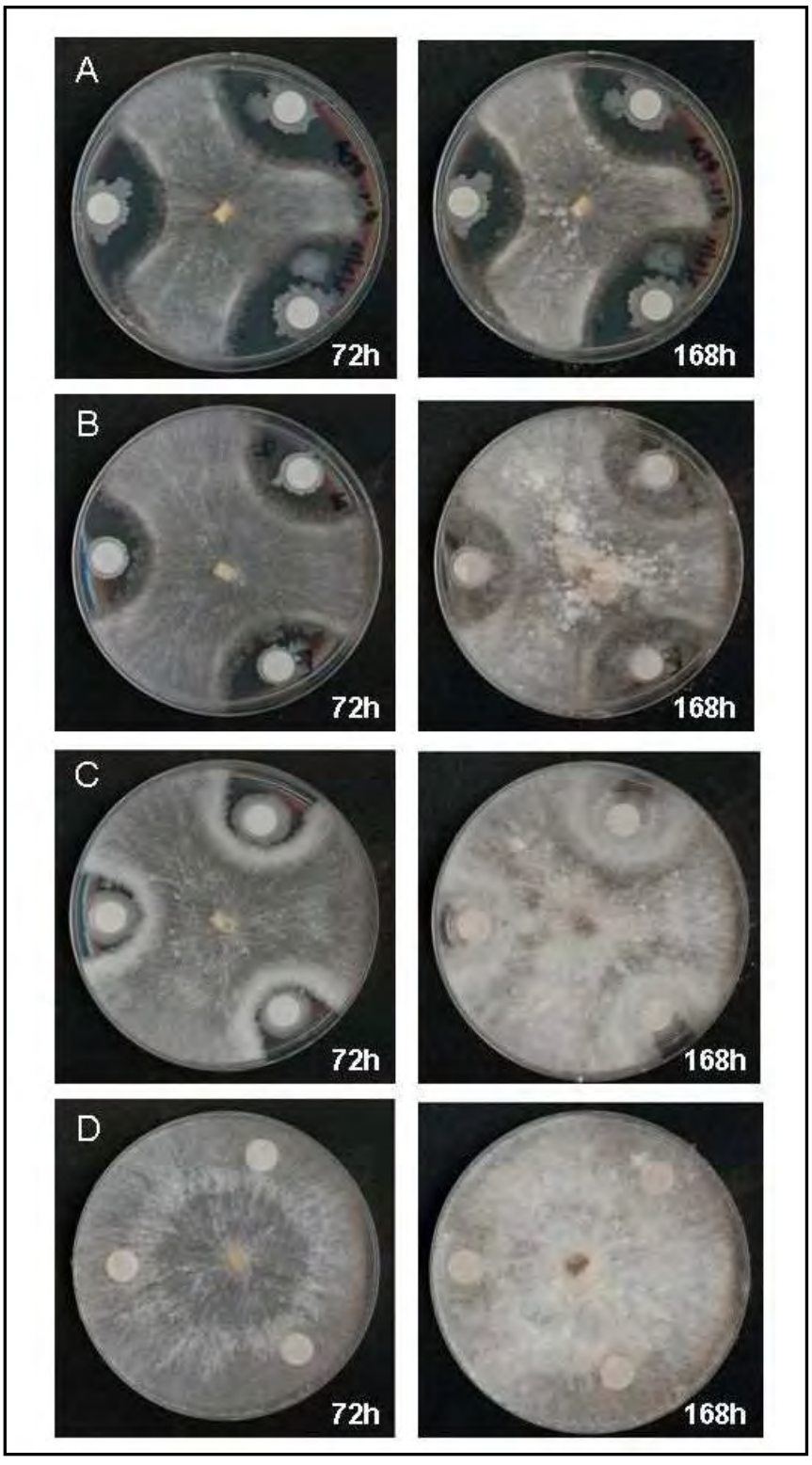


Plate 2.1 Dual-culture bioassays showing examples of *R. solani* antagonism by isolates R16 (used as reference strain, A), CT-R67 (B) and CP-R42 (C) after 72 h and 168 h. (D) is an uninoculated *R. solani* control. Bioassays were performed on PDA plates incubated at 28oC.

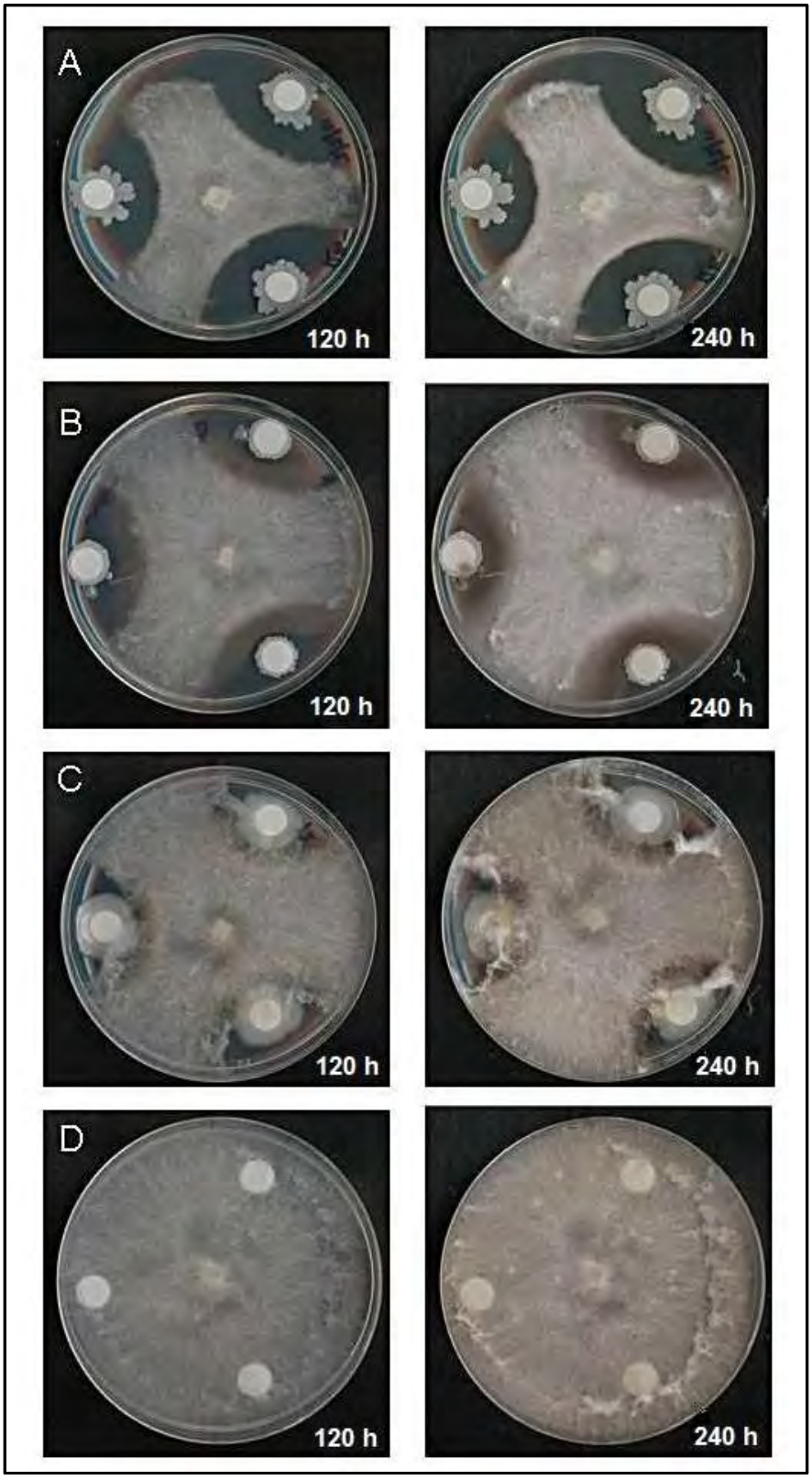


Plate 2.2 Dual-culture bioassays showing examples of *F. oxysporum* antagonism by isolates CL-R53 (A), B81 (reference strain, B) and CP-R25 (C) after 120 h and 240 h. (D) is an uninoculated *F. oxysporum* control. Bioassays were performed on PDA plates incubated at 25^{o}C.

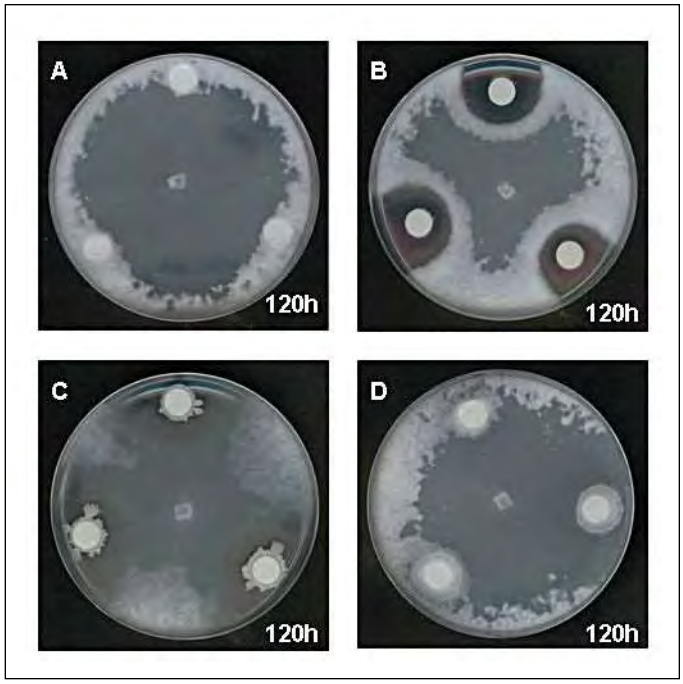


Plate 2.3 Examples of dual-culture bioassays between *P. arrhenomones* and isolates CC-R4 (B), B81 (reference strain, C) and CT-R73 (D) after 120 h. (A) is an uninoculated *P. arrhenomones* control. Bioassays were performed on PDA plates incubated at 25°C.

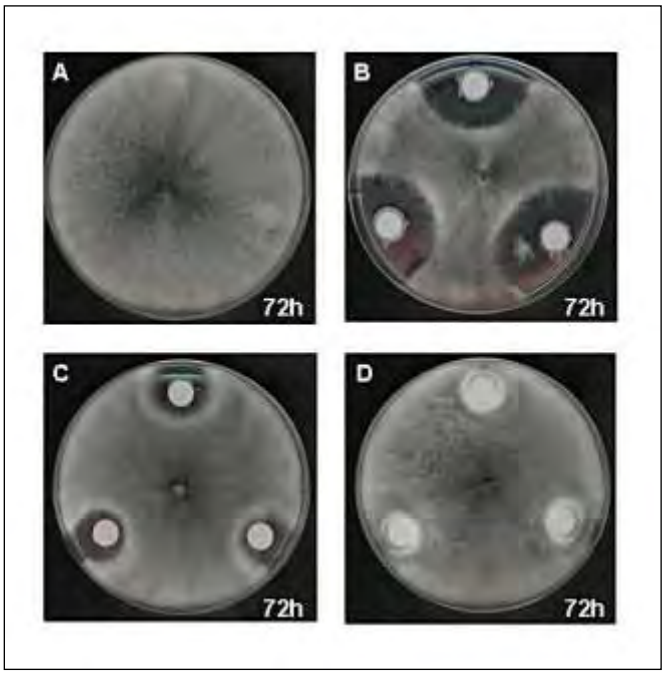


Plate 2.4 Examples of dual-culture bioassays between *B. cinerea* and isolates CP-R23 (B), CL-R64 (C), and CB-R105 (D) after 72 h. (A) is an uninoculated *B. cinerea* control. Bioassays were performed on PDA plates incubated at 28°C.

2.3.2 Morphological characteristics of isolates exhibiting antifungal activity

The morphological characteristics of isolates exhibiting antifungal antagonism are presented in Table 2.4. All isolates were confirmed to be motile, Gram positive endospore-forming rods. To a limited extent isolates could be distinguished based on spore location, cell dimension and colony morphology. The majority of isolates produced centrally or paracentrally located endospores within the vegetative cells. An exception was isolate CC-R4, which produced endospores that were located laterally. For most isolates endospore formation was not associated with a swelling of the mother cell, however, a slight swelling of the mother cell was noted for Isolates B81, CC-R9, CT-R67, CT-R89, CT-R90 and CT-R92. Interestingly, isolates that displayed the greatest levels of antagonism in the dual-culture assays all showed very similar mucoid colony morphologies when cultured on TSA.

Table 2.4 Selected morphological characteristics of isolates exhibiting antifungal activity

Isolate	Spore location	Swelling of mother cell	Cell dimensions (µm)	Colony morphology on TSA
CC-R4	Lateral	none	1.5-2.0 x 0.6	Small, whitish, smooth, transluscent round colonies with entire margins.
CC-R7	Paracentral	none	3.0-4.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CC-R9	Paracentral	slight	3.0-5.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CC-R10	Central/paracentral	none	3.0-4.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CP-R15	Paracentral	none	3.0-5.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CP-R23	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CP-R25	Central/paracentral	none	3.0-4.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CP-R30	Central/paracentral	none	3.0-5.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CP-R41	Paracentral	none	2.0-3.0 x 0.8	Creamy, round to irregular colonies with entire margins.
CP-R42	Central/paracentral	none	3.0-5.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CP-R43	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CP-R45	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CL-R49	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CL-R51	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CL-R53	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CL-R59	Paracentral	none	2.0-3.0 x 0.6	Creamy, round to irregular colonies with entire margins.
CL-R64	Paracentral	none	3.0-5.0 x 0.8	Creamy, round to irregular colonies with entire margins.
CT-R67	Paracentral	slight	3.0-4.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
CT-R73	Central/paracentral	none	3.0-5.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CT-R77	Central/paracentral	none	3.0-5.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CT-R89	Paracentral	slight	2.0-3.0 x 0.8	Creamy, round to irregular colonies with entire margins.
CT-R90	Paracentral	slight	2.0-3.0 x 0.8	Creamy, round to irregular colonies with entire margins.
CT-R92	Paracentral	slight	2.0-3.0 x 0.8	Creamy, round to irregular colonies with entire margins.
CB-R105	Central/paracentral	none	3.0-4.0 x 1.0	Whitish, matt, irregular colonies with entire to undulate margins. Prone to spreading on TSA
CB-R106	Paracentral	none	2.0-4.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
R 16c	Paracentral	none	2.0-3.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.
B81c	Paracentral	slight	3.0-5.0 x 0.8	Cream coloured, membranous colony with underlying mucoid matrix. Colonies become rough and crusty over time. Prone to spreading on TSA.

2.3.3 *In vivo* greenhouse trials

The average heterotrophic plate count (n= 3) for the composted bark medium used in the *in vivo* biocontrol trials was 8.54 (SD± 3.18) x 10^7 CFU x g^{-1} (dry weight). Viable endospore counts for the same medium were 2.19 (SD ± 1.13) x 10^7 CFU x g^{-1} (dry weight). These data indicate that approximately 25% of the CFUs detected using this method possessed viable endospores and may have been in a dormant state at the time of sampling.

The overnight culture (16 h) of R16 used for the preliminary pot trial (influence of pathogen inoculum loading) contained 3.24 x 10^8 cell x ml^{-1}. It was also established that the cells present were in a motile vegetative state and no signs of sporulation were evident. For ease of use, and standardization purposes, the inocula of isolates used as treatments in the subsequent *in vivo* biocontrol trials were also prepared from overnight TSB cultures. Cell counts ranging from 2.4-5.5 x 10^8 cell x ml^{-1} were recorded. No sporulation was evident for eighteen of the isolates assessed whereas the remaining nine isolates showed varying levels of sporulation (indicated as a percentage in parentheses) viz., CC-R7 (~17%), CP-R15 (~15%), CP-R25 (~2%), CP-R30 (~22%), CP-R41 (~9%), CP-R42 (~4%), CT-R73 (~7%), CT-R77 (~3%) and CB-R105 (~6%). With the exception of CP-R41, all isolates exhibiting evidence of sporulation displayed similar colony morphologies (Table 2.4).

2.3.3.1 Influence of pathogen inoculum loading

The influence of *R. solani* inoculum loading on disease severity in cucumber seedlings is shown in Table 2.5. From the positive control it is evident that pathogen loading had a significant negative effect on each of the parameters measured ($P<0.05$). The general trend was that applications of more pathogen inoculum resulted in decreased seedling germination, lower overall survival and mean dry plant biomass, over the course of the experiment. The application of isolate R16 as bacterial drenches reduced disease severity to varying degrees depending on the amount of pathogen applied. In general, increased pathogen loading reduced the overall effectiveness of the bacterial drench applications.

Table 2.5 Influence of *R. solani* inoculum loading on *in vivo* biological control of cucumber seedlings

Treatment	Seedling germination after 72h (%)		Seedling survival after 3 weeks (%)		Mean dry plant biomass after 3 weeks (g)	
1. Seeds (Positive control - no Pathogen added)	85.53	fg	92.20	h	8.62	f
2. Seeds + R16 drench (~2.0 x 10^6 cells per cm^2) (No Pathogen added)	82.20	f	81.13	g	7.93	f
3. Seeds + R16 drench (~2.0 x 10^5cells per cm^2) (No Pathogen added)	95.53	g	95.53	h	12.27	g
4. Seeds + Pathogen (x 1 grain)	64.47	e	43.33	d	3.50	cd
5. Seeds + Pathogen (x 1 grain) + R16 drench (~2.0 x 10^6 cells per cm^2)	62.20	de	56.67	e	5.63	e
6. Seeds + Pathogen (x 1 grain) + R16 drench (~2.0 x 10^5 cells per cm^2)	78.87	f	68.87	f	7.50	f
7. Seeds + Pathogen (x 2 grains)	37.80	ab	17.80	b	2.06	ab
8. Seeds + Pathogen (x 2 grains) + R16 drench (~2.0 x 10^6 cells per cm^2)	45.53	bc	34.47	c	4.32	de
9. Seeds + Pathogen (x 2 grains) + R16 drench (~2.0 x 10^5cells per cm^2)	52.20	cd	35.53	c	4.78	de
10. Seeds + Pathogen (x 3 grains)	33.33	ab	6.67	a	0.71	ab
11. Seeds + Pathogen (x 3 grains) + R16 drench (~2.0 x 10^6 cells per cm^2)	37.80	ab	20.00	b	2.40	bc
12. Seeds + Pathogen (x 3 grains) + R16 drench (~2.0 x 10^5cells per cm^2)	48.87	bc	20.00	b	2.53	bc
F.pr	<0.001		<0.001		<0.001	
CV%	16.10		13.30		23.10	
s.e.d	5.62		3.67		0.69	
l.s.d.	11.27		7.36		1.39	

Values followed by different letters within a column are significantly different (Fisher's unprotected least significant difference test, P=0.05)

The influence of bacterial cell densities on seedling growth and survival were most apparent in pathogen-free treatments and at the lowest pathogen application rate applied. Application rates of ~2 x 10^5 cells per cm^2 were most favourable, resulting in significantly higher levels of seedling survival and overall mean dry plant biomass compared to the higher application rate (~2 x 10^6 cells per cm^2). At the higher pathogen loading rates, no significant differences in these parameters were obtained for the two bacterial biocontrol drench rates used.

2.3.3.2 In vivo screening of endospore-forming isolates for biological control of R. solani

In the first round of greenhouse trials where *R. solani* colonized barley grains were used, very low levels of seed germination (< 20%) were recorded after 72 h for each of the bacterial isolates screened. Seedling survival progressively declined and at one week every treatment, excluding the disease-free control, exhibited >90% seedling mortality; at this point the trial was terminated. Subsequently, a second round of greenhouse trials was undertaken where, *R. solani* inoculum

was applied as colonized agar plugs (3 x 3 mm) rather than as colonized barley grains. As shown below (Table 2.6), the seedling survival results for the disease control from this trial were more commensurate with those obtained when single infected barley grains were used in the first pathogen loading trial (Table 2.5).

Table 2.6 *In vivo* screening showing antagonism of *R. solani* by aerobic endospore-forming bacterial isolates in greenhouse-based biological control trials with cucumber (*Cucumis sativus* L.) cv Ashley

Isolate/Treatment	Mean seedling germination after 72 h (%)	Mean seedling survival after 3 weeks (%)*		Mean (n=6) dry plant biomass after 3 weeks (g)*	
Control (Pathogen free)	61.31	95.10	f	4.50	g
Control (Pathogen applied)	45.97	45.10	a	1.97	a
CC-R4	46.83	75.00	cde	3.13	cdef
CC-R7	51.03	75.00	cde	2.96	bcdef
CC-R9	48.81	76.40	cdef	3.24	cdef
CC-R10	57.86	57.60	b	2.40	ab
CP-R15	59.08	65.30	bcd	2.55	abc
CP-R23	46.94	75.00	cde	3.11	cdef
CP-R25	50.94	70.80	cde	3.07	bcdef
CP-R30	58.64	68.10	bcde	3.03	bcdef
CP-R41	48.94	70.80	cde	3.15	cdef
CP-R42	56.31	64.60	bcd	2.56	abc
CP-R43	40.83	79.90	def	3.45	ef
CP-R45	58.50	79.20	def	3.38	def
CL-R51	58.53	84.00	ef	3.57	f
CL-R53	44.22	64.60	bcd	2.59	abc
CL-R59	44.89	61.10	bc	2.69	bcd
CL-R64	47.69	74.30	cde	3.15	cdef
CT-R67	59.00	81.20	def	3.44	ef
CT-R73	55.25	71.50	cde	2.92	bcdef
CT-R77	43.56	64.60	bcd	2.77	bcde
CT-R89	51.58	61.10	bc	2.55	abc
CT-R90	47.53	66.70	bcde	2.93	bcdef
CT-R92	53.03	67.40	bcde	2.87	bcdef
CB-R105	49.64	70.10	bcde	2.84	bcde
CB-R106	46.75	76.40	cdef	3.06	bcdef
R16 (reference strain)	42.75	77.80	def	3.12	cdef
B81(reference strain)	45.58	70.10	bcde	2.86	bcde
F-Ratio	0.214	<0.001		<0.001	
CV%	10.2	18.3		19.2	
Sed	7.56	7.51		0.45	
l.s.d.	14.95	14.86		0.91	

*Values followed by different letters within a column are significantly different (Fisher's unprotected least significant difference test, P=0.05)

The influence of endospore-forming bacterial isolates on cucumber damping-off in the second greenhouse trial is shown in Table 2.6. The pathogen-free control trays produced healthy seedlings showing no symptoms of damping-off. Conversely, negative controls infected with *R. solani* showed evidence of both pre- and post-emergence damping-off. Diseased seedlings also showed evidence of stem cankers, occurring as reddish brown lesions at the base of stems. As expected, in the pathogen controls seedling survival and mean dry biomass were all negatively affected by the application of *R. solani* ($P<0.001$).

The seedling germination results for most of the treatments, including the controls, exhibited high standard deviation levels and the differences between treatments were not statistically significant (F-Ratio 0.214). Seedling survival ranged from 45.1% to 95.1% for the disease and disease-free, controls respectively. All isolates tested significantly improved the levels of seedling survival compared to the disease control, with survival rates ranging from 57.6% to 84.0% ($P<0.001$) after three weeks. The isolates CC-R9, CP-R43, CP-R45, CL-R51, CT-R67, CB-R106 and R16 resulted in seedling survival levels greater than 75% which were not significantly different from the pathogen free control ($P =0.05$).

The dry plant biomass of the infected control was only 43.8 % of that of the non-infected control. The mean biomass values from each isolate treatment were greater than the diseased control; however, in the case of isolates CC-R10, CP-R15, CP-R42, CL-R53 and CT-R89 the results were not statistically different to those of the infected control ($P=0.05$). Some treatments resulted in dry plant biomass values ranging from 59.8% to 79.3% of that of the pathogen free control ($P<0.001$), which were significantly greater than that of the disease control. Treatments with isolate CL-R51 resulted in the highest dry biomass (3.57g) and, along with isolates CP-R43, CP-R45 and CT-R67 caused mean biomass yields that were greater than 75% of the pathogen free control.

Generally, isolates that achieved high inhibition rankings from *in vitro* bioassays were amongst the best performing isolates when screened *in vivo*. A notable exception was CL-R53 which performed poorly in the biocontrol seedling trial.

2.3.4 Genomic fingerprinting

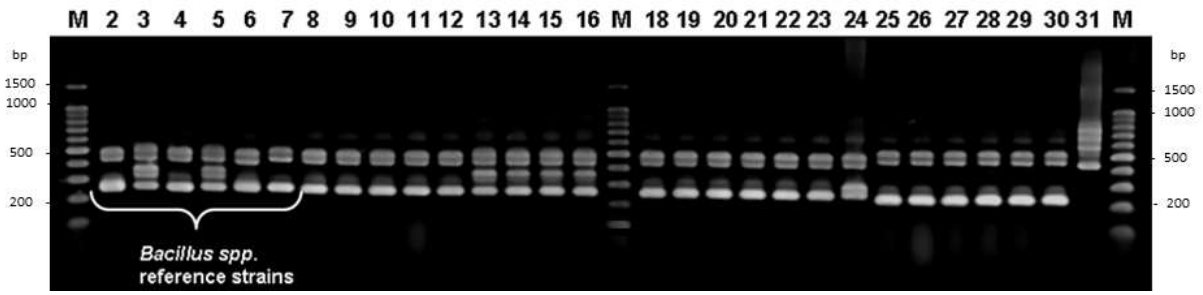
2.3.4.1 ITS-PCR

The results of the ITS-PCR fingerprinting for twenty-one of the root-associated endospore-forming isolates are presented in Figure 2.1. The dendrogram derived from UPGMA cluster analysis indicated that these isolates could be grouped into three clusters based on band numbers, size and intensity. Group I comprised 16 isolates (including strains R16 and B81) which were grouped closely with six *Bacillus* spp. reference strains belonging to the "*Bacillus subtilis* group" of closely related taxa. Group II comprised six organisms that showed virtually identical banding patterns, whilst Group III contained a single representative namely, CC-R4., The ITS-PCR band profiles of four isolates (CC-R10, CP-R15, CT-R73, CB-R106) were omitted from the electrophoretic gel presented in Figure 2.1 due to the space constraints of the gel apparatus used. The ITS profiles for CC-R10, CP-R15 and CT-R73 were consistent with those of isolates placed in Group II, whereas, the ITS profile for CB-R106 was placed within Group I.

Group I strains all displayed ITS profiles that included three major bands with estimated sizes of 260, 425 and 500 bp. Additional bands with approximate sizes of 310 and 360 bp were also present in some of the strains within this grouping. Isolate CL-59 (Lane 24), which had an ITS profile which included a fourth prominent band (310 bp), showed the least similarity (~57 %) to the other isolates in Group I (Figure 2.1). Isolates CP-R23, CL-R49, CL-R51 and CL-R53 also displayed a fourth band (360 bp) in their respective ITS profiles and formed a sub-group with *B. subtilis* DSM3258 and *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^T. The remaining isolates grouped closely with *B. subtilis* subsp. *spizizenii* ATCC6633 (>90% similarity). Strains *B. licheniformis* DSM13^T, *B. pumilus* DSM27^T, *B. subtilis* subsp. *subtilis* DSM10^T and *B. subtilis* subsp. *spizizenii* ATCC6633 all displayed ITS profile similarities that were greater than 85%. It was also interesting to note that the three reference strains of *B. subtilis* included in the study showed some degree of intra-specific variation in their ITS profiles and differed in terms of the presence/absence of two bands with sizes of 310 and 360 bp.

The organisms in Group II also showed three prominent bands but with estimated sizes of 230, 450 and 530 bp. The ITS-PCR profile of Isolate CC-R4 (Group III) was distinct from that of all the other isolates with four prominent bands with sizes of 430, 570, 660 and 770 bp being evident.

A



B

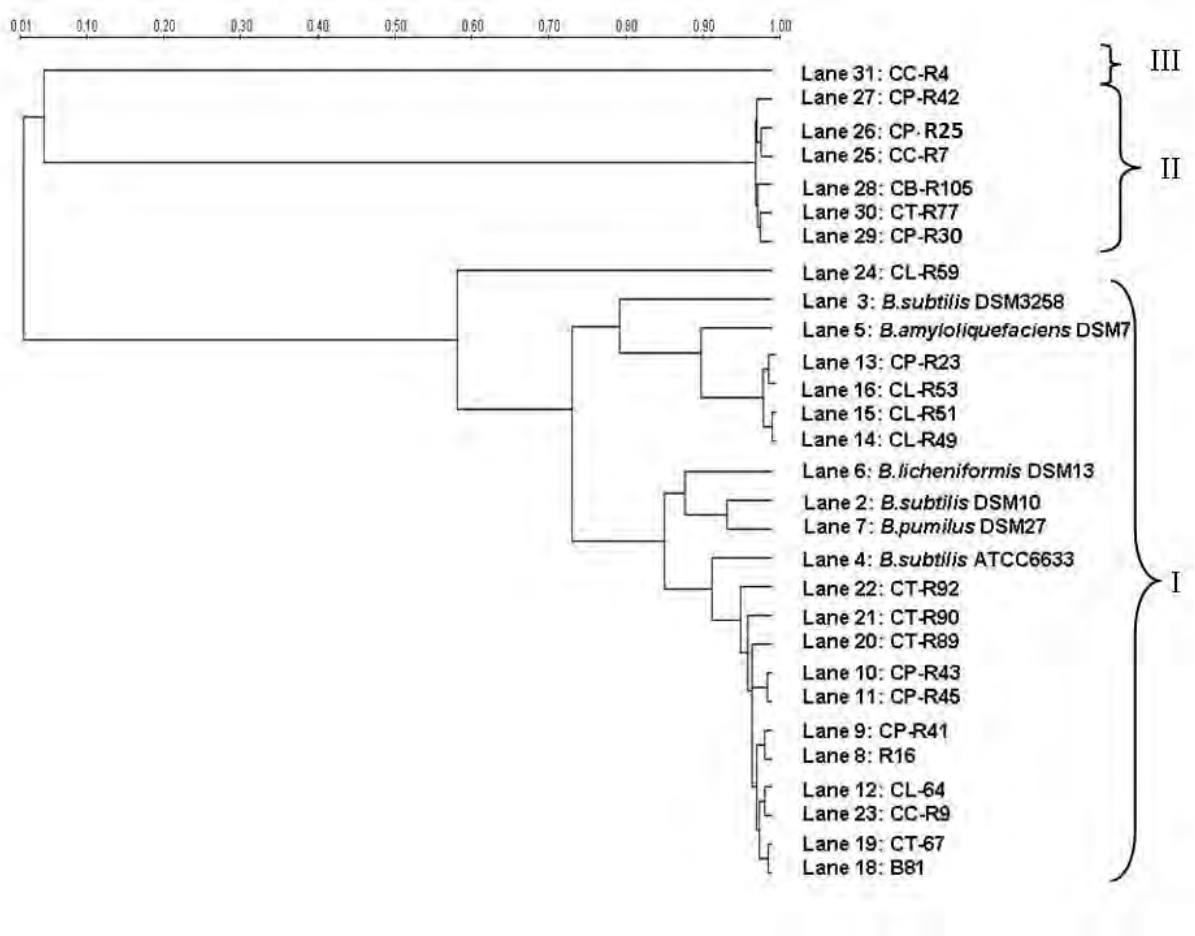


Figure 2.1 ITS-PCR fingerprint profiles (A) of root-associated endospore-forming isolates and reference strains of *Bacillus spp.* Similarities between banding patterns was calculated by Dice Coefficient analysis and a dendrogram (B) depicting relationships between isolates was generated with the unweighted pair-wise group method using arithmetic averages (UPGMA). Lanes M contained a 100 bp DNA ladder (Promega), Lanes 2-7 comprised reference strains *B. subtilis* subsp. *subtilis* DSM10^{T}; *B. subtilis* DSM3258; *B. subtilis* subsp. *spizizenii* ATCC6633; *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^{T}; *B. licheniformis* DSM13^{T} and *B. pumilus* DSM27^{T}; Lanes 8-16 and 18-24, Group I isolates: R16; CP-R41; CP-R43; CP-R45, CL-R64; CP-R23; CL-R49; CL-R51; CL-R53; B81; CT-R67; CT-R89; CT-R90; CT-R92; CC-R9; CL-R59; Lanes 25-30 Group II isolates CC-R7; CP-R25; CP-R30; CP-R42; CT-R77; CB-R105; Lane 31 Goup III Isolate CC-R4.

2.3.4.2 RAPD-PCR

RAPD analysis was performed with four separate primers (OPG-5, OPG-8, OPG-11 and OPG-16) to further distinguish between the isolates grouped by ITS profiling. The RAPD-fingerprints for each set of ITS groupings are presented in Fig. 2.2 (Group I isolates) and 2.3 (Group II and III isolates).

Each of the four arbitrary primers evaluated gave rise to reproducible banding profiles that allowed grouping and matching of strains to be made; however, the levels of genetic heterogeneity detected varied somewhat depending on the primer used and the group of organisms screened. Some variation in band intensity was evident amongst replicate samples, but consistent DNA banding patterns were achieved.

Primers OPG-5, OPG-8 and OPG-11 were judged to yield the best fingerprint profiles for Group I isolates with similar sub-clusters being distinguished. Each of the reference strains produced unique banding patterns with each of the primers used; whereas, amongst the environmental isolates screened, several clusters were identified. Using Primer OPG-11 as a point of reference, the 16 isolates from Group I could be sub-divided into four clusters (A-D) based on banding patterns. A dendrogram representing UPGMA cluster analysis of the OPG-11 RAPD fingerprints is presented in Figure 2.4. Similar clusters were achieved when the other primers were used; a notable exception was Isolate CP-R23 (Lane 10, Fig 2.2) which produced a distinct RAPD profile with Primer OPG-5 but not when OPG-8, OPG-11 or OPG-16 was used. It was interesting to note that banding profiles obtained for the environmental isolates screened were clearly differentiated from reference strains that they were previously grouped with during ITS-PCR profiling.

Cluster A comprised seven strains with representatives isolated from the rhizospheres of bell pepper (CP-R23, CP-R41, CP-R43, CP-R45), lettuce (CL-R64) and cabbage seedlings (CB-R106). Strain R16 which had been isolated from lettuce seedlings in a previous study was also included in this cluster. Cluster B was made up of three strains (CL-R49, CL-R51, CL-R53) all of which had been isolated from lettuce. Cluster C was represented by a single strain (CL-R59) which had also been isolated from lettuce root material. The last grouping, cluster D comprised five strains which had been isolated from cucumber (CC-R9), tomato (CT-R67, CT-R89, CT-

R92) and squash (reference strain B81). The RAPD profile for isolate CT-R90 also fell within cluster D, however, this data was not presented in Figure 2.2 due to the space constraints of the gel apparatus used.

RAPD profiles for the Group II isolates proved to be less consistent and varying levels of heterogeneity were observed (Fig 2.3). Primer OPG-8 gave rise to a single homologous band for each strain. Similarly, Primer OPG-16 produced comparable banding profiles amongst strains but with different intensities and a greater number of bands being evident. Primers OPG-5 and OPG-11 also gave rise to fingerprint profiles with relatively few bands; however greater levels of banding heterogeneity were detected. Several groupings could be distinguished with each of these primers but they were not consistent with one another.

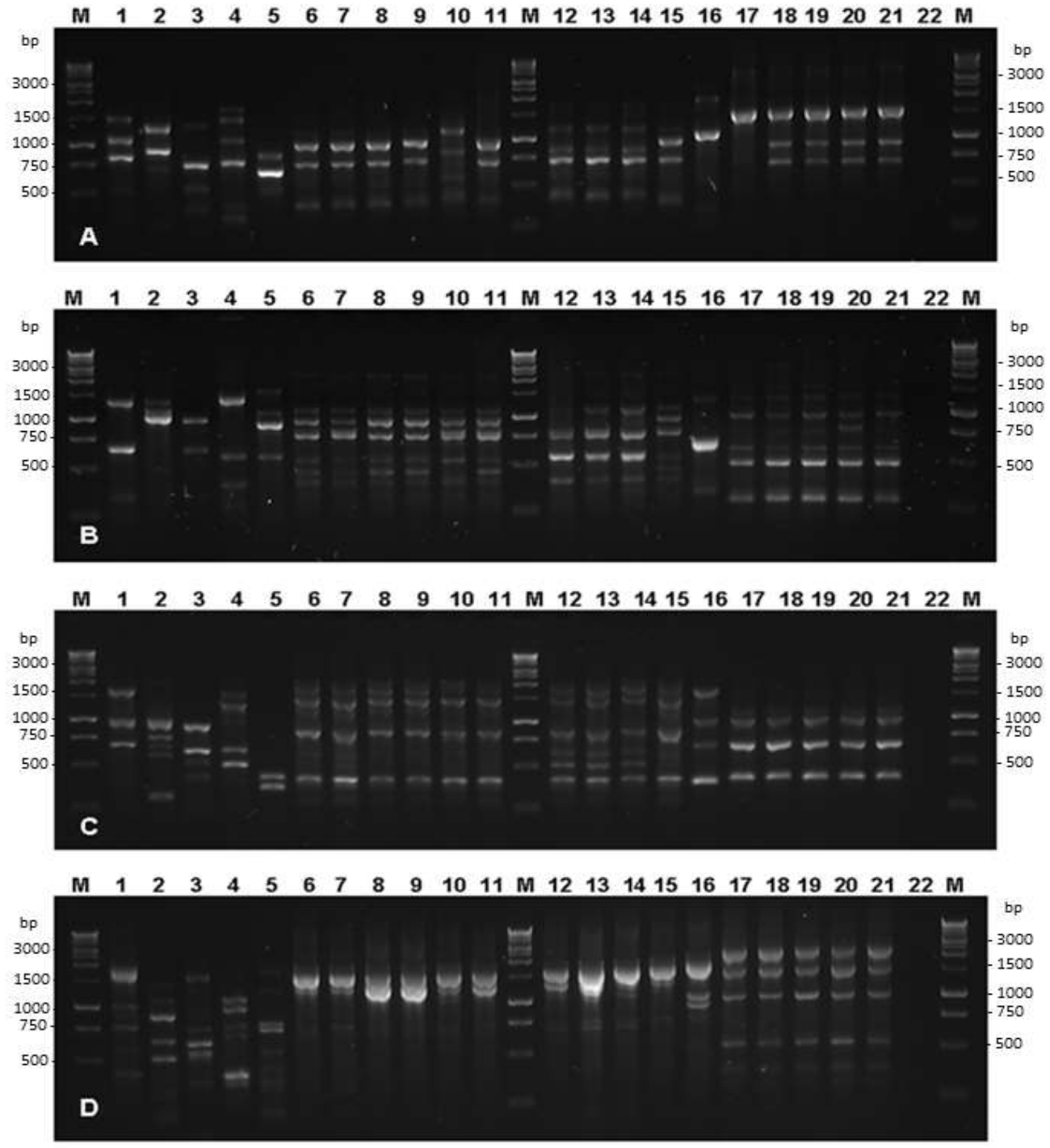


Figure 2.2 RAPD-PCR fingerprints of endospore-forming isolates and *Bacillus* spp. reference strains that ITS-PCR profiling clustered together in Group I. Four separate primers OPG-5 (A), OPG-8 (B), OPG-11 (C) and OPG-16 (D) were used to assess the consistency of the groupings: Lanes M contained a 1-kbp DNA ladder (Promega), Lanes 1-5 comprised reference strains *B. subtilis* subsp. *subtilis* DSM10^{T}; *B. subtilis* subsp. *spizizenii* ATCC6633; *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^{T}; *B. licheniformis* DSM13 and *B. pumilus* DSM27; Lanes 6 to 21 comprised the Group I isolates: R16; CP-R41; CP-R43; CP-R45, CP-R23; CL-R64; CL-R49; CL-R51; CL-R53; CB-R106; CL-R59; CC-R9; CT-R67; CT-R89; CT-R92; B81; Lane 22 was a control lacking DNA template.

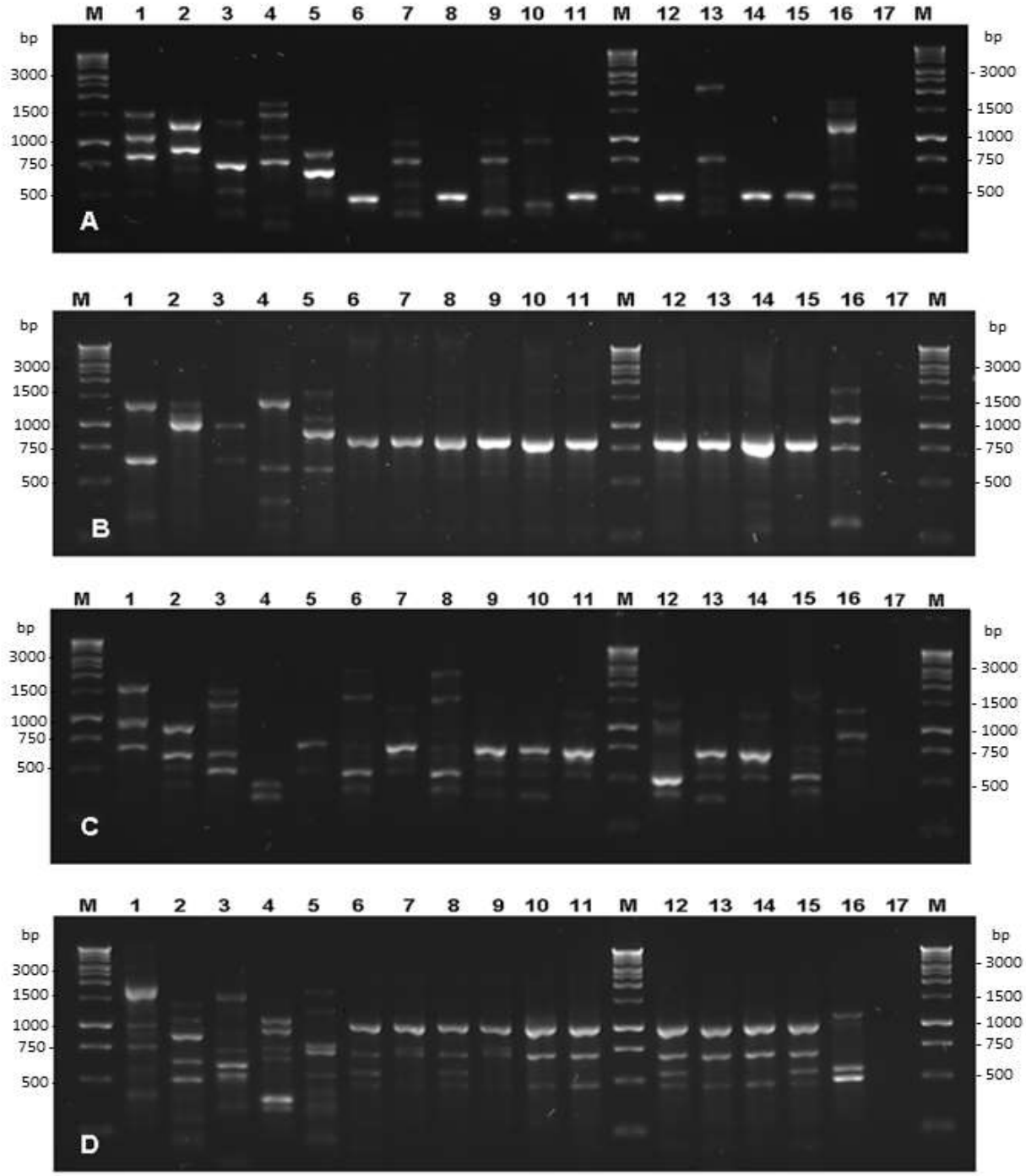


Figure 2.3 RAPD-PCR fingerprints of endospore-forming isolates that ITS-PCR profiling had clustered into Groups II and III. Four separate primers OPG-5 (A), OPG-8 (B), OPG-11 (C) and OPG-16 (D) were used to assess the consistency of the groupings: Lanes M contained a 1-kbp DNA ladder (Promega), Lanes 1-5 comprised reference strains *B. subtilis* subsp. *subtilis* DSM10^T; *B. subtilis* subsp. *spizizenii* ATCC6633; *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^T; *B. licheniformis* DSM13^T and *B. pumilus* DSM27^T; Lanes 6 to 15 Group II isolates: CC-R7; CP-R15; CP-R25; CP-R30, CP-R33; CP-R39; CP-R42; CT-R73; CT-R77; CB-R105; Lane 16 contained the Goup III Isolate CC-R4; Lane 17 was a control lacking DNA template.

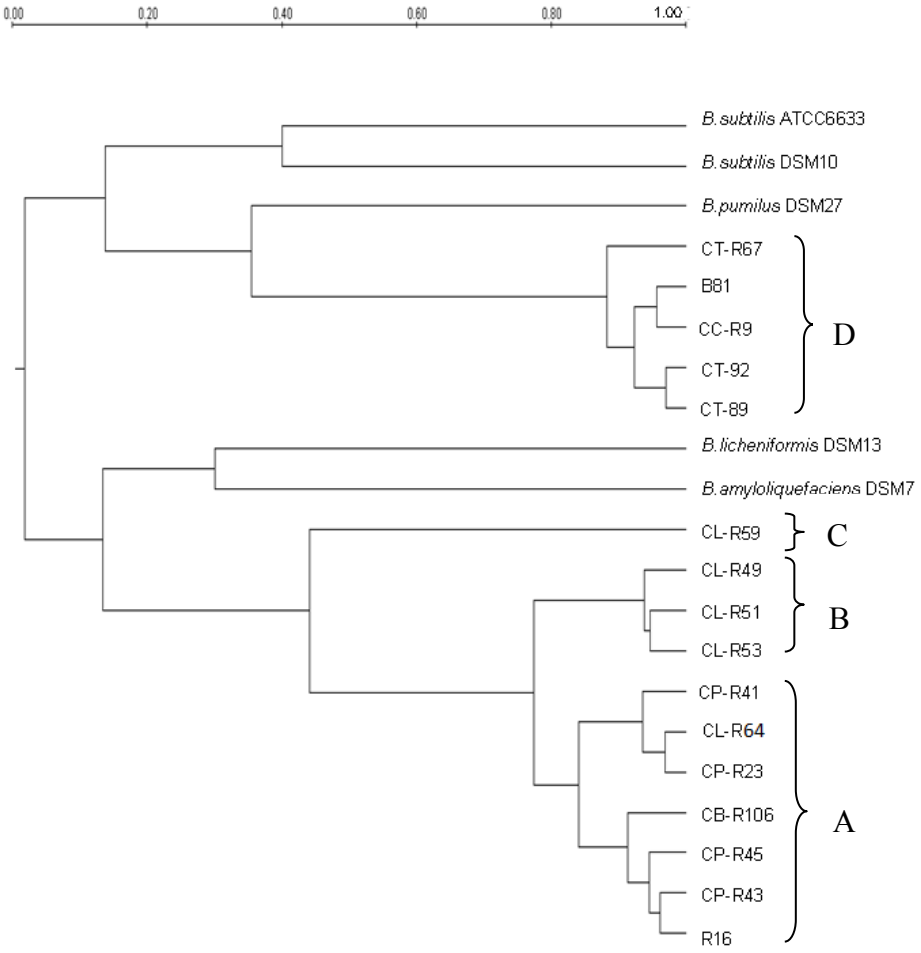


Figure 2.4 UPGMA dendrogram showing clusters (A-D) among Group I isolates and *Bacillus* spp. reference strains inferred from RAPD fingerprint profiles obtained with primer OPG-11.

2.3.4.3 16S rRNA gene sequence analysis

PCR amplification using 16S rRNA specific primers yielded a product of approximately 1200 bp for each of the isolates tested, which was consistent with the results obtained by Garbeva *et al*. (2003). BLAST searches of the Genbank database revealed that the majority of isolates evaluated showed high levels of sequence similarity (≥99.8%) to reference strains within the "*Bacillus subtilis* group" or the "*Bacillus cereus* group" of closely related taxa (Table 2.7). Isolate CC-R4, which was clearly distinguished from the other isolates based on ITS- and RAPD-PCR, showed a

99.2-99.3% similarity to strains of *Brevibacillus laterosporus*. Similar findings were obtained when sequences were aligned to 16S rRNA gene sequences in the SILVA database.

Table 2.7 BLAST results of partial 16SrRNA gene sequences amplified from aerobic endospore-formers exhibiting *in vitro* antagonism towards *R. solani* (accessed November 2014)

Isolate(s)	Product size (bp)	Genbank match	Strain	Accession No.	Similarity (%)	S-score	E-value
R16, CP-R23, CP-R43, CL-R51, CL-R53, CL-R64, CB-R106	1148	*Bacillus amyloliquefaciens subsp. plantarum*	FZB42	NC009725	100	2109	0
			CAU B946	NC016784	100	2109	0
			YAU B6901-Y2	NC017061	100	2109	0
			NAU-B3	NC022530	100	2108	0
			UCMB 5113	NC022081	100	2108	0
		Bacillus amyloliquefaciens	LFB 112	NC023073	100	2108	0
			CC 178	NC022653	100	2108	0
			IT-45	NC020272	100	2108	0
CL-R59	1148	*Bacillus subtilis*	NBRC 14192	AB680581	100	2113	0
		Bacillus subtilis subsp. spizizenii	ATCC 6633	NR118486	100	2113	0
		Bacillus subtilis subsp. inaquosorum	BGSC 3A28	NR104873	100	2113	0
		Bacillus subtilis subsp. subtilis	DSM 10	NR027552	100	2113	0
			NCIB 3610	NC017195	100	2113	0
			168	NC000964	100	2113	0
B81, CC-R9, CT-R67, CT-R89	1148	*Bacillus sp.*	JS	NC017743	100	2122	0
		Bacillus subtilis subsp. spizizenii	NBRC 101239	NR112686	99.9	2119	0
			NRRL B-23049,	NR024931	99.9	2119	0
			ATCC 6633	NR118486	99.8	2113	0
		Bacillus subtilis subsp. subtilis	DSM 10	NR027552	99.8	2113	0
			JCM 1465	NR113265	99.8	2113	0
			NBRC 1379	NR112629	99.8	2113	0
			168	NR102783	99.8	2113	0
		Bacillus subtilis subsp. inaquosorum	BGSC 3A28	NR104873	99.8	2113	0
		Bacillus axarquiensis	LMG 22476	NR115929	99.8	2113	0
		Bacillus malacitensis	CR-95	NR115282	99.8	2113	0
			CECT 5687	NR115930	99.8	2113	0
		Bacillus mojavensis	IFO 15718	NR024693	99.8	2113	0
CC-R7, CP-R25, CP-R42, CT-R73, CT-R77, CB-R105	1148	*Bacillus cereus*	ATCC 14579	NR074540	100	2113	0
			JCM 2152	NR113266	100	2113	0
			NBRC 15305	NR112630	100	2113	0
			IAM 12605	NR115526	100	2113	0
		Bacillus thuringiensis	NBRC 101235	NR112780	100	2113	0
			ATCC 10792	NR114581	100	2113	0
			IAM 12077	NR043403	100	2113	0
		Bacillus toyonensis	BCT-7112 16S	NR121761	100	2113	0
CC-R4	1148	*Brevibacillus laterosporus*	NBRC 15654	NR112727	99.3	2065	0
			DSM 25	NR112212	99.3	2065	0
			IAM 12465	NR037005	99.2	2060	0

Note: S-Score is a measure of similarity of the BLAST query to the sequence shown. E-value is a measure of the reliability of the S-score.

The phylogenetic trees inferred using the NJ and ML methods both displayed very similar topologies (Figures 2.5 and 2.6). Group I, II and III isolates were clearly differentiated from one

another. Isolates falling within clusters A-D (Group I) showed very high levels of sequence similarity (≥99.6%) based on pairwise distance estimates (Jukes-Cantor method). The 16S rRNA gene sequences of cluster A and B isolates were identical (100% similarity) to strains of *B. amyloliquefaciens* and *B. amyloliquefaciens* subsp. *plantarum*. Isolate CL-R59, the sole representative of cluster C, showed 100% sequence similarity to several strains of *B. subtilis* including representatives that have been differentiated at the subspecies level. Cluster D isolates displayed a 99.8 - 99.9% similarity to several species that fall within the "*Bacillus subtilis* group" of related taxa. Interestingly, they showed a 100% sequence similarity to an unidentified strain, *Bacillus* sp. JS, which is a plant growth promoting rhizobacterium that enhances the growth of tobacco and lettuce (Song *et al*., 2012).

All of the Group II isolates evaluated exhibited identical nucleotide sequences indistinguishable (100% similarity) from strains of *B. cereus, B. thuringiensis and B. toyonensis* and were phylogenetically distinct from the Group 1 isolates.

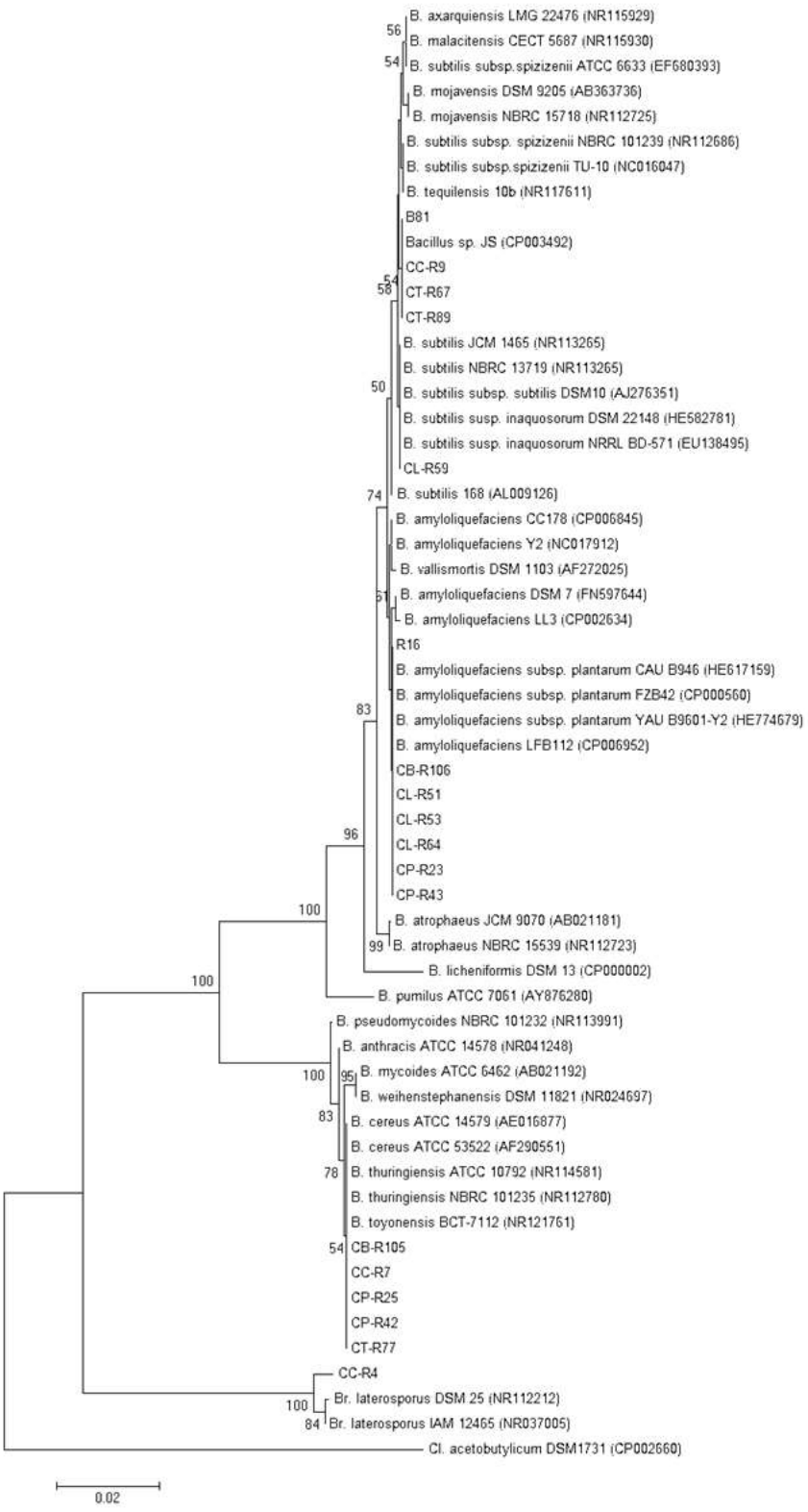


Figure 2.5 Neighbour-joining phylogenetic tree inferring the evolutionary relationship between the endospore-forming isolates and selected reference sequences based on partial 16S rRNA gene sequence analysis. Only bootstrap values >50% are included. The scale bar corresponds to 0.02 nucleotide substitutions per sequence position. Genus abbreviations: B. = *Bacillus*; Br. = *Brevibacillus*; Cl. = *Clostridium*.

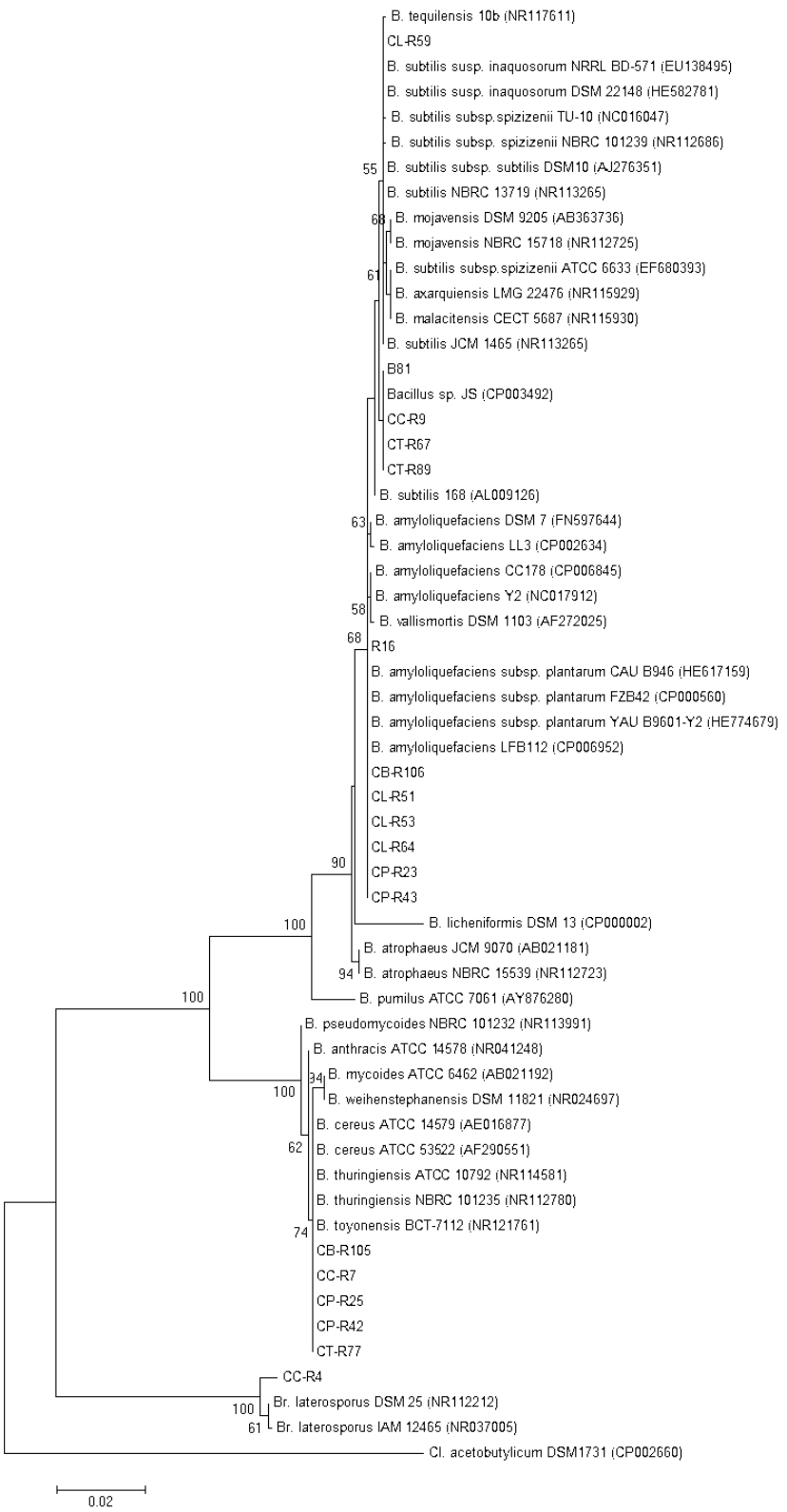


Figure 2.6 Maximum-likelihood phylogenetic tree inferring the evolutionary relationship between the endospore-forming isolates and selected reference sequences based on partial 16S rRNA gene sequence analysis. Only bootstrap values ≥50% are included. The scale bar corresponds to 0.02 nucleotide substitutions per sequence position. Genus abbreviations: *B. = Bacillus; Br. = Brevibacillus; Cl. = Clostridium.*

2.3.4.4 gyrA gene sequences

Further distinctions between Group I isolates were determined by comparing partial gene sequences of the *gyr*A gene that encodes DNA gyrase subunit A (Table 2.8). Within the "*Bacillus subtilis* group" this gene displays a greater degree of genetic variation than the 16S rRNA gene and has successfully been used to distinguish between closely related taxa and ecotypes (Chun and Bae, 2000; Boriss *et al*., 2011).

Table 2.8 BLAST results of sequenced products of partial fragments of the gyrase subunit A (*gyrA*) gene amplified from *Bacillus* spp. by PCR using gene specific primers (accessed November 2014)

Isolate(s)	Product size (bp)	Genbank match	Strain	Accession No.	Similarity (%)	S-score	E-value
R16, , CP-R43, CL-R64, CB-R106	882	*B. amyloliquefaciens*	SQR9	CP006890	99.4	1598	0
		B. amyloliquefaciens subsp. *plantarum*	AS43.3	CP003838	99.3	1592	0
			FZB42	CP000560	98.6	1559	0
CP-R23, CL-R51, CL-R53	882	*B. amyloliquefaciens*	SQR9	CP006890	99.6	1609	0
			CC178	CP006845	99.3	1592	0
		B. amyloliquefaciens subsp. *plantarum*	FZB42	CP000560	99.3	1592	0
CL-R59	882	*B. subtilis* subsp. *subtilis*	BSP1	CP003695	100	1626	0
			BSn5	CP002468	99.7	1615	0
			BAB-1	CP004405	99.4	1598	0
B81, CC-R9, CT-67, CT-R89	882	*Bacillus* sp.	JS	CP003492	99.3	1598	0
		B. subtilis subsp. *subtilis*	168	CP010052	95	1382	0
		B. subtilis subsp. *spizizenii*	TU-B-10	CP002905	94.5	1360	0

Note: S-Score is a measure of similarity of the BLAST query to the sequence shown. E-value is a measure of the reliability of the S-score.

The phylogenetic trees inferred using the NJ and ML methods both displayed very similar topologies (Figures 2.7 and 2.8). From these it was evident that partial *gyrA* sequences provided clearer distinctions between the groups separated by RAPD-PCR fingerprinting than did the tree for 16S rRNA gene sequences. The *gyrA* genes of isolates from clusters A and B, which exhibit minor differences in each of their respective nucleotide sequences, again showed high levels of similarity (98.6-99.4%) to strains of *B. amyloliquefaciens* and *B. amyloliquefaciens* subsp. *plantarum* (Table 2.8). Similarly, the partial *gyrA* gene sequences of isolate CL-R59 showed a close match (≥99.4%) to several strains of *B. subtilis* subsp. *subtilis.* Isolates placed within cluster D continued to show a high level of similarity (99.3%) to the *gyrA* gene of *Bacillus* sp. JS. Other matches with strains of *B. subtilis* subsp. *spizizenii* and *B. subtilis* subsp. *subtilis* were observed but in these instances similarity levels were lower (94.5 and 95 % respectively). From the phylogenetic trees it was evident that cluster D isolates formed a distinct sub-cluster within the clade that grouped different strains and sub-species of *B. subtilis.* On the basis of this information isolates B81, CC-R9, CT-R67 and CT-R89 were putatively identified as strains of *B. subtilis*.

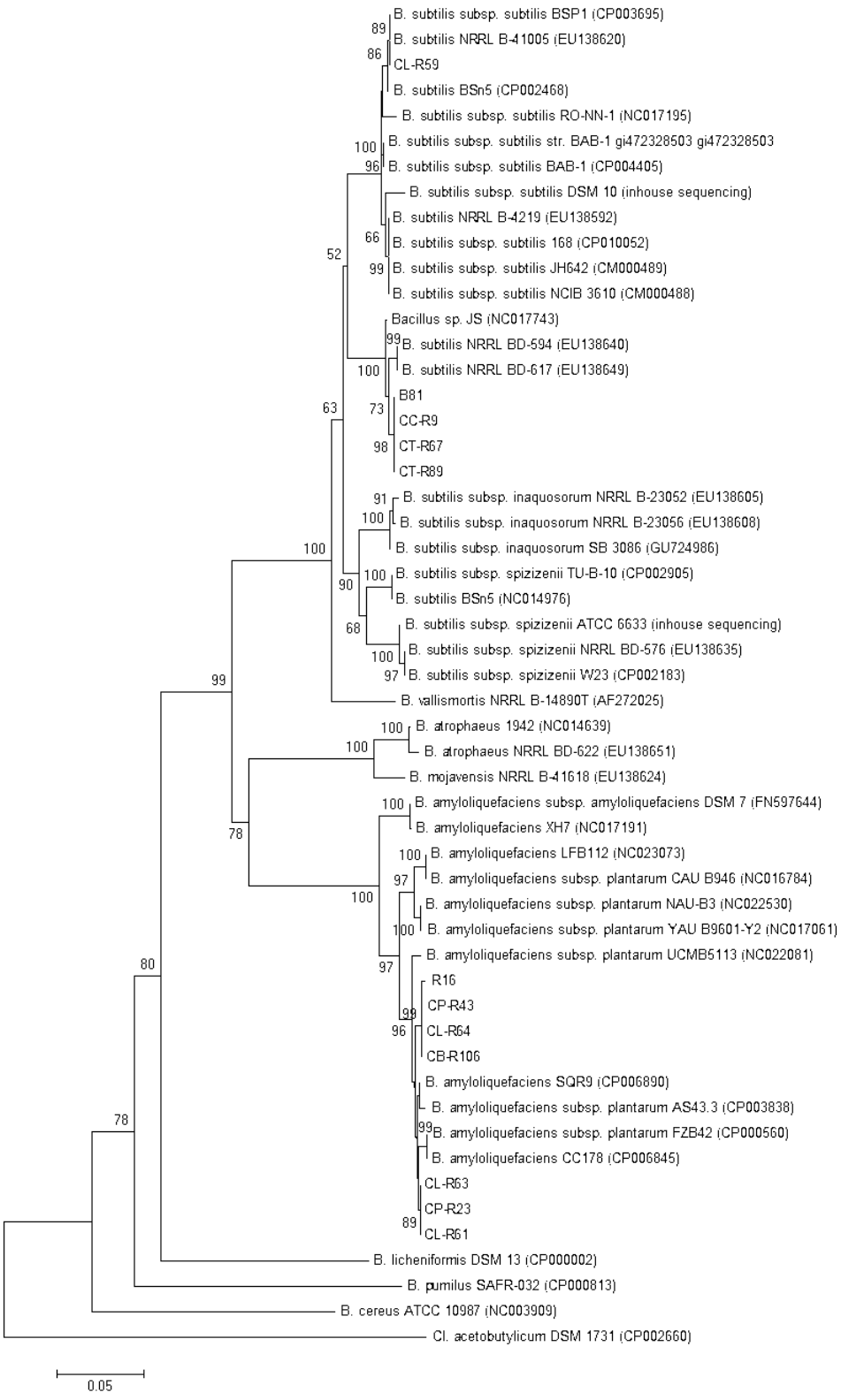


Figure 2.7 Neighbour-joining phylogenetic tree inferring the evolutionary relationship between the endospore-forming isolates and selected reference sequences based on partial *gyrA* nucleotide sequences. Only bootstrap values ≥50% are included. The scale bar corresponds to 0.05 nucleotide substitutions per sequence position. Genus abbreviations: *B. = Bacillus; Br. = Brevibacillus; Cl. = Clostridium.*

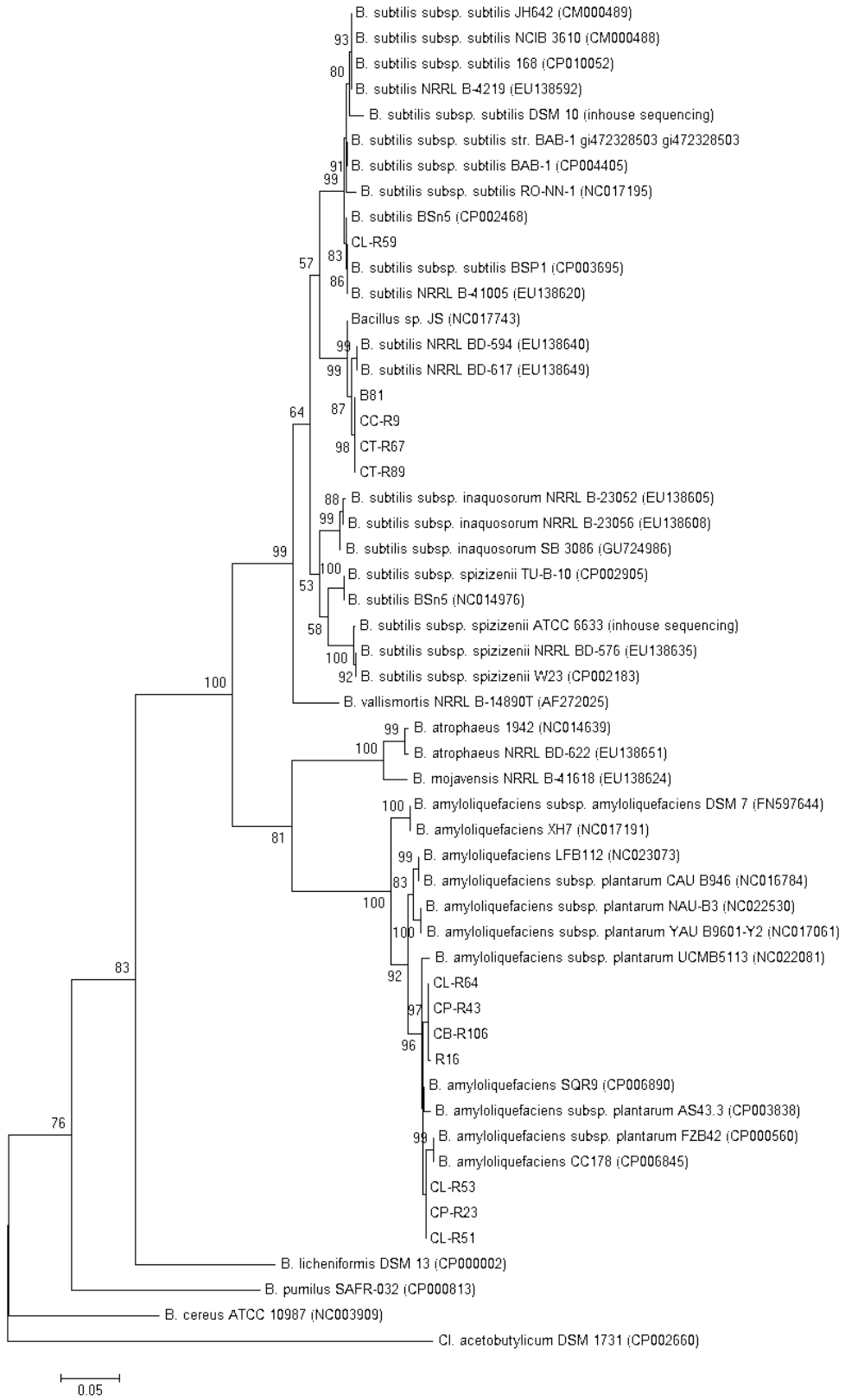


Figure 2.8 Maximum-likelihood phylogenetic tree inferring the evolutionary relationship between the endospore-forming isolates and selected reference sequences based on partial *gyrA* nucleotide sequences. Only bootstrap values ≥50% are included. The scale bar corresponds to 0.05 nucleotide substitutions per sequence position. Genus abbreviations: *B. = Bacillus; Br. = Brevibacillus; Cl. = Clostridium.*

2.4 Discussion

The application of biocontrol microorganisms to the propagation and cultivation of plants within greenhouses and nurseries has been regarded as an important focus area for developing environmentally friendly practices for controlling plant diseases (Paulitz and Bélanger, 2001). The degree to which environmental conditions can be controlled within such structures favours biological control strategies, theoretically allowing for greater consistency in efficacy to be achieved (Schroth and Becker, 1990). Composted bark media have been used globally as growing media and are regarded as promising sources from which to isolate and screen microrganisms for biocontrol traits (Hoitink and Fahy, 1986; Scheuerell *et al*., 2005; Suàrez-Estrella *et al*., 2013). In particular, aerobic endospore-formers that exhibit antagonism of phytopathogens have been identified as promising candidates for biological control applications. Several studies have previously identified strains of *Bacillus* spp. that antagonize *R. solani* and afford some biological control protection *in vivo* in plant types such as tomato (Asaka and Shoda, 1996; Szczech and Shoda, 2004), cucumber (Huang *et al*., 2012), lettuce (Chowdhury *et al*., 2013), cotton (Brannen and Kenny, 1997), chili pepper (Mojica-Marin *et al*., 2008) and maize (Ugoji and Laing, 2008). Taking into account that *Bacillus* species are prominent members of compost microflora communities (Strom, 1985), this study was undertaken to screen for potential biocontrol strains that antagonize *R. solani* associated with cucumber damping-off disease. *In vitro* and *in vivo* screening methods were used in conjunction with various genomic fingerprinting approaches. The major aim of the study was to determine diversity levels amongst isolates and identify closely related groups; thereby providing information that could assist in selecting promising organisms for further evaluation.

In order to maximize the pool of potential candidate organisms to be screened, endospore-forming bacteria from the rhizosphere were isolated from five plant types known to be susceptible to *Rhizoctonia* damping-off disease. These plant species were also chosen as representative crop plants that are commonly propagated at nurseries in KwaZulu-Natal, South Africa using composted pine bark media. This approach was adopted to target organisms from locations that are ecologically relevant to the sites of their intended use (Cook and Baker, 1983).

2.4.1 *In vitro* screening

Approximately 6.25% of the 400 isolates tested exhibited some degree of antagonism towards *R. solani* in the preliminary dual-culture bioassays performed. The formation of clear inhibition zones around isolates in these assays was considered to be an indication of antibiosis. Although a number of mechanisms (e.g. siderophore production, lytic enzymes) have been associated with fungal antagonism *in vitro*, antibiotic production is considered a major contributor to biocontrol activity by *Bacillus* spp. (Raaijmakers *et al*., 2002; Stein, 2005; Ongena and Jacques, 2008). This assertion is further supported by studies indicating that antibiotic-deficient mutants lose biocontrol efficacy (Asaka and Shoda, 1996). Dual-culture bioassays have, therefore, been widely used as a simple, reproducible means to screen for antimicrobial activity *in vitro* and are used as a convenient starting point from which to select isolates for further biological control evaluation (Kloepper and Schroth, 1981; Anith *et al*., 2003; Pliego *et al*., 2011).

Results from the dual-culture bioassays showed that isolates differed in their ability to antagonize the test organisms used in the study (Table 2.3). The extent and stability of inhibition zones over time varied between isolates. Approximately fifty-five percent of the isolates screened inhibited all the four fungal species tested, indicating that the inhibitory compound(s) produced by these isolates have "broad" fungicidal activity. Of these, seven isolates retained prominent zones of inhibition against *R. solani* even after 168 h of incubation, which attests to the environmental stability of the compound(s) produced by these organisms. However, for four isolates no inhibition was detected after 168 hours incubation indicating that the active compound(s) produced lost activity over the same incubation period.

Several studies have reported that strains of *B. amyloliquefaciens* and *B. subtilis* are able to inhibit a broad range of fungal pathogens (Kim *et al*., 1997; Yoshida *et al*., 2001; Ahmad *et al*., 2008; Arguelles-Arias *et al*., 2009). Isolates demonstrating this trait are of particular interest since they could be effective biological control agents against more than one fungal pathogen (Berg *et al*., 2001). *Bacillus* spp. are known to produce a wide range of bioactive compounds, with varied chemical structures and properties, and hence, cannot be expected to behave identically under *in vitro* test conditions (Stein, 2005; Ongena and Jacques, 2008). Furthermore, some strains produce more than one type of antibiotic compound which can act

synergistically under *in vitro* conditions (Chen *et al*., 2009, Liu *et al.*, 2014). When antibiotic substances are produced by Gram positive bacteria *in vitro,* they are usually excreted and diffuse into the surrounding medium, resulting in a concentration gradient that decreases with increasing distance from the producer colony or cell. A number of biological, physical and chemical parameters influence the size of the inhibition zone formed in agar-based diffusion assays. For example, the solubility, molecular weight, hydrophobicity, charge and stability of the active compound(s) produced will influence its (their) rate of diffusion in agar. In addition, the antibiotic production is also governed by various regulatory mechanisms controlling gene expression, which in turn are influenced by the pH, temperature and nutrient composition of the test medium. The duration of the assay, the rate of growth of the test organism, as well as the timing of its application can also influence the outcome of an assay (Perez *et al*., 1992; Leifert *et al*., 1995; Bernal *et al*., 2002; Caldeira *et al*., 2008). The ranking of dual-culture bioassays purely on the basis of zone of inhibition should therefore be viewed with caution. The results from this study indicated that additional parameters such as the stability of the inhibitory response *in vitro* and the spectrum of antifungal activity also provided useful information which could assist in ranking the performance of the *in vitro* bioassays.

Overall, the results show that *in vitro* screening can serve as a useful starting point in a search for biological control candidates; however, this screening approach has several short-comings. Dual-culture bioassays are inherently selective and therefore have the potential to exclude promising organisms that exhibit alternative modes of action (Pliego *et al*., 2011). *In vitro* bioassays provide little or no information regarding the taxonomic affiliations of isolates or the types of antimicrobial compound(s) produced. Furthermore, these types of assays do not take into account biotic or abiotic factors such as the competing microflora, nutrient status, water availability, redox conditions and pH that influence host-antagonist-pathogen interactions within the rhizosphere environment. Nonetheless, the "ranking" of candidate organisms based on the level of growth inhibition observed *in vitro* has still been used widely as a criterion for selecting isolates for further testing (Idris *et al*., 2007; Pliego *et al*., 2011). This approach assumes that the extent of *in vitro* inhibition correlates closely with biocontrol efficacy *in vivo*. It also overlooks ecological considerations associated with gene expression and antibiotic production *in situ*, as well as the fact that different compounds will behave differently under *in vitro* test conditions. This is borne out by a number of biological control

studies that have reported instances of poor correlation between *in vitro* inhibition levels and greenhouse performance (Reddy and Hynes, 1994; Bevivino *et al*., 1998; Cavaglieri *et al*., 2005; Idris *et al*., 2007, Liu *et al*., 2014). Evidence of *in vitro* antagonism by an isolate is therefore not a reliable indicator of success in subsequent greenhouse biocontrol trials and thus, *in vivo* screening is an essential component of any biocontrol screening programme.

2.4.2 *In vivo* screening

The *in vivo* screening experiments carried out in this study were performed in a greenhouse to simulate "real world" conditions as closely as possible. It was evident from the *Rhizoctonia* infested controls (Table 2.5 and 2.6) that the bark medium was not naturally suppressive, suggesting that the resident microbial populations did not play an important role in any of the positive biocontrol results obtained.

The first pot trial showed that the level of pathogen infestation played an important role in influencing disease severity. At the higher levels of pathogen loading tested, the mitigating effect of the bacterial antagonist (R16) applied was noticeably diminished. Although single barley grains colonized by *Rhizoctonia* gave rise to the lowest levels of disease incidence in the first trial, when applied to seedling trays with smaller well volumes, it soon became evident that the pathogen loading rate was too great for the system, resulting in extensive damping-off in all treatments. These results highlighted the empirical nature of this *in vivo* screening approach. Standardization of the amount of pathogen inoculum added was therefore identified as one of the biggest challenges faced in setting up the biocontrol trials. This problem was further compounded by the fact that *R. solani* does not produce conidia and only rarely produces basidiospores (González-García *et al.*, 2006). Consequently, the pathogen dosage had to be applied as vegetative biomass rather than in the form of more easily quantifiable reproductive structures thereby causing variation.

In a greenhouse biocontrol trial similar to the ones described in this study, Yobo *et al*. (2004) found that lower levels of disease severity resulted when the pathogen was seeded in the form of colonized agar plugs. Consequently, when the greenhouse trial was repeated during the present investigation, this approach was adopted and data that showed significant differences between treatments (P=0.05) were obtained, with a number of isolates being identified that provided significant levels of biocontrol against *R. solani* damping-off compared to the

diseased controls (Table 2.6). Overall, seedling survival and dry biomass data proved more valuable than germination data in assessing biocontrol potential and identifying promising biocontrol candidates. Seven isolates (CC-R9, CP-R43, CP-R45, CL-R51, CT-R67, CB-R106 and R16) allowed for seedling survival levels greater than 75%, which were not significantly different from the positive control ($p=0.05$, Table 2.6). Treatment with four of these isolates (CP-R43, CP-R45, CL-R51 and CT-R67) also resulted in final dry plant biomass levels that were between 75 % and 79.3% of the positive control's final value ($P<0.001$). Generally, isolates that achieved high *in vitro* inhibition rankings against *R. solani* and exhibited prolonged antifungal activity over the course of the dual-culture bioassay were amongst the best performing isolates when screened *in vivo*. A notable exception was CL-R53, which performed poorly in the biocontrol seedling trial; possible explanations for this are discussed later.

The choice of bacterial inoculant application is an important variable that has been found to impact biocontrol plant trials (Koch *et al*., 1998). Several reports indicate that *Bacillus* sp. drenches provide higher levels of biocontrol against phytopathogens than seed coating with the same bacterium (Georgakopoulos *et al*., 2002; Szczech and Shoda, 2006). For this reason drenching was adopted as the preferred means of applying microbial inocula in the *in vivo* pot trials. Application rates of at least $1x10^6$ cells x ml^{-1} or higher have been recommended for *Bacillus* spp. (Asaka and Shoda, 1996; Collins and Jacobsen, 2003). The results from pot trials conducted in this study established that an application rate of ~2 $x10^5$ cells per cm^2 (or ~10^7 cells per treatment) was adequate for detecting biocontrol effects during *in vivo* screening.

In the preliminary pot trial a possible growth promotion effect was also noted when R16 drenches (~2 $x10^5$ cells per cm^2) were applied to pathogen-free treatments. However, this trend was not evident when a higher application rate (~2 $x10^6$ cells per cm^2) was used. No obvious reason for this discrepancy was apparent. Various growth promotion mechanisms such as nutrient mineralization and/or the production of plant growth stimulators have been reported for *Bacillus* spp. previously (Kloepper, 1993). It is possible that at high cell concentrations a threshold density was reached whereby the bacterial inoculant (R16 in this study, Table 2.5) competed with the host plant for nutrients. An alternative explanation is that

the regulation of plant growth promoting mechanisms may be cell density dependent thereby accounting for the differences observed.

The level of sporulation within an inoculum together with the viability of spores present is another variable that can influence the outcomes of biocontrol plant trials. For example, the application of vegetative cells of *B. subtilis* BacB has been shown to provide significantly better control of *Cercospora* leaf spot in sugar beet under greenhouse conditions than did an equivalent spore formulation (Collins and Jacobsen, 2003). For ease of use, the inocula used in the *in vivo* pot trials were prepared from overnight (16 h) TSB cultures. Preliminary studies using isolate R16 revealed that broth cultures contained motile vegetative cells with no evidence of sporulation. However, in the subsequent pot trial nine of the twenty-seven isolates evaluated exhibited varying levels of sporulation ranging from about 2-22%. Interestingly, most of these were isolates that all displayed very similar colony morphologies (Table 2.4). Representatives of these (e.g. CP-R15, CP-R42, CT-R77) were amongst the poorest performers in terms of influencing seedling survival and plant biomass production. These findings suggest that sporulation levels and/or the metabolic state of cells in an inoculum could be a major contributing factor of an isolate's performance in biocontrol trials. This variable warrants further investigation in future studies in order to optimize the efficacy of biocontrol screening trials.

Results from the *in vivo* pot trial also showed that a number of the strains isolated from the different plant species performed as well as, or better than, those isolated from cucumber roots. The composted bark medium had not previously been used to grow cucumber plants and would thus not have been enriched for a cucumber-specific rhizosphere population. Thus, the isolates obtained from this study may reflect populations of endospore-formers naturally present in the bark medium that are attracted to the rhizosphere environments of various plant species by the release of root exudates that stimulate microbial root colonization (Somers *et al*., 2004). This suggests that some of the rhizobacteria isolated from the roots of different plants exhibit broad-spectrum rhizosphere competency. This approach to isolating antagonistic rhizobacteria has met with some success previously. For example, Berg *et al*. (2001) isolated rhizobacterial strains from different plant species, susceptible to *Verticillium dahliae*, that were effective in antagonizing the same pathogen in strawberries cultivated under greenhouse conditions.

The results from *in vivo* screening showed that several promising biocontrol candidates could be short-listed for further evaluation. However, a major drawback of this screening approach is the laborious, resource intensive, and often fickle nature of the work involved; particularly when large numbers of isolates were screened. The value of identifying and grouping closely related strains for dereplication purposes was therefore recognized. Such information would be useful in rationalising large numbers of bacterial isolates into smaller, more manageable groups, thereby assisting the selection of candidate organisms for further evaluation.

2.4.3 Genomic fingerprinting

Molecular fingerprinting methods potentially offer a simple and consistent means of differentiating between rhizosphere isolates exhibiting biological control traits. In this study ITS-PCR and RAPD-PCR were evaluated as a means of establishing the levels of genetic diversity within a collection of aerobic endospore-former isolates that exhibit *in vitro* antagonism of *R. solani.* Both methods have previously been used to distinguish inter- and intra-specific diversity amongst members of the genus *Bacillus* and neither requires extensive preparative steps. These methods have the added advantage that they are suitable for screening unidentified isolates without the necessity for prior information relating to their genetic make-up.

ITS-PCR fingerprint profiles proved useful for establishing preliminary differentiation between isolates and for grouping taxonomically related species into clusters for further analysis. Three major groupings were distinguished based on UPGMA analysis of the banding profiles (Fig. 2.1). Group I included reference strains belonging to the "*Bacillus subtilis"* group of related taxa. Their relationship to environmental isolates with comparable banding profiles was subsequently confirmed by 16S rRNA gene sequence analysis (Table 2.7). The band profiles obtained for Group II isolates showed similar organization to those of Group I, but were clearly distinguished from the latter based on length polymorphisms. Comparisons to sequences in the NCBI Genbank and SILVA databases revealed that the 16S rRNA gene sequences of Group II isolates were identical (100% similarity) to strains of *B. cereus*, *B. thuringiensis* and *B. toyonensis*. The ITS-PCR profile for Isolate CC-R4 (Group III) was distinct from all of the other isolates screened and 16S rRNA gene sequence analysis revealed a close match strains of *Brevibacillus laterosporus.* This finding was consistent with

earlier microscopic observations that endospores of Isolate CC-R4 are laterally situated within the cells, a characteristic trait of *B. laterosporus* strains (Shida *et al*., 1996).

Similar ITS-PCR banding profiles to those recorded here for strains of *B. subtilis* and *B. cereus* have been reported in the literature (Daffonchio *et al*., 1998; Daffonchio *et al*., 2000; Martinez and Sineriz, 2004). The multiple bands observed were attributed to the presence of multiple copies of the ribosomal operon; a feature previously described for members of the genus *Bacillus* (Daffonchio *et al*., 1998). Polymorphism has also been attributed to sequence variation and the occurrence of one or more copies of the tRNA gene within the ITS region (Nagpal *et al*., 1998; Shaver *et al*., 2002). Daffonchio *et al*. (2003) also showed that cross-hybridization of different ribosomal operons can lead to the formation of heteroduplex artifacts. They found that improved differentiation of species and strains could be achieved using ITS-homoduplex-heteroduplex (HHP) analysis; a technique that resolves ITS-PCR products on a polyacrylamide gel allowing more complex banding patterns to be determined.

A major drawback of the ITS-PCR method is its limited ability to detect inter- and intra-specific variation amongst closely related species/organisms. This problem was recognized by Daffonchio *et al*. (1998) who had to use additional typing methods, such as single-strand conformation polymorphism and/or RAPD-PCR fingerprinting to further differentiate between closely related members of the genus *Bacillus.* RAPD-PCR, which is considered a relatively simple and robust typing method, was therefore used to further separate the isolates screened in this study. This method generated strain-specific fingerprint profiles which allowed the clonal relationships of strains to be determined (Logan and De Vos, 2009). Previously, this approach has been successfully used to type strains of *B. cereus* (Nilsson *et al*., 1998), *B. licheniformis* (Daffonchio *et al*., 1998) and *B. thuringiensis* (Brousseau *et al*., 1993; Konecka *et al.*, 2007).

RAPD-PCR profiles supported the ITS-PCR findings but also revealed further levels of genetic diversity within each of the ITS groupings. Each of the four arbitrary primers used produced reproducible banding patterns that allowed for grouping and matching of the isolates. However, the levels of genetic heterogeneity detected varied somewhat depending on the primer used and the group of organisms screened. This observation supports the notion

that the use of several different primers in RAPD-PCR improves the likelihood of detecting subtle differences between closely related strains (Nilsson et al., 1998).

RAPD profiles for the Group II isolates ("*Bacillus cereus* "group) exhibited varying levels of homology depending on the primer used. Some inconsistency was also apparent in the clusters produced by each primer. Some of the observed intra-specific diversity could be attributed to the fact that strains of *B. cereus* and *B. thuringiensis* can have a large number of strain specific genes that may account for up to 15-16% of the genome (Økstad and Kolstø, 2011). This variability in gene content amongst different strains can be explained by the Pan-genome concept which proposes that bacterial genomes have a "core" component of essential genes as well as a more variable set of strain specific genes arising from localized environmental adaptations. Members of these two genera are also known to house a diverse array of one or more plasmids of varying size (2-600 Kb) (Rasko *et al*., 2005; Kolstø *et al*., 2009).

Four distinct groups were distinguished amongst the 16 isolates that were clustered within the "*Bacillus subtilis"* group (Group I) as a result of the ITS-PCR profiling. Clonal similarities were evident in a number of the strains isolated from different plant species. This finding possibly reflects the rhizosphere competence of these strains. Interestingly, strains R16 and B81, which had been isolated from rhizosphere soil several years prior to this study, were amongst the isolates placed within two of these clusters. Furthermore, isolates representative of clusters A, B and D were amongst the best performers in the *in vivo* biocontrol pot trial. In general, the members of these clusters consistently performed better than those of the "*Bacillus cereus"* group (Group II). Several exceptions were noted, such as CL-R53 (cluster B) and CT-R89 (cluster D) which both performed poorly in the *in vivo* trial. Intra-strain variability that affects rhizosphere competency and/or biocontrol properties might account for the poor biocontrol performance observed for these isolates. For future studies, a comparison of these isolates with related strains that performed well in the *in vivo* screening trial could provide a very useful basis from which to identify traits associated with enhanced biocontrol performance.

Although biocontrol activity has been positively correlated to antibiotic production it is also impacted by an organism's ability to colonize roots and persist within the rhizosphere after

seed inoculation (Berg and Smalla, 2009; Compant *et al.*, 2010). For *Bacillus* spp. an ability to colonize roots has been linked to chemotaxis, swarming motility and biofilm formation (Dietel *et al.*, 2013; Zhang *et al.*, 2014). Though poorly understood, the production of antifungal lipopeptide compounds within the rhizosphere has also been positively correlated with biofilm formation and was found to be influenced by nutrient status and oxygen availability (Nihorimbere *et al.*, 2012). Significantly, the composition and relative abundance of lipopeptides produced within a rhizosphere environment were found to differ considerably from the lipopeptide signature produced *in vitro* under laboratory conditions (Nihorimbere *et al*., 2012). The lipopeptide surfactin has been shown to play an important role in biofilm formation by *Bacillus* spp. within rhizosphere and phylloplane environments (Nihorimbere *et al*., 2012; Zeriouh *et al.*, 2014). For example, mutants deficient in the ability to produce surfactin were found to negatively impact the synthesis of exopolysaccharide and TasA protein, major components of the extracellular matrix of biofilms (Zeriouh *et al.*, 2014). The mechanisms regulating surfactin production appear to be intricately linked with systems controlling cellular differentiation, competence development, quorum sensing and biofilm formation (Hamoen *et al*., 2003; Duitman *et a*l., 2007). Factors that affect bacterial distribution and influence root colonization are therefore important determinants of the *in vivo* performance of biocontrol candidates. Deficiencies in any of these traits could account for the poor *in vivo* performance of strains like CL-R53 that showed strong *in vitro* antagonism of *R. solani*.

A criticism of RAPD-PCR has been that it is prone to poor reproducibility, particularly between different laboratories (MacPherson *et al*., 1993; Rademaker *et al*., 2005). However, standardization of key parameters, such as DNA-preparation, amplification conditions and the equipment used, have been shown to minimise the short-comings sometimes encountered with this method (Damiani *et al*., 1996). In this study, good reproducibility was achieved when DNA template concentrations were kept within a 50-100 ng concentration range. Nilsson *et al*. (1998) found that reproducible banding profiles among *B. cereus* strains could be obtained using crude DNA template derived directly from colony pick-offs. They did acknowledge however, that individual bands varied in intensity and additional faint bands were sometimes visible. This was attributed to variations in template DNA concentration which increase the likelihood of non-specific amplification occurring, particularly when PCR conditions with low levels of stringency are used (Roux, 1995). This approach shows promise

as a simple, fast and cost effective means of screening large numbers of isolates by circumventing the need for laborious purification steps commonly associated with obtaining chromosomal DNA from bacterial isolates (Nilsson *et al*., 1998).

2.4.4 16s rRNA and *gyrA* gene sequence analysis

16S rRNA gene sequence analysis is useful for classifying and distinguishing phylogenetic clusters amongst members of the genus *Bacillus* (Ash *et al*., 1991). Sequence similarities ranging from 98.2 to 99.0% have been recommended as the threshold from which to differentiate bacterial species (Stackebrandt and Ebers, 2006; Meier-Kolthoff *et al*., 2013; Kim *et al*., 2014). However, within the genus *Bacillus* a number of distinct 16S RNA gene sequence groups have been identified that contain several closely related species that possess 16S rRNA gene sequences that are virtually indistinguishable (>99% similarity) from one another (Fox *et al*., 1992; Logan and De Vos, 2009; Rooney *et al*., 2009). Specifically, these groups include representatives of the "*Bacillus subtilis"* group and "*Bacillus cereus"* group of closely related taxa. The phylogenetic analysis of highly conserved housekeeping, or other protein encoding genes loci can provide higher resolution than 16S rRNA gene sequences and has been recommended as a useful means of differentiating closely related *Bacillus* species (Chun and Bae, 2000; Logan *et al.*, 2009). Chun and Bae (2000) showed that comparative sequence analysis of *gyr*A gene fragments coding for DNA gyrase subunit A was able to differentiate members of the "*Bacillus subtilis"* group that could not be distinguished by means of phenotypic testing or 16s rRNA gene sequence analysis. For this reason phylogenetic analysis of partial *gyr*A genes was undertaken to further differentiate Group I isolates that showed high levels of similarity (≥99%) when 16S rRNA gene sequence analysis was performed.

16S rRNA gene sequence analysis revealed that most of the of isolates evaluated fell within either the "*Bacillus subtilis"* group or "*Bacillus cereus"* group of closely related taxa (Table 2.7, Fig. 2.5 and 2.6). Isolate CC-R4 was the exception, being identified as a strain of *Brevibacillus laterosporus*. These findings were not unexpected and are consistent with the biological control literature which contains a mass of data linking representatives from each of these groups to biological control and/or plant growth promotion traits (Ongena *et al*., 2010; Borriss, 2011; Cawoy *et al*., 2011). Members of the "*Bacillus subtilis"* group, in particular, appear to have received the greatest attention in this regard; conversely, reports

linking *Br. laterosporus* to biocontrol applications appear to be relatively sparse (de Oliveira *et al*., 2004; Saikia *et al.*, 2011). Various strains of the "*Bacillus cereus"* group, including *B. cereus* UW85, also exhibit plant growth promoting traits and have been extensively studied by some groups (Emmert and Handelsman, 1999; Emmert *et al*., 2004). Although *B. thuringiensis* is widely associated with insect biocontrol, there are some reports suggesting that certain strains are also antagonistic to phytopathogens (Zhao *et al*., 2007; Mojica-Marín *et al*., 2008). The association of *B. cereus* with toxin production, food poisoning and opportunistic infections, has lessened their appeal for development as commercial inoculants (Calvo *et al*., 2010). On the basis of this, and the fact that isolates grouped within the "*Bacillus cereus"* group did not perform strongly in the *in vivo* pot trial, these isolates were excluded from further investigation and characterization.

Although 16S rRNA gene sequencing of Group I isolates revealed - as expected on microbiological grounds - very high levels of sequence similarity (≥99%), representatives of each RAPD fingerprint cluster could be distinguished and matched to distinct species or strains based on sequence data obtained from the NCBI Genbank and SILVA databases. Cluster A and B isolates were all matched to strains of *B. amyloliquefaciens* and *B. amyloliquefaciens* subsp. *plantarum* (100% similarity). Significantly, whole genome studies on three of the matching strains, namely, strains FZB42, CAUB946 and YAUB9601-Y2 have recently been undertaken, due to interest in their biological control and/or plant growth promoting traits (Chen *et al*., 2007; Blom *et al.*, 2012; Hao *et al*., 2012). *B. amyloliquefaciens* subsp. *plantarum,* FZB42, in particular, has been studied extensively and has been developed commercially as a plant growth promoter/biofertilizer (Borriss *et al*., 2006). It is interesting to note that the RAPD fingerprints of isolates from these clusters were clearly distinguishable from the phylogenetically related reference strain *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^{T}; suggesting that the RAPD fingerprinting method can differentiate strains at the subspecies level. Furthermore, strain CL-59 (cluster C) which matched closely to strains of *B. subtilis* subsp. *subtilis,* including the type strain DSM10^{T}, also produced RAPD fingerprints distinct from this reference strain; possibly indicating further intraspecific diversity. Isolates grouped within cluster D exhibited a ≥99.8% similarity to a number of species that fall within the "*Bacillus subtilis"* group, but no clear species delineation was evident. However, it is interesting to note that these isolates showed 100% sequence

similarity to *Bacillus* sp. J2, a plant growth promoting rhizobacterium found to enhance the growth of tobacco and lettuce (Song *et al*., 2012).

From *gyrA* sequence analysis it was evident that Group I isolates displayed greater levels of genetic variation than the corresponding 16S rRNA gene sequences had indicated, allowing for clearer distinctions to be made when evolutionary distances were inferred (Fig. 2.7 and 2.8). It was also apparent that the isolates were grouped according to their RAPD fingerprint clusters and these were consistent with the species matched during 16S rRNA gene sequence analysis (Tables 2.7 and 2.8). Isolates from clusters A and B were again very closely related to strains of *B. amyloliquefaciens* and *B. amyloliquefaciens* subsp. *plantarum* and could be distinguished from reference strain *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^{T}. These findings give credence to the notion that these isolates could represent members of bona-fide rhizosphere populations rather than being incidental or transitory inhabitants.

Borriss *et al.* (2011) used phylogenetic analysis of partial *gyr*A and *che*A gene sequences as a basis to sub-divide *B. amyloliquefaciens* into two separate subspecies; namely, *B. amyloliquefaciens* subsp. *plantarum* and *B. amyloliquefaciens* subsp. *amyloliquefaciens.* Distinction between these sub-species was also based on their potential to synthesize non-ribosomal lipopeptides and polyketide compounds. The difference between strains of *B. amyloliquefaciens* was first recognised by Reva *et al.* (2004) who showed that plant associated strains were better adapted to plant colonization than other strains within the species. They also proposed that these strains represented a distinct ecotype.

Plant associated *B. amyloliquefaciens* strains have been increasingly associated with biological control and plant growth promotion applications in the literature (Chen *et al*., 2009; Alvarez *et al*., 2011). Antagonism of plant pathogens has been widely associated with their ability to produce a range of lipopeptide compounds (Arguelles-Arias *et al*., 2009; Zhang *et al*., 2012). A number of these secondary metabolites have also been linked to rhizosphere competence, plant-microbe interactions and persistence within the rhizosphere (Ongena *et al*., 2007; Raaijmakers *et al*., 2010; Nihorimbere *et al*., 2012). Interestingly, Boriss *et al.* (2011) speculated that based on g*yrA* sequence evidence many strains of *B. subtilis* described in the biocontrol literature are in fact strains of *B. amyloliquefaciens*.

Phylogenetic analysis of cluster D *gyr*A sequences revealed that these isolates, along with *Bacillus* sp. J2, formed a distinct sub-cluster within the *B. subtilis* clade (Fig. 2.7 and 2.8). This phylogenetic group was clearly delineated from recognized subspecies of *B. subtilis,* which were separated into distinct groupings, supported by high bootstrap values. Further characterization of cluster D isolates is therefore warranted to ascertain whether or not these strains might represent an ecotype or possibly even a new subspecies of *B. subtilis*. Minimum standards for describing novel aerobic endospore-forming bacteria have been recommended by Logan *et al.* (2009) and advocate a polyphasic approach which integrates the characterization of morphological, physiological and biochemical traits with chemotaxonomic characters, phylogenetic analysis and DNA–DNA hybridization (DDH) relatedness studies with closely related species. Multi-locus sequence typing (MLST) has also gained favour as an effective method for delineating species (Sokorin *et al.*, 2006; Madslien *et al.,* 2012). The advent of high throughput DNA sequencing technologies has also allowed for direct computational comparisons of genomes to be made. Methods such as average nucleotide identity (ANI) and genome BLAST distance phylogeny (GBDP) have been proposed as possible replacement for DDH which is considered to be more labour-intensive and error-prone (Kim *et al.*, 2014).

Overall, this study showed that ITS-PCR and RAPD-PCR could be used to group closely related organisms and further establish the levels of diversity apparent amongst isolates sampled from different host plant species. As expected, RAPD-PCR proved to be more effective than ITS-PCR in distinguishing between closely related strains and there was evidence suggesting that intra-species differences could be distinguished amongst isolates. 16S rRNA and *gyr*A gene sequences supported the groupings constructed using fingerprinting results and provided evidence that several of the bacterial groupings distinguished amongst the isolates were similar to biocontrol organisms reported in the literature. This information provides a useful insight into the prevalence, distribution and possible rhizosphere competency of related strains. When used in conjunction with other *in vitro* and *in vivo* screening methods, such information could be useful in short-listing candidate biological control organisms for further evaluation and characterization.

2.5 References

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

Alvarez, F., Castro, M., Príncipe, A., Borioli, G., Fischer, S., Mori, G. and Jofré, E. (2011) The plant-associated *Bacillus amyloliquefaciens* strains MEP_218 and ARP_23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of *Sclerotinia* stem rot disease. Journal of Applied Microbiology, 112, 159-174.

Anith, K.N., Radhakrishnan, N.V. and Manmohandas, T.P. (2003) Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (*Piper nigrum*). Microbiological Research, 158, 91-97.

Arguelles-Arias, A., Ongena, M., Halimi, B., Lara, B., Brans, A., Joris, B. and Fickers, P. (2009) *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microbial Cell Factories, 8, 63-71.

Asaka, O. and Shoda, M. (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Applied and Environmental Microbiology, 62, 4081-4085.

Ash, C., Farrow, J.A.E., Wallbanks, S. and Collins, M.D. (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-rRNA sequences. Letters in Applied Microbiology, 13, 202-206.

Bais, H.P., Fall, R. and Vivanco, J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology, 134, 307-319.

Barnes, E.H. (1979) Atlas and Manual of Plant Pathology 2nd Edition. Plenum Press, New York, USA, 327 p.

Berg, G., Fritze, A., Roskot, N. and Smalla, K. (2001) Evaluation of potential biocontrol rhizobacteria, from different host plants of *Verticillium dahliae* Kleb. Journal of Applied Microbiology, 91, 963-971.

Berg, G., Marten, P. and Ballin, G. (1996) *Stenotrophomonas maltophilia* in the rhizosphere of oilseed rape: occurrence, characterisation and interaction with phytopathogenic fungi. Microbiological Research, 151, 19-27.

Berg, G. and Smalla, K. (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere, FEMS Microbiol Ecology, 68, 1-13.

Bernal, G., Illanes, A. and Ciampi, L. (2002) Isolation and partial purification of a metabolite from a mutant strain of *Bacillus* sp. with antibiotic activity against plant pathogenic agents. Electronic Journal of Biotechnology, 5, 7-8.

Bevivino, A., Sarrocco, S., Dalmastri, C., Tabacchioni, S., Cantale, C. and Chiarini, L. (1998) Characterization of a free-living maize-rhizosphere population of *Burkholderia cepacia*:

Effect of seed treatment on disease suppression and growth promotion of maize. FEMS Microbiology Ecology, 27, 225-237.

Blom, J., Rueckert, C., Niu, B., Wang, Q. and Borriss, R. (2012) The complete genome of *Bacillus amyloliquefaciens* subsp. *plantarum* CAU B946 contains a gene cluster for nonribosomal synthesis of Iturin A. Journal of Bacteriology, 194, 1845-1846.

Borriss, R. (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents, In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses. Springer, Heidelberg, Germany, pp. 41-76.

Borriss, R., Bochow, H. and Junge, H. (2006) Use of *Bacillus subtilis*/*amyloliquefaciens* FZB strains for plant growth promotion and biocontrol. In: 7th International Workshop on Plant Growth Promoting Rhizobacteria, Program and abstract book, Noordwijkerhout, Netherlands, 15 p.

Borriss, R., Chen, X-H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., Fan, B., Pukall, R., Schumann, P., Sprőer, R., Junge, H., Vater, J., Pűhler, A. and Klenk, H-P. (2011) Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM7^{T} and FZB42^{T}: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. *nov*. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. *nov*. based on complete genome sequence comparisons. International Journal of Systematics and Evolutionary Microbiology, 61, 1786 -1801.

Brannen, P.M. and Kenney, D.S. (1997) Kodiak – a successful biological-control product for suppression of soil-borne plant pathogens of cotton. Journal Industrial Microbiology and Biotechnology, 19, 169-171.

Brousseau, R., Saint-Onge, A., Préfontaine, G., Masson, L. and Cabana, J. (1993) Arbitrary primer polymerase chain reaction, a powerful method to identify *Bacillus thuringiensis* serovars and strains. Applied and Environmental Microbiology. 59, 114-119.

Caldeira, A.T., Feio, S.S., Arteiro, J.M.S., Coelho, A.V. and Roseiro, J.C. (2008) Environmental dynamics of *Bacillus amyloliquefaciens* CCMI1051 antifungal activity under different nitrogen patterns. Journal of Applied Microbiology, 104, 808-816.

Calvo, P., Ormeño-Orrillo, E., Martínez-Romero, E. and Zúñiga, D. (2010) Characterization of *Bacillus* isolates of potato rhizosphere from Andean soils of Peru PERU and their potential PGPR characteristics. Brazilian Journal of Microbiology, 41, 899-906

Cavaglieri, L., Passone, A. and Etcheverry M. (2004) Screening procedures for selecting rhizobacteria with biocontrol effects upon *Fusarium verticillioides* growth and fumonisin B1 production. Research in Microbiology, 155, 747-754.

Cavaglieri, L., Orlando, J., Rodríguez, M.I., Chulze, S. and Etcheverry, M. (2005) Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and at the maize root level. Research in Microbiology, 156, 748-754.

Cawoy, H., Bettiol, W., Fickers, P. and Ongena, M. (2011) *Bacillus*-based biological control of plant diseases. In: Stoytcheva, M. (ed.) Pesticides in the Modern World - Pesticides Use and Management, InTech, Rijeka, Croatia, pp. 273-302.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut, P.R., Vater, J., Sűssmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G. and Borriss, R. (2007) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nature Biotechnology, 25, 1007-1014.

Chen, X.H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S. and Borriss, R. (2009) Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. Journal of Biotechnology, 140, 38-44.

Choudhary, D. K. and Johri, B. N. (2009). Interactions of *Bacillus* spp. and plants – With special reference to induced systemic resistance (ISR). Microbiological Research, 164, 493-513.

Chowdhury, S.P., Dietel, K., Rändler, M., Schmid, M., Junge, H., Junge, H., Borriss, R., Hartmann, A. and Grosch, R. (2013) Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. PLoS ONE, 8, e68818. doi:10.1371/journal.pone.0068818.

Chowdhury, S.P., Hartmann, A., Gao, X. and Borriss, R. (2015) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42: a review. Frontiers in Microbiology, 6, 780. doi:10.3389/fmicb.2015.00780.

Chun, J. and Bae, K.S. (2000) Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial *gyrA* gene sequences. Antonie van Leeuwenhoek, 78, 123-127.

Chung, W.C., Huang, J.W. and Huang, H.C. (2005) Formulation of a soil biofungicide for control of damping-off of Chinese cabbage (*Brassica chinensis*) caused by *Rhizoctonia solani*. Biological Control, 32, 287-294.

Collins, D.P. and Jacobsen, B.J. (2003) Optimizing a *Bacillus subtilis* isolate for biological control of sugar beet C*ercospora* leaf spot. Biological Control, 26, 153-161.

Compant, S., Clément, C. and Sessitsch, A. (2010) Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, 42, 669-678.

Cook, R.J. and Baker, K.F. (1983) The nature and practice of biological control of plant pathogens. American Phytopathological Society, St Paul, Minnesota, USA, 539 p.

Daffonchio, D., Borin, S., Frova, G., Manachini, P. and Sorlini, C. (1998) PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveal a different intraspecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. International Journal of Systematics and Evolutionary Microbiology, 48, 107-116.

Daffonchio, D., Cherif, A. and Borin, S. (2000) Homoduplex and heteroduplex polymorphisms of the amplified ribosomal 16S-23S internal transcribed spacers describe genetic relationships in the "*Bacillus cereus* group". Applied and Environmental Microbiology, 66, 5460-5468.

Daffonchio, D., Cherif, A., Brusetti, L., Rizzi, A., Mora, D., Boudabous, A. and Borin, S. (2003) Nature of polymorphisms in 16S-23S rRNA gene intergenic transcribed spacer fingerprinting of *Bacillus* and related genera. Applied and Environmental Microbiology, 69, 5128-5137.

Damiani, G., Amedeo, P., Bandi, C., Fani, R., Bellizzi, D. and Sgaramella, V. (1996) Bacteria identification by PCR-based techniques. In: Adolph, K.W. (ed.) Microbial Genome Methods. CRC Press, Boca Raton, USA, pp. 167-178.

de Bruijn, F.J., Rademaker, J., Schneider, M., Rossbach, U. and Louws, F.J. (1996) Rep-PCR genomic fingerprinting of plant-associated bacteria and computer-assisted phylogenetic analyses. In: Stacey, G., Mullin, B. and Gresshoff, P. (eds) Biology of Plant-Microbe Interaction, Proceedings of the 8th International Congress of Molecular Plant-Microbe Interactions, APS Press, pp. 497-502.

de Oliveira, E.J., Rabinovitch, L., Monnerat, R.J., Passos, L.K.J. and Zahner, V. (2004) Molecular characterization of *Brevibacillus laterosporus* and its potential use in biological control. Applied and Environmental Microbiology, 70, 6657-6664.

Dietel, K., Beator, B., Budiharjo, A., Fan, B. and Borriss, R. (2013) Bacterial traits involved in colonization of *Arabidopsis thaliana* roots by *Bacillus amyloliquefaciens* FZB42. The Plant Pathology Journal, 29, 59-65.

Duitman, E. H., Wyczawski, D., Boven, L. G., Venema, G., Kuipers, O. P. and Hamoen, L. W. (2007) Novel methods for genetic transformation of natural *Bacillus subtilis* isolates used to study the regulation of the mycosubtilin and surfactin synthetases. Applied and Environmental Microbiology, 73, 3490-3496.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Reseach, 32, 1792-1797.

Emmert, E.A.B. and Handelsman, J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiology Letters, 171, 1-9.

Emmert, E.A.B., Klimowicz, A.K., Thomas, M.G. and Handelsman, J. (2004) Genetics of Zwittermicin A production by *Bacillus cereus*. Applied and Environmental Microbiology, 70, 104-113.

Felici, C., Vettori, L., Toffanin, A. and Nuti, M. (2008) Development of a strain-specific genomic marker for monitoring a *Bacillus subtilis* biocontrol strain in the rhizosphere of tomato. FEMS Microbiology Ecology, 65, 289-298.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39, 783-791.

Fox, G.E., Wisotzkey, J.D. and Jurtshuk, P. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. International Journal of Systematic Bacteriology, 42, 166-170.

Francis, I., Holsters, M. and Vereecke, D. (2010) The Gram-positive side of plant-microbe interactions. Environmental Microbiology, 12, 1-12.

Freitas, D.B., Reis, M.P., Lima-Bittencourt, C.I., Costa, P.S., Assis, P.S., Chartone-Souza, E. and Nascimento, A.M.A. (2008) Genotypic and phenotypic diversity of *Bacillus* spp. isolated from steel plant waste . BioMed Central Research Notes, 1, 92. doi:10.1186/1756-0500-1-92.

Garbeva, P., van Veen, J.A. and van Elsas, J.D. (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. Microbial Ecology, 45, 302-316.

Georgakopoulos, D.G., Fiddaman, P., Leifert, C. and Malathrakis, N.E. (2002) Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. Journal of Applied Microbiology, 92, 1078-1086.

González-García, V., Portal Onco, M.A. and Rubio Susan, S.V. (2006) Review: Biology and systematics of the form genus *Rhizoctonia*. Spanish Journal of Agricultural Research, 4, 55-79.

Guttierez, W.A., Shew, H.D. and Melton, T.A. (1997) Sources of inoculum and management for *Rhizoctonia solani* damping-off on tobacco transplants under greenhouse conditions. Plant Disease, 81, 604-606.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95-98.

Hamoen, L.W., Venema, G. and Kuipers, O.P. (2003) Controlling competence in *Bacillus subtilis*:shared use of regulators. Microbiology, 149, 9-17.

Hao, K., He, P., Blom, J., Rueckert, C., Mao, Z., Wu, Y., He, Y. and Borriss, R. (2012) The genome of plant growth-promoting *Bacillus amyloliquefaciens* subsp. *plantarum* strain YAU B9601-Y2 contains a gene cluster for mersacidin synthesis. Journal of Bacteriology, 194, 3264-3265.

Heuer, H., Krsek, M., Baker, P., Smalla, K. and Wellington, E.M. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied and Environmental Microbiology, 63, 3233-3241.

Hoitink, H.A.J. and Fahy, P.C. (1986) Basis for the control of soilborne plant pathogens with composts. Annual Review of Phytopathology, 24, 93-114.

Huang, J.W. and Huang, H.C. (2000) A formulated container medium suppressive to *Rhizoctonia* damping-off of cabbage. Botanical Bulletin of Academia Sinica, 41, 49-56.

Huang, X., Zhang, N., Yong, X., Yang, X. and Shen, Q. (2012) Biocontrol of *Rhizoctonia solani* damping-off disease in cucumber with *Bacillus pumilus* SQR-N43. Microbiological Research, 167, 135-143.

Idris, H.A., Labuschagne, N. and Korsten, L. (2007) Screening rhizobacteria for biological control of *Fusarium* root and crown rot of sorghum in Ethiopia. Biological Control, 40, 97-106.

Jenson, M.A., Webster, J.A. and Straus, N. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Applied and Environmental Microbiology. 59, 945-952.

Katara, J., Deshmukh, R., Singh, N.K. and Kaur, S. (2013) Diversity analysis of *Bacillus thuringiensis* isolates recovered from diverse habitats in India using random amplified polymorphic DNA (RAPD) markers. Journal of Biological Sciences, 13, 514-520.

Kim, D.S., Cook, R.J. and Weller, D.M. (1997) *Bacillus* spp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology, 87, 551-558.

Kim, M., Oh, H-S., Park, S-C. and Chun1, J. (2014) Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. International Journal of Systematic and Evolutionary Microbiology, 64, 346-351.

Kinsella, K., Schulthess, C.P., Morris, T.F. and Stuart, J.D. (2009) Rapid quantification of *Bacillus subtilis* antibiotics in the rhizosphere. Soil Biology and Biochemistry**,** 41, 374-379.

Kloepper, J.W. (1993) Plant growth-promoting rhizobacteria as biological control agents. In: Metting B (ed.) Soil Microbial Technologies. Marcel Dekker, New York, pp. 255-274.

Kloepper, J.W., Ryu, C.M. and Zhang, S.A. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology, 94, 1259-1266.

Kloepper, J.W. and Schroth, M.N. (1981) Plant growth promoting rhizobacteria on radishes, In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Angers, France, pp. 879-882.

Koch, E., Kempf, H.J. and Hessenmüller, A. (1998) Characterization of the biocontrol activity and evaluation of potentialgrowth-promoting properties of selected rhizobacteria. Journal of Plant Disease Protection, 105, 567-580.

Kolstø, A.B., Tourasse, N.J. and Økstad, O.A. (2009) What sets *Bacillus anthracis* apart from other *Bacillus* species? Annual Review of Microbiology, 63, 451-476.

Konecka, E., Kaznowski, A. Ziemnicka, J. and Ziemnicki, K. (2007) Molecular and phenotypic characterization of *Bacillus thuringiensis* isolated during epizootics in *Cydia pomonella*. Journal of Invertebrate Pathology, 94, 56-63.

Krasilnikov, P. and Arnold, R. (2009) Soil classification of the Republic South Africa, in: P. Krasilnikov, P. Marti, I.J., Arnold, R. and Shoba, S. (eds), A Handbook of Soil Terminology, Correlation and Classification, EarthScan, London. UK, pp. 310-315.

Kumar, A., Prakash, A. and Johri, B.N. (2011) *Bacillus* as PGPR in crop ecosystem, In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses, Springer-Verlag, Berlin, Germany, pp. 37-56.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Danchin, A. *et al* (1997) The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature, 390, 249-256.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H. and Harbour, A. (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology, 78, 97-108.

Liu, J., Hagberg, I., Novitsky, L., Hadj-Moussa, H. and Avis, T. (2014) Interaction of antimicrobial cyclic lipopeptides from *Bacillus subtilis* influences their effect on spore germination and membrane permeability in fungal plant pathogens. Fungal Biology, 118, 855–861. doi: 10.1016/j.funbio.2014.07.004.

Logan, N.A., Berge, O., Bishop, A.H., Busse, H-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L., Salkinoja-Salonen, Seldin, L. and Ventosa, A. (2009) Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. International Journal of Systematic and Evolutionary Microbiology, 59, 2114-2121.

Logan, N.A. and De Vos, P. (2009). Genus *Bacillus* Cohn 1872. In: De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B (eds). Bergey's Manual of Systematic Bacteriology, 2nd Edition, Volume 3. Springer, New York, USA, pp. 21-127.

MacPherson, J.M., Eckstein, P.E., Scoles, G.J., and Gajadhar, A.A. (1993) Variability of the random amplified polymorphic DNA assay among thermalcyclers, and effects of primer and DNA concentration. Molecular Cellular Probes, 7, 293-299.

Madslien, E.H., Olsen, J.S., Granum, P.E. and Blatny, J.M. (2012) Genotyping of *B. licheniformis* based on a novel multi-locus sequence typing (MLST) scheme. BMC Microbiology, 12, doi:10.1186/1471-2180-12-230.

Marten, P., Smalla, K. and Berg, G. (2000) Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*. Journal of Applied Microbiology 2000, 89, 463-471.

Martínez, M.A. and Siňeriz, F. (2004) PCR fingerprinting of rRNA intergenic spacer regions for molecular characterization of environmental bacteria isolates. In: Spencer, J.F.T. and Ragout de Spencer, A.L. (eds) Methods in Biotechnology: Environmental Microbiology – Methods and Protocols, Humana Press, Totowa, pp.159-166.

Matarante, A., Baruzzi, F., Cocconcelli, P.S. and Morea, M. (2004) Genotyping and toxigenic potential of *Bacillus subtilis* and *Bacillus pumilus* strains occurring in industrial and artisanal cured sausages. Applied and Environmental Microbiology, 70, 5168-5176.

McSpadden-Gardener, B.B. (2004) Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems. Phytopathology, 94, 1252-1258.

Meier-Kolthoff, J. P., Auch, A. F., Klenk, H. P. and Gőker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics, 14:60. doi:10.1186/1471-2105-14-60.

Mohanty, S., Choudhury, P. K, Dash, A., Samanta, M. and Maiti, N. K. (2011) Genotypic and phenotypic diversity of *Bacillus* spp. isolated from freshwater ecosystems. Aquaculture Research and Development, 2, 1-4. doi:10.4172/2155-9546.1000112.

Mojica-Marín, V., Luna-Olvera, H.A., Sandoval-Coronado, C.F., Pereyra-Alférez, B., Morales-Ramos, L.H., Hernández-Luna, C.E. and Alvarado-Gomez, O.G. (2008) Antagonistic activity of selected strains of *Bacillus thuringiensis* against *Rhizoctonia solani* of chili pepper. African Journal of Biotechnology, 7, 1271-1276.

Müller, S., Strack, S. N., Hoefler, B. C., Straight, P. D., Kearns, D. B. and Kirby, J. R. (2014) Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. Applied and Environmental Microbiology, 80, 5603-5610.

Nakamura, L.K., Roberts, M.S. and Cohan, F.M. (1999) Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. *nov*. and *Bacillus subtilis* subsp. *spizizenii* subsp. *nov*. International Journal of Systematic Bacteriology, 49, 1211-1215.

Nagorska, K., Bikowski, M. and Obuchowki, M. (2007) Multicellular behaviour and production of a wide vaiety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochimica Polonica, 54, 495-508.

Nagpal, M.L., Fox, K.F. and Fox, A. (1998) Utility of 16S-23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? Journal of Microbiological Methods, 33, 211-219.

Nelson, L.M. (2004) Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. Plant Management Network, online doi:10.1094/CM-2004-0301-05-RV.

Nihorimbere, V., Cawoy, H., Seyer, A., Brunelle, A., Thonart, P. and Ongena, M. (2012) Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. FEMS Microbiology Ecology, 79, 176-191.

Nilsson, J., Svensson, B., Ekelund, K. and Christiansson, A. (1998) A RAPD-PCR method for large-scale typing of *Bacillus cereus*. Letters in Applied Microbiology, 27, 168-172.

Økstad, O.A. and Kolstø, A.B. (2011) Genomics of *Bacillus* Species. In: Wiedmann, M. and Zhang, W. (eds), Genomics of Foodborne Bacterial Pathogens. Food Microbiology and Food Safety, Springer Science and Business Media LLC, Philadelphia, PA, USA, pp. 29-53.

Ongena, M., Adam, A., Jourdan, E., Paquot, M., Brans, A., Joris, B., Arpigny, J.L. and Thonart, P. (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environmental Microbiology, 9, 1084-1090.

Ongena, M., Henry, G. and Thonart, P. (2010) The roles of cyclic lipopeptides in the biocontrol activity of *Bacillus subtilis.* In: Gisi, U., Chet, I. and Gullino, M.L. (eds), Recent Developments in Management of Plant Diseases, Plant Pathology in the 21st Century, Volume 1, Springer, Dordrecht, Germany, pp. 59-70.

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology, 16, 115-125.

Paulitz, T.C. and Bélanger, R.R. (2001) Biological control in greenhouse systems. Annual Review Phytopathology, 39,103-133.

Perez, C., Suarez, C. and Castro, G.R. (1992) Production of antimicrobials by *Bacillus subtilis* MIR 15. Journal of Biotechnology, 26, 331-336.

Pérez-García, C., Romero, D., Zerioub, H. and de Vicente, S. (2011) Biological control of phytopathogenic fungi by aerobic endospore-formers. In: Logan, N.A. and De Vos, P. (eds) Endospore-forming Soil Bacteria, Soil Biology Volume 27, Springer-Verlag, Berlin Heidelberg, Germany, pp.157-180.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. and Urdaci, M.C. (2002) Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology, 153, 269-276.

Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F. M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil, 340, 505-520.

Priest, F.G. (1989) Isolation and identification of aerobic endospore-forming bacteria. In: Harwood, C.R. (ed.), Biotechnology Handbooks – *Bacillus*. Vol 2. Plenum Press, NewYork, pp. 27-51.

Pruesse, E., Peplies, J. and Glöckner, F.O. (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. Bioinformatics, 28, 1823-1829.

Raaijmakers, J.M., Vlami, M. and de Souza, J.T. (2002) Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek, 81, 537-547.

Raaijmakers, J.M., De Bruijn, I., Nybroe, O. and Ongena, M. (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiology Reviews, 34, 1037-1062.

Rademaker, J.L.W., Aarts, H.J.M. and Vinuesa, P. (2005) Molecular typing of environmental isolates. In: Osborn, M. and Smith, C. (eds). Molecular Microbial Ecology, Taylor & Francis New York, USA, pp. 97-126.

Rasko, D., Altherr, M., Han, C. and Ravel, J. (2005) Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiology Reviews, 29, 303-329.

Reddy, M.S. and Hynes, R.K. (1994) Relationship between *in vitro* growth inhibition of pathogens and suppression of pre-emergence damping-off and post-emergence root rot of white beans seedlings in the greenhouse by bacteria. Canadian Journal of Microbiology, 40, 113-119.

Reva, O.N., Dixelius, C., Meijer, J. and Priest, F.G. (2004) Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. FEMS Microbiology and Ecology, 48 (2), 249-259.

Roberts, M.S., Nakumura, L.K. and Cohan, F.M. (1994) *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition, International Journal of Systematic Bacteriology, 44, 256-264.

Rooney, A.P., Price, N.P.J., Ehrhardt, C., Swezey, J.L. and Bannan, J.D. (2009) Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. *nov*. International Journal of Systematic and Evolutionary Microbiology, 59, 2429-2436.

Roux, K.H. (1995) Optimization and troubleshooting in PCR. Genome Research, 4, S185-S194.

Sadfi, N., Chérif, M., Hajlaoui, M.R., Boudabbous, A. and Bélanger, R. (2002) Isolation and partial purification of antifungal metabolites produced by *Bacillus cereus.* Annals of Microbiology, 52, 323-337.

Saikia, R., Gogoi, D.K., Mazumder, S., Yadav, A., Sarma, R.K., Bora, T.C. and Gogoi, B.K. (2011) *Brevibacillus laterosporus* strain BPM3, a potential biocontrol agent isolated from a natural hot water spring of Assam, India. Microbiological Research, 166, 216-225.

Sambrook, J. and Russell, D.W. (2006) Standard ethanol precipitation of DNA in microcentrifuge tubes. Cold Spring Harbour Protocols, doi:10.1101/pdb.prot4456.

Scheuerell, S.J., Sullivan, D.M. and Mahafee, W.F. (2005) Suppression of seedling damping-off caused by *Pythium ultimum*, *P.irregulare*, and *Rhizoctonia solani* in contained media amended with a diverse range of Pacific Northwest compost sources. Phytopathology, 95, 306-315.

Schroth, M.N. and Becker, J.O. (1990) Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. In: Hornby, D. (ed) Biological Control of Soilborne Plant Pathogens. CAB International, Wallingford, UK, pp. 389-414.

Shaver, Y.J., Nagpal, M.L., Rudner, R., Nakamura,L.K., Fox, K.F. and Fox, A. (2002) Restriction fragment length polymorphism of rRNA operons for discrimination and intergenic spacer sequences for cataloging of *Bacillus subtilis* sub-groups. Journal of Microbiological Methods, 50, 215- 223.

Shida, O., Takagi, H., Kadowaki, K. and Komagata, K. (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. International Journal Systematic Bacteriology, 46, 939-946.

Slepecky R.A. and Hemphill H.E. (2006) The genus *Bacillus* – nonmedical. In: Dworkin M., Falkow S., Rosenberg E. and Schleifer K. (eds). The Prokaryotes. A Handbook of the Biology of Bacteria. Springer Science and Business Media, New York, USA, pp. 530-555.

Song, J.Y., Kim, H.A., Kim, J.S., Kim, S.Y., Jeong, H., Kang, S.G., Kim, B.K., Kwon, S.K., Lee, C.H., Yu, D.S., Kim, B.S., Kim, S.H., Kwon, S.Y., Kim, J.F. (2012) Genome sequence of the plant growth-promoting rhizobacterium *Bacillus* sp. strain JS. Journal of Bacteriology, 94, 3760-3761.

Sorokin, A., Candelon, B., Guilloux, K. and Galleron, N. (2006) Multiple-locus sequence typing analysis of *Bacillus cereus* and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of psychrotrophic strains. Applied and Environmental Microbiology. 72, 1569-1578.

Sorokulova, I.B., Reva1, O.N., Smirnov, V.V., Pinchuk, I.V., Lapa, S.V. and Urdaci, M.C. (2003) Genetic diversity and involvement in bread spoilage of *Bacillus* strains isolated from flour and ropy bread. Letters in Applied Microbiology, 37, 169-173.

Stackebrandt, E. and Goebel, B. (1994) Taxonomic note. A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. International Journal of Systematic Bacteriology. 44, 846-849.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology, 56, 845-857.

Strom, P.F. (1985) Effect of temperature on bacterial species diversity during thermophilic solid waste composting. Applied and Environmental Microbiology, 50, 899-905.

Suárez-Estrella, F., Jurado, M.M., Vargas-García, M.C., López, M.J. and Moreno, J. (2013) Isolation of bio-protective microbial agents from eco-composts, Biological Control, 67, 66-74.

Szczech, M. and Shoda, M. (2004) Biocontrol of *Rhizoctonia* damping-off of tomato by *Bacillus subtilis* combined with *Burkholderia cepacia*. Journal of Phytopathology, 152, 549-556.

Szczech, M. and Shoda, M. (2006) The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*. Journal of. Phytopathology, 154, 370-377.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

Ugoji, E. and Laing, M. (2008) Rhizotron studies on *Zea mays* L. to evaluate biocontrol activity of *Bacillus subtilis*. World Journal of Microbiology and Biotechnology, 24, 269-274.

Utkhede, R.S. and Rahe, J.E. (1983) Interactions of antagonist and pathogen in biological control of onion white rot. Phytopathology, 73, 890-893.

Welsh, J. and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18, 7213-7218.

Wright, E.S., Yilmaz, L.S. and Noguera, D.R. (2012) DECIPHER, a search-based approach to chimera identification for 16S rRNA Sequences. Applied and Environmental Microbiology, 78, 717-725.

Wu, X.Y., Walker, M.J., Hornitzky, M. and Chin, J. (2006) Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. Journal of Microbiological Methods, 64, 107-119.

Xu, D. and Côté, J. C. (2003) Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3′ end 16S rDNA and 5′ end 16S–23S ITS nucleotide sequences. International Journal of Systematics and Evolutionary Microbiology, 53, 695-704.

Yobo, K.S., Laing, M.D., Hunter, C.H. and Morris, M.J. (2004) Biological control of *Rhizoctonia solani* by two *Trichoderma* species isolated from South African composted soil. South African Journal Plant and Soil, 21, 139-144.

Yobo, K.S., Laing, M.D. and Hunter, C.H. (2009) Effects of single and dual applications of selected *Trichoderma* and *Bacillus* isolates on performance of dry bean seedlings grown in composted pine bark growth medium under shade house conditions materials and methods. Journal of Plant Nutrition, 32, 1271-1289.

Yoshida, S., Hiradate, S., Tsukamoto, T., Hatakeda, K. and Shirata, A. (2001) Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology, 91, 181-187.

Yu, G.Y., Sinclair, J. B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry, 34, 955-963.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000) A greedy algorithm for aligning DNA sequences. Journal of Computational Biology, 7, 203-14.

Zhang, S-M., Wang, Y-X., Meng, L-Q., Li, J., Zhao, X-Y., Cao, X., Chen, X-L., Wang, A-X. and Li, J-F. (2012) Isolation and characterization of antifungal lipopeptides produced by endophytic *Bacillus amyloliquefaciens* TF28. African Journal of Microbiology Research, 6, 1747-1755.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q. and Zhang, R. (2014) Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. Plant and Soil, 374, 689-700.

Zhao, C., Luo, Y., Song, C., Liu, Z., Chen, S., Yu, Z. and Sun M. (2007) Identification of three Zwittermicin A biosynthesis-related genes from *Bacillus thuringiensis* subsp. *kurstaki* strain YBT-1520. Archives of Microbiology, 187, 313-319.

Zeriouh, H., Vicente, A., Pérez-García, A. and Romero, D. (2014) Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. Environmental Microbiology, 16, 2196-2211.

CHAPTER THREE

DETECTION AND SCREENING FOR ANTIFUNGAL LIPOPEPTIDES FROM SELECTED *BACILLUS* SPP.

3.1 Introduction

The ability to produce antifungal metabolites is widely regarded as an important trait of "so called" rhizobacteria exhibiting biocontrol or plant growth promoting attributes (Silo-Suh *et al*., 1994; Ongena and Jacques, 2008; Borriss, 2011; Pérez-García *et al*., 2011; Zhang *et al*., 2012; Gond *et al*., 2015). Therefore, strategies that target organisms which produce specific antifungal compounds have been advocated as a convenient means of selectively screening for potential biocontrol candidates (de Souza and Raaijmakers, 2003; Joshi and McSpadden-Gardener, 2006; Ramarathnam *et al*., 2007; Athukorala *et al*., 2009; Pliego *et al*., 2011).

Members of the genus *Bacillus* are known to produce an impressive array of bioactive compounds with a number of them showing marked antifungal activity (Stein, 2005; Ongena and Jacques, 2008). Cyclic lipopeptides belonging to the surfactin, fengycin and iturin families of biosurfactants are amongst the most frequently reported antifungal compounds produced by *Bacillus* spp. Production of these compounds has been linked to strains that have been developed commercially as biocontrol agents or which yielded promising results under greenhouse and field conditions (Ongena and Jacques, 2008; Borriss, 2011). Several other compounds such as zwittermycin A (an aminopolyol) and kanosamine (an aminoglycoside), which are produced by strains of *B. cereus*, also show antifungal activity *in vitro*. However, evidence linking these compounds to biocontrol activity *in vivo* is less prevalent (Stabb *et al*., 1994).

Structurally, lipopeptides consist of a cyclic peptide moiety with an attached hydrophobic fatty acid side chain. Surfactin and iturin compounds occur as lipoheptapeptides; with the former having a β-hydroxy fatty acid side chain and the latter a β-amino fatty acid side chain (Magnet-Dana and Peypoux, 1994). Fengycin occurs as a lipodecapeptide and has a β-hydroxy fatty acid side chain. These compounds are synthesized non-ribosomally and occur as families of closely related isoforms which can vary in terms of the length of the fatty acid side chain and composition of the peptide ring structure (Finking and Marahiel, 2004). The ability to produce lipopeptide antifungal compounds appears to be associated mainly with

representatives of the *B. subtilis* group of closely related taxa, including *B. amyloliquefaciens* (Ongena and Jacques, 2008; Cawoy *et al*., 2015); although some reports suggest that strains of *B. cereus* and *B. thuringiensis* are also capable of doing so (Kim *et al*., 2004; Ramarathnam *et al*., 2007). Some strains are able to synthesize multiple lipopeptides and, significantly, this appears to be a feature common to organisms that have been developed for commercial biocontrol purposes (Chen *et al*., 2009; Borriss, 2011).

Lipopeptides have amphipathic properties and are membrane-active; while iturins and fengycins display strong antifungal activity, the antifungal activity of surfactin is limited (Carrillo *et al*., 2003; Jacques, 2011). Besides their antibiotic role, surfactins and fengycin have also been linked to the stimulation of host defense mechanisms by eliciting induced sytemic resistance in plants (Ongena *et al*., 2007; Jourdan *et al*., 2009). Surfactins are also recognized for their role in promoting bacterial attachment to surfaces and biofilm formation (Zeriouh *et al*., 2014). These attributes are key to initial colonization and the persistence of bacteria within a rhizosphere environment (Danhorn and Fuqua, 2007; Raaijmakers *et al*., 2010; Nihorimbere *et al*., 2012).

Chemical and molecular methods to detect specific classes of lipopeptides have been successfully developed. For example, Vater *et al*. (2002) used matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as a rapid and sensitive method to characterize biosurfactants from either whole cells or crude culture filtrates of *B. subtilis* C-1 and proposed it as a suitable method for screening isolates. Polymerase chain reaction (PCR) based methods have also been developed to detect lipopeptide biosynthesis associated genes and used to screen for surfactin, fengycin, bacillomycin and iturin production capabilities amongst *Bacillus* isolates (Ramarathnam *et al*., 2007; Hsieh *et al*., 2008; Athukorala *et al*., 2009).

Characterization of the active compound(s) involved in fungal antagonism is important for understanding the underlying mechanisms involved in biocontrol. Thus, the current study was undertaken to ascertain the lipopeptide producing capabilities of selected isolates showing antagonism towards *R. solani* under *in vitro* and *in vivo* test conditions. Electrospray-ionization-time of flight (ESI-TOF) mass spectrometry (MS) was used to characterize lipopeptides purified from culture supernatants. Subsequently, various PCR screening

protocols were assessed to determine their suitability for screening isolates for the presence of genes associated with lipopeptide biosynthesis. In addition to this, specific PCR primers targeting fengycin synthetase genes of *B. amyloliquefaciens* strains were designed and evaluated.

3.2 Materials and methods

3.2.1 Bacterial strains

Bacterial isolates obtained from rhizosphere samples and selected on the basis of their *in vitro* antagonism of *R.solani* (Chapter 2) were used in this study. Reference strains *B. amyloliquefaciens* R16 and *B. subtilis* B81, which both exhibited broad spectrum antifungal activities *in vitro* were used to develop and establish methods for lipopeptide extraction and characterization. Subsequently, a further ten isolates, viz. *Brevibacillus laterosporus* CC-R4, *B. amyloliquefaciens* CP-R23, CP-R43, CP-R45, CL-R51and CL-R53; *B. subtilis* CC-R9, CT-R67 and CT-R92; and, *B. cereus* CB-R105 were evaluated for their ability to produce these substances. Pure cultures were maintained on TSA plates or stored in TSB amended with 20% ($^{v}/_{v}$) glycerol and kept at -80°C, as described previously (Chapter 2).

The above isolates, together with isolates *B. cereus* CP-R25, CP-R42 and CT-R73; and, *B. subtilis* CL-R59, CT-R64, and CT-R89 were selected for PCR based screening of biosynthetic gene markers associated with surfactin, fengycin, iturin and bacillomycin production. Four lipopeptide-producing strains *viz. B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^{T}, *B. subtilis* DSM3258, *B. subtilis* subsp. *subtilis* DSM10^{T} and *B. subtilis* subsp. *spizizenii* ATCC 6633 were included in the screening process. Genomic DNA from each of these organisms was obtained as described in Chapter 2 and stored at -20^{o}C until needed.

3.2.2 Extraction of antifungal compounds

The isolates were cultured in Landy medium (glucose, 15 g; L-glutamic acid, 5 g; $MgSO_4$**.** $7H_2O$, 1.02 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MnSO_4$**.**H_2O, 5 mg; $CuSO_4$**.**$5H_2O$, 0.16 mg; and, $FeSO_4$**.**$7H_2O$, 150 µg; 1000 ml distilled water; pH 6.0) in order to promote the production of lipopeptide antibiotics (McKeen *et al*., 1986). The medium was dispensed as 10 ml and 100

ml aliquots into 50 ml and 500 ml Erlenmeyer flasks, respectively, and autoclaved at 121°C (100 KPa) for 15 min.

Starter cultures were established by inoculating 10 ml Landy medium with a loop of pure culture biomass from a 24 h agar plate and incubating overnight at 30°C with agitation (150 rpm). One milliliter of this culture was used to inoculate 100 ml of Landy medium and incubated for 72 h under the same conditions. The culture medium was then centrifuged at 12,000 ×g for 30 min at 4^{o}C (JA-10 rotor, Avanti J-26XPI, Beckman). One millilitre of cell-free supernatant was withdrawn and filter-sterilized (0.22 µm cellulose acetate filter, Pall Acrodisc, Port Washington, USA) for use in an agar-well diffusion bioassay to test for antifungal activity against *R. solani in vitro*. Lipopetide compounds were extracted from the supernatants of all positive isolates by acid precipitation (Vater *et al*., 2002; Hsieh *et al*., 2008). The pH of the cell-free supernatants was adjusted to pH 2.0-2.1 with 1N HCl before storing at 4^{o}C for a minimum of 4 h. This was followed by a centrifugation step (12,000 x g for 30 min) after which the supernatant was removed and the precipitate extracted twice with methanol (2 x 2.5 ml). Methanol extracts were then tested *in vitro* for antifungal activity using filter-disc bioassays. The extracted supernatants were also tested for antifungal activity using the agar-well diffusion approach. The pH of each extracted supernatant was adjusted to pH 7.4 with 5N NaOH before testing.

The methanol extracts were stored at -20^{o}C until analysed by thin-layer chromatography (TLC) and reverse-phase Ultra Performance Liquid Chromatography (UPLC) in conjunction with electrospray ionization–mass spectrometry (ESI-MS). UPLC and ESI-MS analysis was performed at the Sasol Mass Spectrometry Laboratory, Discipline of Chemistry, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

3.2.3 Agar-well and filter-disc diffusion assays

For the agar-well diffusion assay agar plugs were punched out using the back of a Pasteur pipette along the periphery of a PDA plate to form wells 6 mm in diameter and approximately 5-7 mm deep. A drop of molten agar was added to the bottom of each to seal it. Filter sterilized culture supernatant (50 µl) was placed in each well and a PDA plug (4x4 mm) colonised with *R. solani* mycelium was placed at the centre of each plate. Plates were incubated at 28^{o}C and checked daily for signs of mycelial growth inhibition. Controls were

established whereby sterile Landy medium (50 µl) was added to wells instead of culture extracts.

For the filter-disc diffusion assay, aliquots (2 x 10 µl) of methanol extract were sequentially applied to a sterile 8 mm filterpaper-disc (Oxoid, Cambridge, UK) and after the methanol was evaporated off, the disc was transferred to a PDA plate and the bioassay run as described for the agar-well diffusion bioassay. To determine whether methanol residues influenced the bioassay results, filterpaper-discs impregnated with pure methanol and evaporated to dryness before placing on *R. solani*-inoculated PDA plates, served as controls.

3.2.4 Thin Layer Chromatography

Methanol extracts were examined by thin-layer chromatography (TLC) using Silica Gel DC 60 F_{254} aluminium plates (5x10 cm) (Merck, Darmstadt, Germany). Samples (10 µl) were spotted on the plates and separated using an n-propanol-water (70:30 $^{v}/_{v}$) solvent system following standard procedures (Brenner *et al*., 1965). Compound separation was detected using three methods: by viewing the plates under UV illumination; by spraying them with atomized distilled H_2O to detect hydrophobic regions associated with lipopeptide compounds; or, by spraying plates with 2% ($^{w}/_{v}$) ninhydrin in ethanol and heating them at 110°C for 5-10 min to detect compounds with free amino groups present (Yu *et al*., 2002).

TLC plates (5x15 cm) (Silica Gel DC 60, Merck) were also used in bioautography overlay assays in order to detect zones/bands of activity. In this instance single samples (200 µl) were spotted across the entire width of a plate to form a single line. Developed plates were placed in sterile glass Petri dishes (diameter 20 cm) and overlaid with molten PDA until they were covered by a thin layer (1-2 mm) of agar. Agar strips (80 x 4 mm) were then cut out of PDA plates colonized by *R. solani* and arranged along the longitudinal axis of each TLC plate. The plates were incubated at 28^{o}C and viewed daily for signs of inhibition.

TLC was also used as a means of partially purifying the lipopeptide extracts, obtaining fractions for assessing antifungal activity and mass determination analysis using UPLC in conjunction with ESI-MS. TLC plates were prepared and run as described for the overlay assays. After drying with a hairdryer to remove all traces of solvent, the plates were sprayed with dH_2O to detect hydrophobic regions associated with lipopeptide or biosurfactant

compounds. The compounds were recovered by scraping the silica gel off the plates at positions corresponding to each hydrophobic region and transferring the solid matrix material to a 1.5 ml microfuge tube. Methanol (200 µl) was added to each tube which was vortexed for 2 min and left to stand for at least 1 h at 4^{o}C, to facilitate the extraction and recovery of the active compounds. Thereafter, the methanol extract was separated from the silica gel by centrifugation (12,000 x g for 5 min) and stored at -20^{o}C until required. Aliquots (60 µl) from each methanol extract were evaluated for antifungal activity towards *R. solani* using the filter-disc agar-diffusion bioassay.

3.2.5 Purification and characterization of lipopeptide compounds

Methanol extracts from crude lipopeptide fractions (2 µl) and partially purified TLC fractions (7 µl) were loaded onto a Waters Acquity BEH C_{18} column (2.1 x 100 mm, particle size of 1.7 µm, 35^{o}C) and separated under Ultra Performance Liquid Chromatography conditions (UPLC, Waters Acquity, Milford, MA, USA). The solvents used were 0.2 % ($^{v}/_{v}$) acetic acid (**A**) and methanol (**B**) run at a flow rate of 0.35 ml min^{-1}. Initially, the system was run isocratically for 30 min with Eluents A and B kept at a ratio of 9:1; this was followed with a linear gradient that increased from 10% to 100% ($^{v}/_{v}$) methanol over the time period 30-38 min; thereafter, the previous isocratic elution setting was resumed (38.5-40 min). Eluted compounds were detected by electrospray-ionization mass-spectrometry (ESI-MS) and their molecular weights determined using a Waters LCT Premier, Time-of-Flight mass spectrometer system fitted with an electrospray ionization source (Waters). The positive mode (ESI+) was used to ionize the samples and the fragments detected in the *m/z* range 900 - 2000. A capillary voltage of 5000 kV was applied throughout and the cone voltage was set at 35 V. A desolvation temperature of 350^{o}C was used and a desolvation gas flow rate of 400 l h^{-1} was maintained.

Commercial preparations of purified iturin A (purity ≥95%) and surfactin (purity ≥98%) (Sigma-Aldrich, Chemie GmbH, München, Germany) were used as standards and made up to 1000 mg l^{-1} by dissolving appropriate amounts in methanol. These standands were diluted 100-fold before aliquots (2 µl) were analysed by UPLC in conjunction with ESI-MS.

3.2.6 Detection of gene markers associated with lipopeptide biosynthesis

PCR primers targeting specific sequences associated with lipopeptide biosynthesis genes were used to screen bacterial rhizosphere isolates for their potential to produce iturin, bacillomycin, fengycin or surfactin compounds (Table 3.1). All primers were synthesized and supplied by Inqaba Biotec™ (Hatfield, Pretoria, South Africa).

Table 3.1 Specific primer sequences used to detect genes associated with lipopeptide biosynthesis in *Bacillus* spp.

Lipopeptide Compound	Target gene	Primer Designation	Primer Sequence (5' - 3')	Frament size	Reference
Iturin A	*ituD*	ITUD1-F	ATGAACAATCTTGCCTTTTTA	~ 1200 bp	Hsieh *et al.,* (2008)
		ITUD1-R	TTATTTTAAAATCCGCAATT		
Bacillomycin D	*bmyC*	BACC1-F	GAAGGACACGGCAGAGAGTC	~ 875 bp	Ramarathnam *et al.,* (2007)
		BACC1-R	CGCTGATGACTGTTCATGCT		
Fengycin	*fenD*	FEND1-F	TTTGGCAGCAGGAGAAGTTT	~ 964 bp	Ramarathnam *et al.,* (2007)
		FEND1-R	GCTGTCCGTTCTGCTTTTTC		
Fengycin	*fenD*	FENG-F	GGAACGTCACGGAAATGAAG	~ 500 bp	Current study
		FENG-R	CAGCTGAAACGGTACGCTGT		
Surfactin	*srfA*	SUR3-F	ACAGTATGGAGGCATGGTC	~ 440 bp	Athukorala *et al.,* (2009)
		SUR3-R	TTCCGCCACTTTTTCAGTTT		

The *ituD* gene coding for a malonyl CoA-transacylase involved in iturin A biosynthesis, was screened for according to the method of Hsieh *et al*. (2008). The *fenD* gene coding for a fengycin synthetase and a gene region linked to bacillomycin D synthetase C (*bmyC*) were screened for according to a modified method of Ramarathnam *et al*. (2007) using the primer pairs FEND1-F/FEND1-R and BACC1-F/BACC1-F, respectively. Primers SUR3-F and SUR3-R (Athukorala *et al*., 2009) were used to screen for a biosynthetic gene associated with surfactin production following a PCR reaction sequence adapted from Ramarathnam *et al*. (2007). In addition to these, a *fenD* specific primer set FENG-F/FENG-R was developed to target fengycin producing strains of *B. amyloliquefaciens* that yielded negative results when the FEND1-F/FEND1-R primer set was used (Section 3.2.7). All PCR reactions (25 µl) were carried out in sterile 200 µl thin-walled PCR tubes using GoTaq® PCR reagents (Promega, Madison, USA) in a Bioer thermal-cycler (XP Cycler, Bioer, Hangzhou, PRC).

Amplification of *ituD* gene fragments was achieved using a reaction sequence with the following temperature profile: initial denaturation at 94°C for 4 min; followed by 30 cycles each consisting of a denaturation step at 94°C for 1 min, an annealing step at 50°C for 1 min, and an extension step at 72°C for 1.5 min; followed by a final extension step at 72°C for 10 min. Samples were subsequently kept at 4°C until electrophoretic analysis. Each PCR reaction consisted of 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, 1.25 U Taq polymerase, 25-50 ng of DNA template, 1 × PCR reaction buffer and made up to final volume of 25 µl with sterile nuclease-free water. PCR reactions lacking DNA template were used as negative controls. Isolate CP-R23 which was putatively identified as a strain of *B. amyloliquefaciens* subsp. *plantarum* (Chapter 2) and found to produce iturin isoforms when grown in Landy medium was used as a positive control.

Gene fragments associated with surfactin synthetase, bacillomycin D synthetase C and fengycin synthetase D were all amplified using a common PCR protocol adapted from Ramarathnam *et al*. (2007). The PCR reaction was similar to that described above for *ituD* amplification except that the $MgCl_2$ concentration was increased to 2.0 mM per reaction. The PCR reaction sequence involved an initial denaturation at 94°C for 3 min, followed by 25 cycles each comprising a denaturation step at 94°C for 1 min, an annealing step at 60°C for 30 s and an extension step at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. Thereafter, samples were cooled and stored at 4°C until electrophoretically analysed. In establishing this protocol it was found that by reducing the numbers of PCR reaction cycles from 35 to 25, artifacts were largely eliminated. PCR reactions lacking DNA template were used as negative controls. DNA from reference strain *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7^T was used as a positive control for surfactin synthase and fengycin synthetase gene fragment detection (Boriss *et al*., 2011). Isolate R16, putatively identified as a strain of *B. amyloliquefaciens* subsp. *plantarum* (Chapter 2) and found to produce bacillomycin D when grown in Landy medium, was also used as a positive control.

PCR amplification of each targeted gene fragment was confirmed by agarose gel (1.5 % $^w/_v$) electrophoresis, as described previously (Section 2.2.2.2, Chapter 2). As before, selected amplicons were sequenced by Inqaba BiotechTM (Hatfield Pretoria, RSA). Sequence data were then subjected to a BLAST-N database search (http://www.ncbi.nlm.nih.gov) using the blastn algorithm (default parameters, 12 November 2013) and matched to existing nucleotide

sequences in GenBank, allowing for putative matches and identifications to be made. The sequences determined in this study were deposited in GenBank under accession numbers KU495928-KU495932 and KU504257-KU504270.

The phylogenetic relationships of *fenD* and *srfD* gene fragments obtained using primer sets FenD1-F/FenD1-R and Sur3-F/Sur3-R respectively, were compared to homologous nucleotide sequences sourced from GenBank using Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods described previously (Section 2.2.2.4, Chapter 2) (Mega 6, Version 6.06) (Tamura *et al.,* 2013).

3.2.7 Design of primers to detect fengycin synthetase gene *fenD* associated with *B. amyloliquefaciens* strains

Nucleotide sequence data associated with the *fenD* gene of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7^T (7.7 kbp, Accession no. FN597644, Region: 2031260-2038936) (Boriss *et al*., 2011) and *B*. *amyloliquefaciens* subsp. *plantarum* $FZB42^T$ (10.7 kbp, Accession no. CP000560.1, Region: 1935150-1945925) (Chen *et al.*, 2007) were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The sequences were aligned using BioEdit (Version 7.1.3.0) software and assessed visually for regions of homology. A segment, 649 bp in length, which corresponded to region: 1939324-1939972 of the complete genome of *B*. *amyloliquefaciens* subsp. *plantarum* $FZB42^T$ was chosen to design primer pairs using Primer3® web-based software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Several primer sets were generated based on a number of criteria including primer length (20 bp), melting temperatures (Tm c. 60^{o}C), Tm differences between primers (< 2^{o}C), GC content (50-55 %) and anticipated amplicon size (~ 350 -500 bp). The specificity of the primer sets generated were then determined by a Blast primer search. A primer set FENG-F/FENG-R, which targeted a 500 bp region of the *fenD* gene, was selected for further evaluation (Table 3.1). The PCR protocol described above, [adapted from Ramarathnam *et al*. (2007)], was used as the starting point in assessing the efficacy of the selected primers. Several variables such as annealing temperature, $MgCl_2$ concentration and number of PCR reaction cycles, were assessed empirically during optimization of the PCR conditions. Selected PCR amplicons were visualized, sequenced and subjected to a BLAST-N database search as described above.

3.3 Results

Isolates R16 and B81 were selected for further characterization of their antifungal metabolites as representatives of two groups of *Bacillus* spp. differentiated on the basis of RAPD fingerprinting and showing broad spectrum antifungal activity *in vitro*. Organisms placed within these two RAPD groupings were amongst the best performing isolates in the *in vivo* biocontrol pot trials against *R. solani* (Chapter 2). Initially, agar-diffusion assays were used to detect antifungal activity in cell-free supernatants of cultures from R16 and B81 (Plate 3.1). Antifungal activity associated with R16 supernatant was clearly distinguished whereas for B81 supernatant the inhibitory response was less distinct. In each instance the isolates were cultured for 72 h prior to sampling in order to promote secondary metabolite production during stationary growth phase.

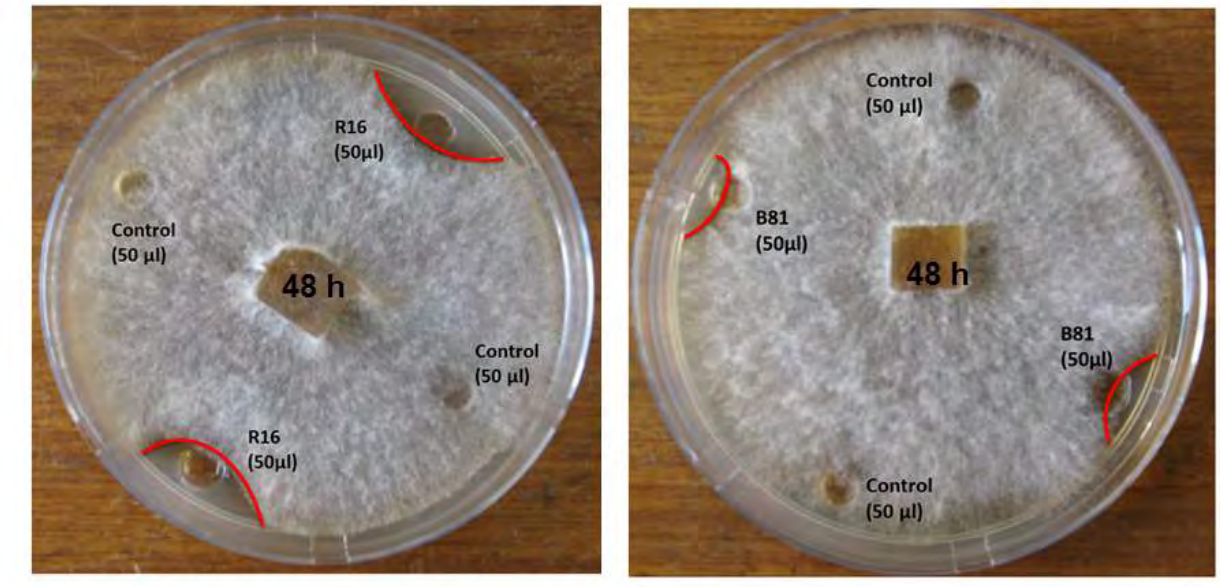


Plate 3.1 Agar-diffusion bioassays showing antifungal activity of cell-free culture supernatants of isolates R16 (A) and B81 (B) grown in Landy medium (72 h), against *R. solani*. The periphery of each zone of mycelial inhibition detected is demarcated by a red line. Controls comprised 50 µl sterile Landy's medium.

Lowering the pH of each supernatant to 2.0 resulted in the precipitation of an insoluble fraction which could be removed by centrifugation. Methanol extracts of the precipitated fractions from both isolates also showed antifungal activity towards *R. solani* in filter-disc diffusion bioassays (Plate 3.2). Control discs impregnated with methanol were overgrown with mycelium, indicating that methanol residues did not have an adverse effect on the assay. After precipitate removal, the pH of each extracted supernatant was adjusted to pH 7.4 and assayed again for antifungal activity (Plate 3.3). In both instances, fungal mycelium overgrew agar-wells containing extracted supernatant indicating a loss of antifungal activity; thereby confirmng the success of the extraction procedure used.

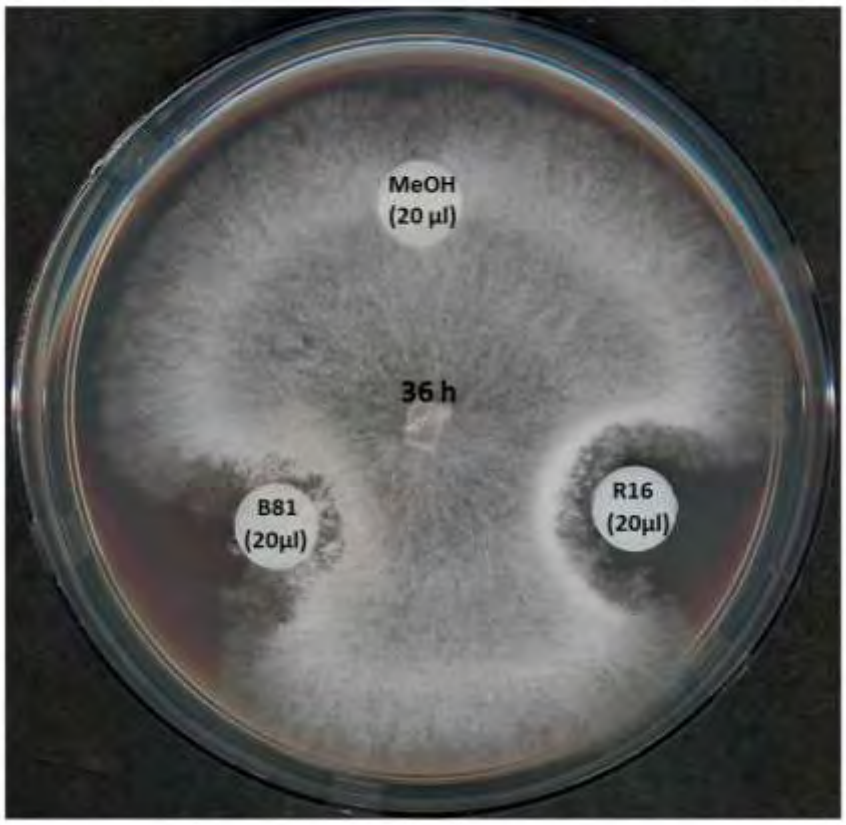


Plate 3.2 Filter-disc bioassay showing antifungal activity of methanol extracts containing active compound(s) derived from cultures of isolates R16 and B81 grown in Landy medium.

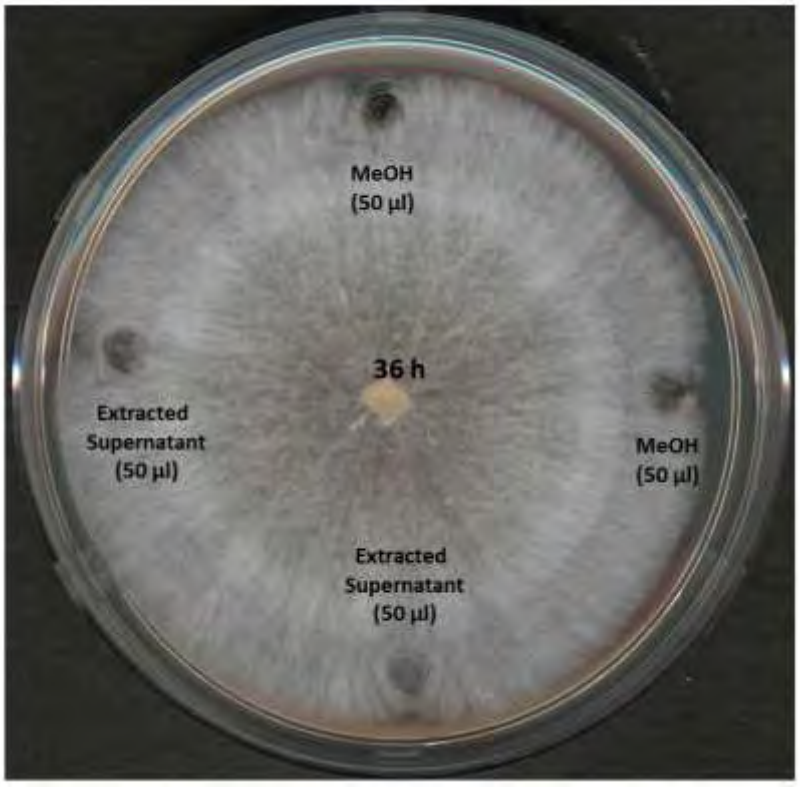


Plate 3.3 Agar-diffusion bioassay showing the effect of extracted supernatant (pH 7.4) from isolates R16 and B81 towards *R. solani* compared to methanol controls.

TLC analysis of each methanolic extract revealed several discrete bands present on plates (Plate 3.4). Isolate R16 extract yielded four prominent hydrophobic regions with Rf values of 0.88, 0.74, 0.64 and 0.53 when TLC plates were sprayed with water. These zones were also discernible under UV; however, the first region (Rf 0.88) could be further differentiated into three bands with Rf values of 0.93, 0.87 and 0.81. Five hydrophobic regions were detected in the case of isolate B81 (Rf 0.88, 0.77, 0.72, 0.64 and 0.53), three of which (Rf 0.77, 0.72 and 0.53) were very faint. These zones were also discernible under UV and the Rf 0.88 hydrophobic region could be further differentiated into two bands with Rf values of 0.88 and 0.84. The iturin A standard yielded a single discrete band (Rf 0.76) which displayed hydrophobic properties and was also visible under UV. Interestingly, when ninhydrin was used to visualize bands the iturin A standard was not detected (Plate 3.5). For R16 and B81 only two of the bands distinguished previously (viz., Rf 0.64 and 0.53) were visualized when ninhydrin was applied. In addition to these, a region corresponding to an Rf of 0.38 was also distinguished.

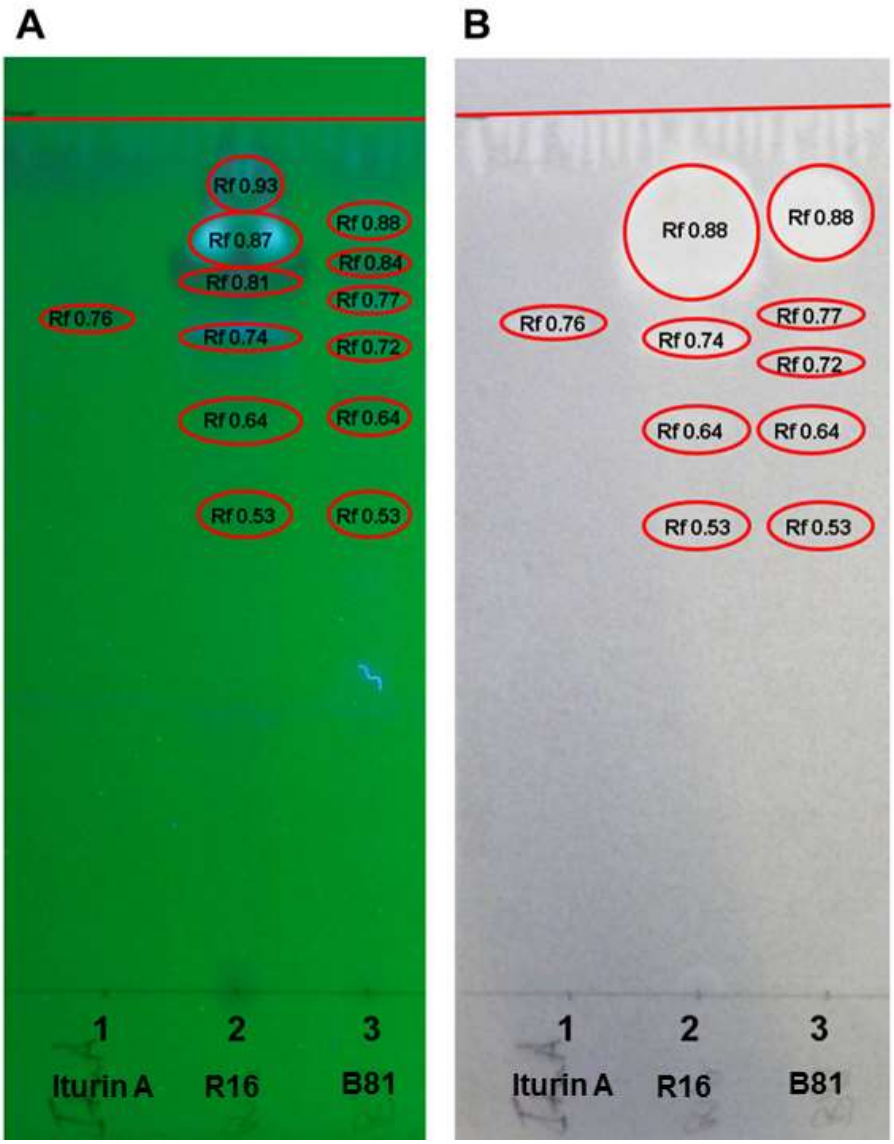


Plate 3.4 Thin layer chromatography of methanol extracts containing antifungal compound(s) derived from cultures of isolates R16 and B81 grown in Landy's medium. Compounds were detected by viewing the TLC plate under UV light (A) and by spraying the surface with dH_2O (B) to detect hydrophobic regions. Iturin A was included in the analysis as a standard.

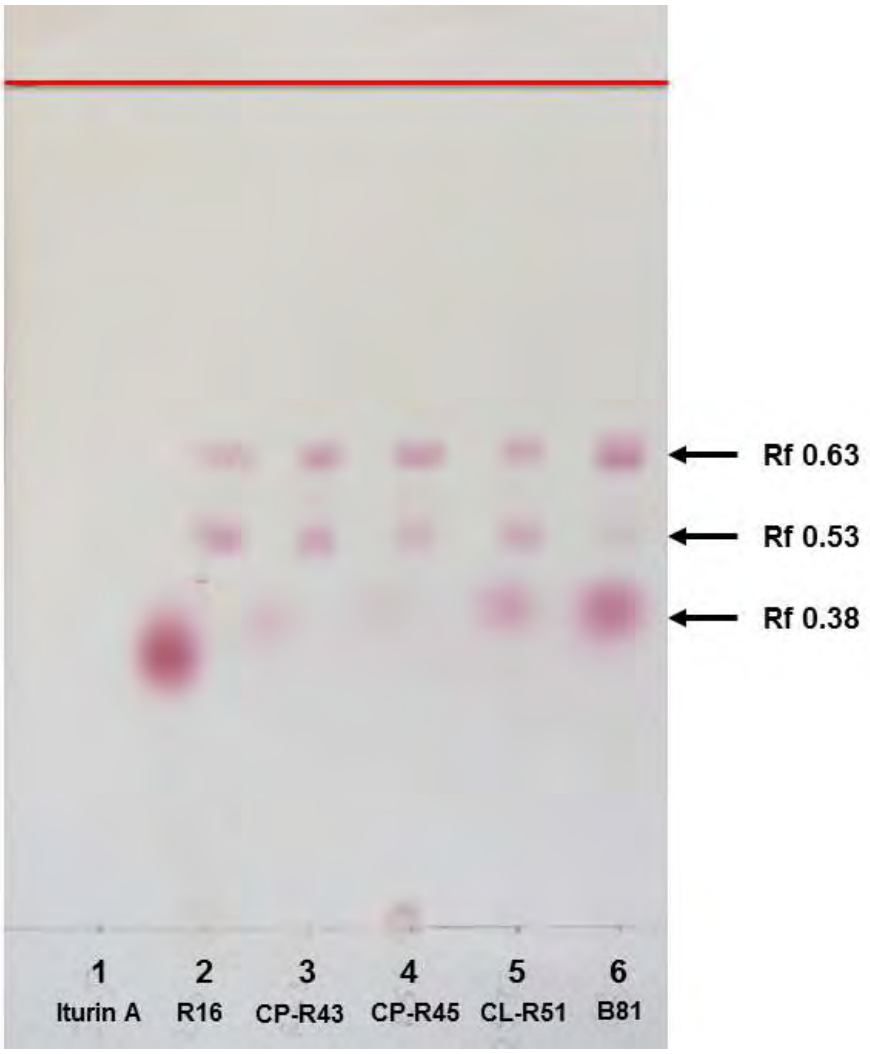


Plate 3.5 Ninhydrin detection of TLC resolved compounds containing free amino groups. Methanol extracts containing antifungal compound(s) derived from cultures of isolates R16, CP-R43, CL-R51, CL-R53 and B81 were analyzed by TLC using an n-propanol-water (70:30 $^{v}/_{v}$) solvent system. Iturin A was used as a standard.

When bioautography assays were performed on developed TLC plates overlaid with agar, the R16 extract showed evidence of antifungal activity in a region that corresponded to the band with an Rf value of 0.74 (Plate 3.6A). In contrast, extracts from isolate B81 showed no obvious signs of antagonism and these plates were completely overgrown by *R. solani* (Plate 3.6B). Subsequently, zones corresponding to hydrophobic regions resolved for each extract were scraped off developed TLC plates, extracted with methanol and then assayed for antifungal activity. Three fractions of the R16 extract, with Rf values 0.74, 0.64 and 0.53, all showed antagonism towards *R. solani* when 120 µl of the methanolic extract was applied to each filter disc in the bioassay (Plate 3.7). The zone of inhibition associated with the Rf 0.74 compound appeared to be more prominent compared to the other two fractions. When the B81 extract was evaluated, only the fraction with an Rf value of 0.63 showed clear evidence of mycelial inhibition. The Rf 0.53 fraction showed some evidence of mycelial inhibition earlier in the assay (36 h) but this was no longer evident when plates were rated after 48 h.

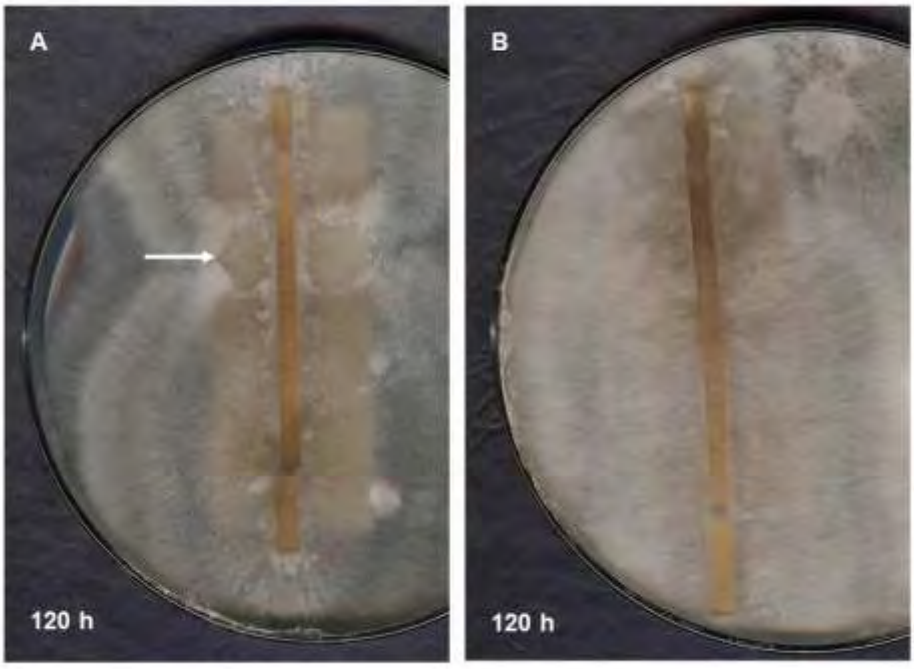


Plate 3.6 Bioautogram overlay assay used to detect zones of fungal growth inhibition associated with compound(s) extracted from R16 (A) and B81 (B) cultures that were separated by TLC. Zones of inhibition are indicated by the symbol (→).

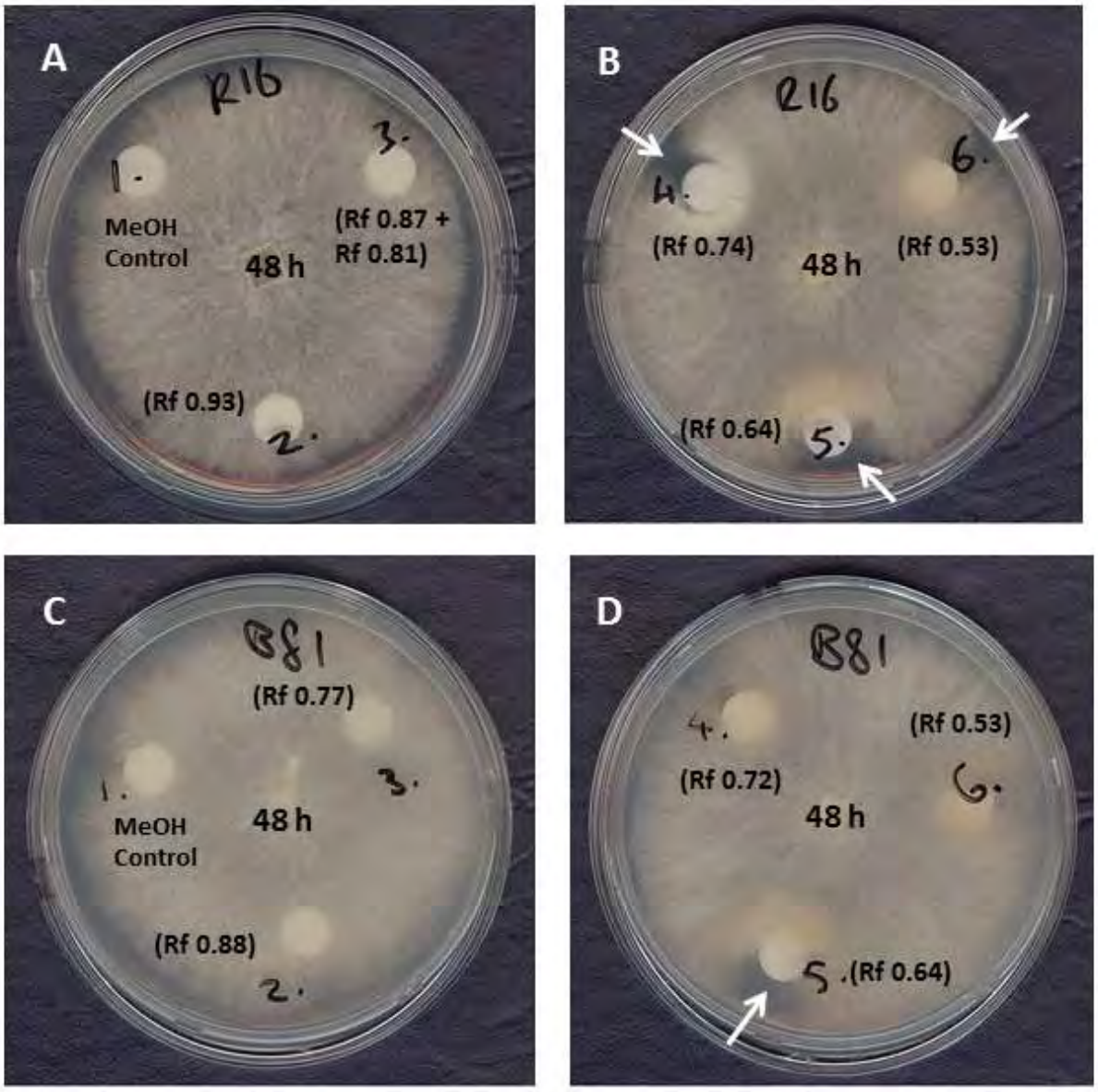


Plate 3.7 Filter-disc bioassays of hydrophobic bands extracted from TLC plates into methanol (120 µl). Fractions for isolates R16 (A and B) and B81 (C and D) are identified by their respective Rf values. Evidence of fungal inhibition is indicated by the symbol (→).

3.3.1 UPLC and ESI-TOF MS analysis of lipopeptide fractions

Each TLC fraction was analyzed by reversed phase UPLC in conjunction with ESI-TOF mass spectrometry for the presence of lipopeptide compounds. Commercial preparations of surfactin and iturin A were used as standards to determine if either of these compounds was produced by the test organisms.

Initially, UPLC chromatograms and related mass spectra for each standard were generated (Appendix A). As expected, multiple peaks were resolved from each standard and the identity of the major peaks was determined by comparing their mass spectra with those reported in the literature (Vater *et al*, 2002; Koumoutsi *et al*., 2004) (Table 3.2). In both instances, each standard comprised several isoforms which were resolved as separate peaks according to their hydrophobicities.

Table 3.2 Mass peaks identified in commercial surfactin and iturin A standards by high-resolution UPLC separation and ESI-TOF mass spectrometry analysis.

Compound	Mass peaks detected (m/z)*	Assignment**
Surfactin		
	994.69, 1016.68	C12-surfactin $[M+H, Na]^+$
	1008.71, 1030.69	C13-surfactin $[M+H, Na]^+$
	1022.73, 1044.69	C14-surfactin $[M+H, Na]^+$
	1036.73, 1058.68	C15-surfactin $[M+H, Na]^+$
	1050.76, 1072.74	C16-surfactin $[M+H, Na]^+$
Iturin A		
	1043.62, 1065.6	C14-iturin $[M+H, Na]^+$
	1057,65, 1079.63	C15-iturin $[M+H, Na]^+$
	1071.67, 1093.66	C16-iturin $[M+H, Na]^+$
	1085.69, 1107,67	C17-iturin $[M+H, Na]^+$

*Mass numbers of the main monoisotopic isoforms are presented
**Mass peak data were assigned to isoform species on the basis of matching data reported in the literature (Vater *et al*., 2002; Koumoutsi *et al*., 2004)

These isoforms reflect differences in the length of the β-amino- and β-hydroxy fatty acid side chains found in iturins (C14-C17) and surfactins (C12-C16), respectively (Magnet-Dana and Peypoux, 1994; Peypoux *et al*., 1999). Each peak was found to comprise several ionic species of an isoform with the protonated species $[M+H]^+$ and sodium adducts $[M+Na]^+$ being present at the highest relative intensities. Isoforms were detected by identifying mass fragments that differed by 14 mass units or multiples thereof, since this mass difference reflects the presence or absence of a (CH_2) group in a fatty acid side chain (Vater *et al*, 2002).

Sodium adducts were distinguished on the basis that they yield peaks 22 mass units higher than the $[M+H]^+$ peak. Both standards also contained several minor unidentified peaks which were attributed to impurities in the standards, or from the leaching of compounds from the glass and plastic containers used to prepare and store the standards. Interestingly, surfactin isoforms were detected in the iturin A standard at low relative intensities (Appendix B).

Lipopeptides detected in each TLC fraction from isolate R16 and B81 are presented in Table 3.3. Surfactin isoforms were the major compounds detected in the first TLC fraction (Rf 0.88) of each isolate. Surfactin was also the main component of fractions Rf 0.77 and Rf 0.72 emanating from B81, but was present at lower intensities.

Table 3.3 Lipopetide biosurfactants produced by isolates R16 and B81 identified by UPLC-ESI-TOF MS analysis of methanol extracts from TLC fractions.

Fraction	Isolate	Mass peak (m/z)**	Assignment*
Rf 0.88	- B81	994.65, 1016.63	C12-surfactin $[M+H, Na]^+$
	R16, B81	1008.67, 1030.65	C13-surfactin $[M+H, Na]^+$
	R16, B81	1022.68, 1044.66	C14-surfactin $[M+H, Na]^+$
	R16, B81	1036.69, 1058.66	C15-surfactin $[M+H, Na]^+$
	R16 -	1050.72, 1072.7	C16-surfactin $[M+H, Na]^+$
	R16 -	1064.73, 1086.72	C17-surfactin $[M+H, Na]^+$
Rf 0.74	R16 -	1031.55, 1053.52	C14-bacillomycin $[M+H, Na]^+$
	R16 -	1045.56, 1067.52	C15-bacillomycin $[M+H, Na]^+$
	R16 -	1059.58, 1081.50	C16-bacillomycin $[M+H, Na]^+$
	R16 -	1073.6, 1095.57	C17-bacillomycin $[M+H, Na]^+$
Rf 0.64	R16 -	1477.84, 1499.82	Ala-6-C17 fengycin $[M+H, Na]^+$
	R16, B81	1491.85, 1513.84	Val-6-C16 fengycin $[M+H, Na]^+$
	R16, B81	1505.87, 1527.85	Val-6-C17 fengycin $[M+H, Na]^+$
Rf 0.53	R16 -	1449.80, 1471.78	Ala-6-C15 fengycin $[M+H, Na]^+$
	R16, B81	1463.82, 1485.80	Ala-6-C16 fengycin $[M+H, Na]^+$
	R16, B81	1477.83, 1499.82	Ala-6-C17 fengycin $[M+H, Na]^+$

*Mass peak data were assigned to isoform species on the basis of matching data reported in the literature (Vater *et al*., 2002; Koumoutsi *et al*., 2004).

**Only mass peaks of the major peaks present in each TLC fraction are reported.

Neither isolate produced iturin; however, four major peaks were resolved from the Rf 0.74 fraction of R16 (Fig. 3.1) matching mass spectra consistent with C14-C17 isoforms of bacillomycin D, a member of the iturin family of lipopeptides (Moyne *et al*., 2001; Koumoutsi *et al*., 2004, Koumoutsi *et al*., 2007). Sodium adducts were the most prevalent species in each of the isoforms present. Surfactin isoforms were also detected in the Rf 0.74 fraction but at much lower relative intensities.

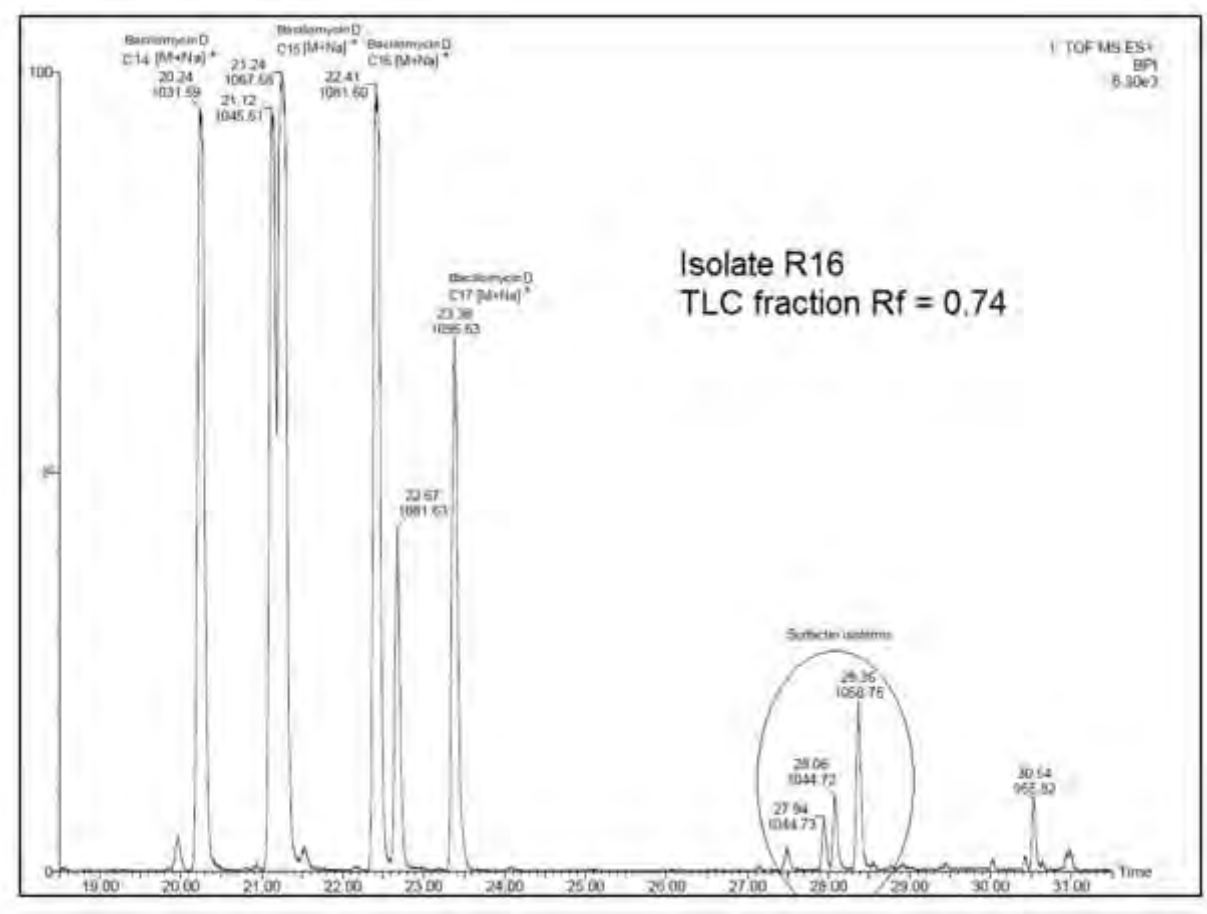
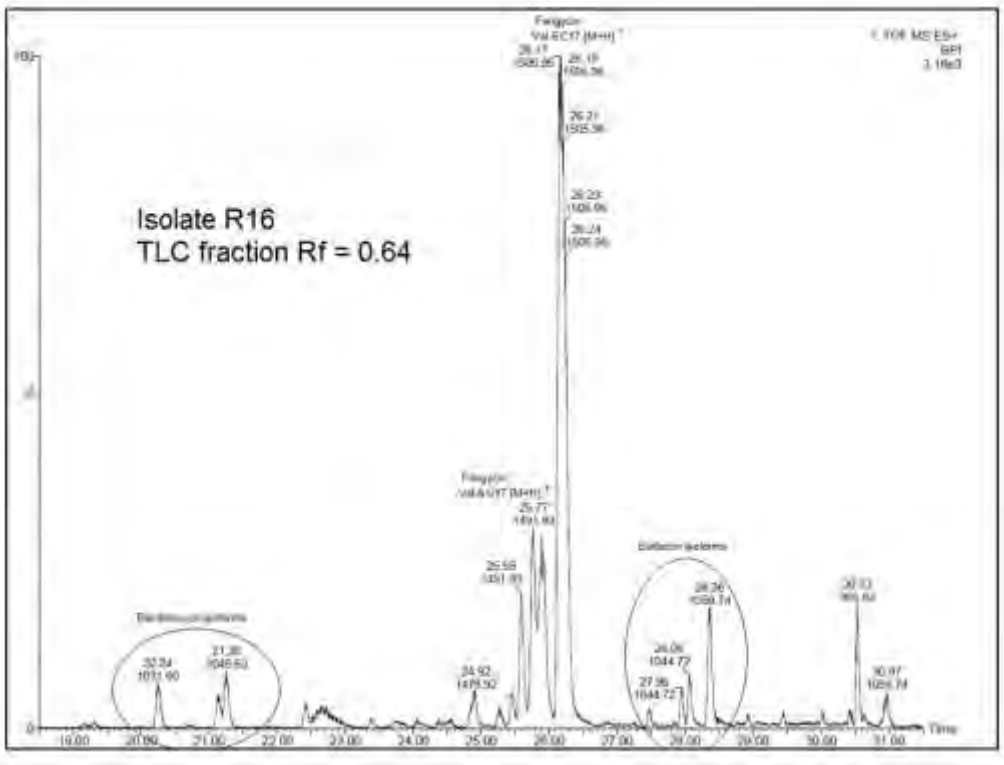


Figure 3.1 UPLC analysis of methanol extracts from isolate R16 TLC fraction Rf 0.74.

Fractions Rf 0.64 and Rf 0.53, produced similar results for both isolates, yielding mass spectra consistent with isoforms of the lipopeptide fengycin (Table 3.3) (Fig. 3.2 A and B) (Vater *et al*., 2002). Protonated $[M+H]^+$ isoforms of fengycin were the most prevalent species present. Based on *m/z* values, two structural analogs (Val-6 and Ala-6) of fengycin were presumptively distinguished. The Val-6 fengycin analogs (C16 and C17) were the most prevalent isoforms present in the Rf 0.64 fractions (Fig. 3.2 A); whereas, Ala-6 C16 and C17 analogs were the predominant fenycin isoforms present in the Rf 0.53 fraction (Fig. 3.2 B).

Surfactin isoforms were detected at low relative intensities in both fengycin fractions, while traces of bacillomycin D were also distinguished in the Rf 0.64 fraction. Both groups of compounds were judged to be residual "contaminants" left over from the TLC separation procedure.

(A)



(B)

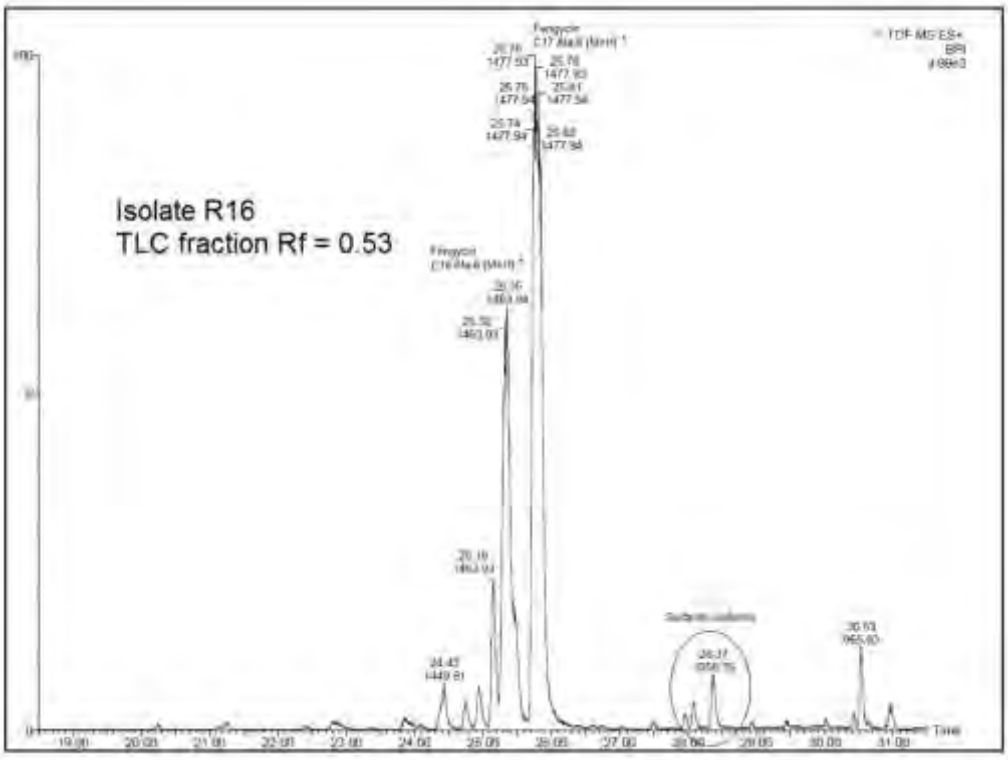


Figure 3.2 UPLC ESI-TOF MS analysis of methanol extracts from isolate R16 TLC fractions Rf 0.64 (A) and Rf 0.53 (B).

When the methanolic extracts of the crude lipopeptide fractions from R16 and B81 were analysed by UPLC and ESI-TOF MS, all of the major lipopeptide components identified previously could be detected (Table 3.4). Surfactin peaks showed the highest intensities, whereas fengycin isoforms were detected at much lower levels, in both isolates (Fig. 3.3 A and B).

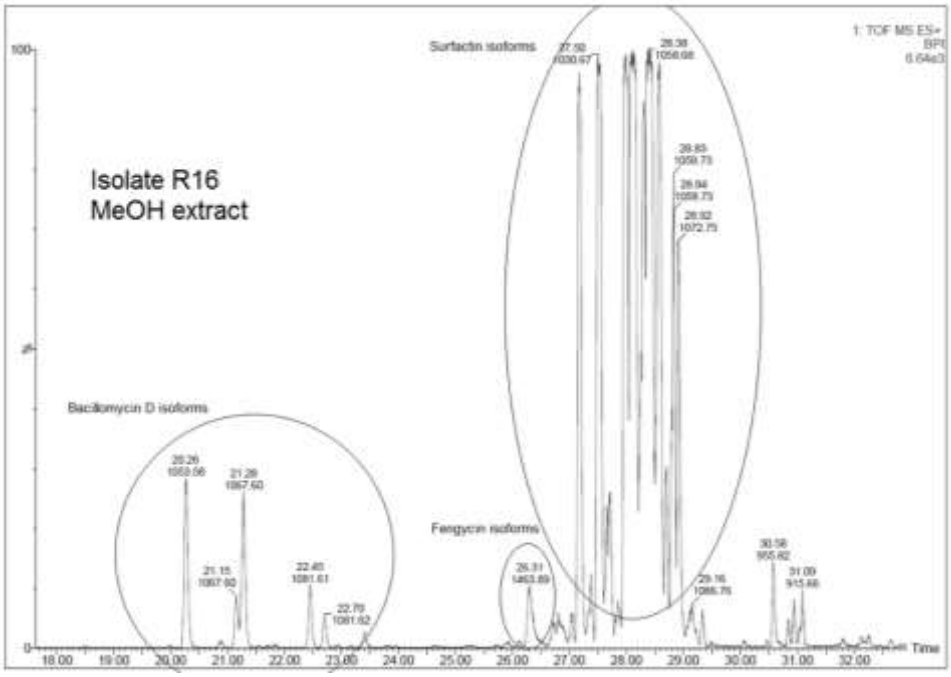
Table 3.4 ESI-TOF mass spectrometric analysis of lipopeptide extracts from selected *Bacillus* spp. isolates obtained from culture filtrates precipitated with HCl and extracted with methanol

Compound	Main Mass Peaks* (m/z)	Species	Isolates
Bacillomycin D	1053.55,1067.57, 1081.59, 1095.60	C14-17 $[M+Na]^+$	R16, CP-R43, CP-R45, CL-R51, CL-R53
Iturin A	1065.6, 1079.61, 1093.62, 1107.64	C14-17 $[M+Na]^+$	CP-R23
Fengycin	1463.81, 1477.84, 1491.85, 1505.87,	Ala-6 C16-17 $[M+H]^+$ Val-6 C16-17 $[M+H]^+$	R16, B81, CP-R23, CP-R43, CP-R45, CL-R51, CL-R53, CT-R67, CT-R89
Surfactin	1030.70, 1044.71, 1058.71, 1072.76	C13-16 $[M+Na]^+$	R16, B81, CP-R23, CP-R43, CP-R45, CL-R51, CL-R53, CT-R67, CT-R89

*Only the mass peaks of the main isoforms present have been reported

Subsequently, a further 9 isolates were evaluated for their ability to produce lipopeptides when grown in Landy medium; the assignment of major peaks associated with crude lipopeptide fractions from seven of the isolates is presented in Table 3.4. Isolate CC-R4 was unable to grow in defined Landy medium; consequently, further characterization of the antifungal compound(s) produced by this organism was not pursued. Isolate CB-R105, which was identified as a strain of *B. cereus* (Chapter 2), grew profusely in Landy medium but failed to yield an insoluble lipopeptide fraction when the cell-free supernatant was acidified. It was also noted that cell-free filtrate of CB-R105 supernatant lacked antifungal activity when tested *in vivo*. The pH of the Landy medium did not exceed pH7.4 after 48 h whereas for isolates which produced lipopeptide compounds the medium became basic, ranging from pH 8.1 to 8.4 after 48 h.

(A)



(B)

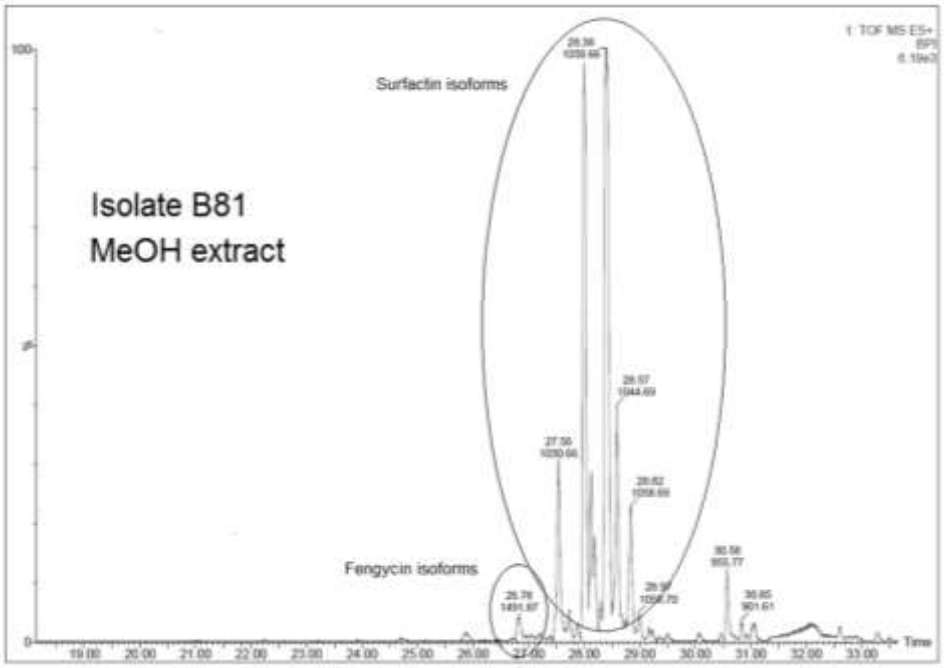


Figure 3.3 UPLC ESI-TOF MS analysis of lipopeptide extracts from isolates R16 (A) and B81 (B).

Isolates CP-R43, CP-R45, CL-R51 and CL-R53 all produced lipopeptide profiles that were very similar to that of isolate R16; whereas the profiles of isolates CT-R67 and CT-R89 were similar to B81. Interestingly, CP-R23 was found to be distinct from the other lipopeptide

producers in that it produced iturin isoforms, with elution times and mass spectra consistent with the iturin A standard (Fig. 3.4).

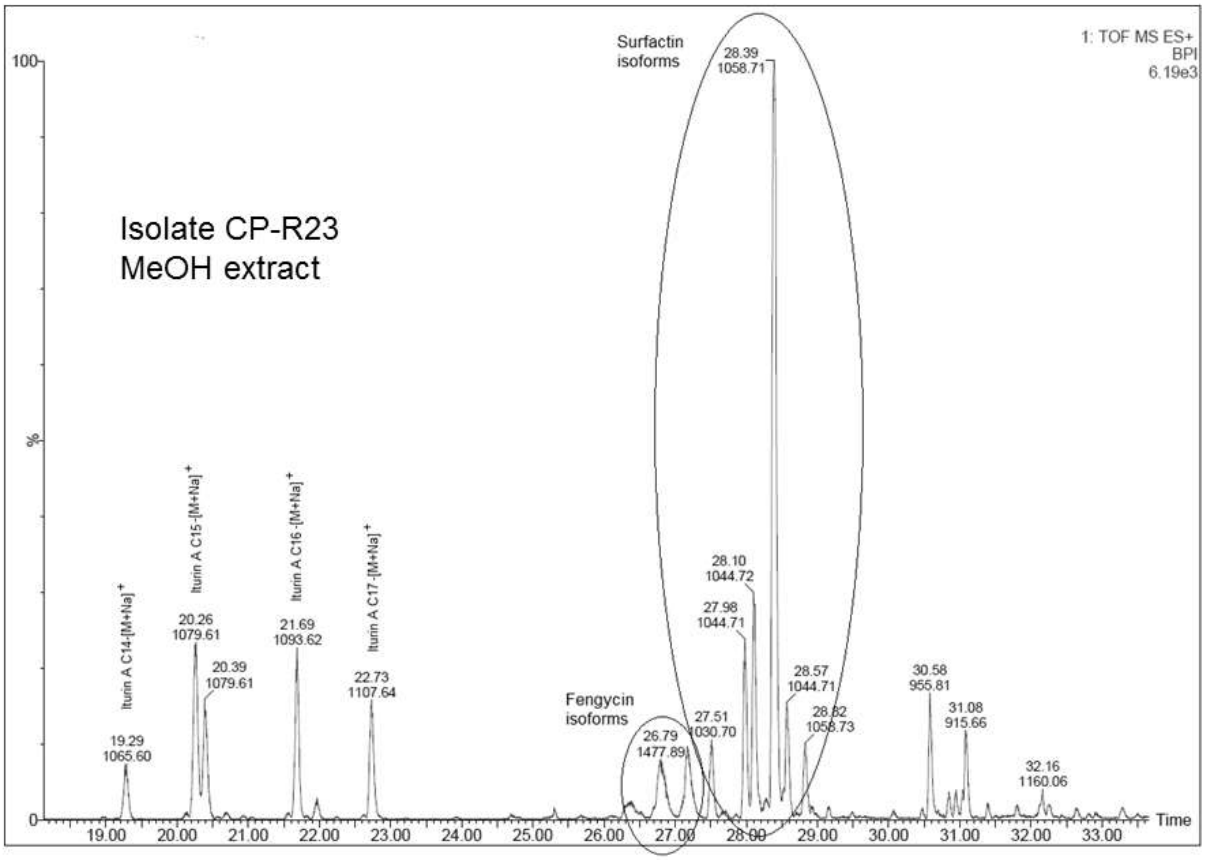


Figure 3.4 UPLC ESI-TOF MS analysis of lipopeptide extract from isolate CP-R23.

3.3.2 PCR Screening for genes associated with lipopeptide biosynthesis

The results of PCR amplification of lipopeptide biosynthetic gene fragments are shown in Figures 3.5-7. Six of the 21 isolates screened (viz., R16, CP-R43, CP-R45, CL-R51, CL-R53 and CT-R64) gave rise to a ~850 bp fragment when BACC1F/1R primers, targeting the *bmyC* gene of the bacillomycin D biosynthetic gene cluster, were used (Fig. 3.5(A)). Two sequence types demonstrating 99% similarity were distinguished when sequences were aligned and similarity matrices generated using Clustal X and BioEdit software. It was interesting to note that these isolates also separated along the same lines when RAPD-PCR fingerprinting methods were used to distinguish between them. An NCBI BLAST search of the GenBank database established that the sequenced products had high levels of sequence homology (>98% similarity) to the bacillomycin D synthetase C genes (*bmyC*) of *B. amyloliquefaciens* subsp. *plantarum* strains FZB42 (Accession no. CP000560) and YAU B6901-Y2 (Accession no. HE774679)(Table 3.5). These results concurred with the ESI-TOF MS findings which identified isolates R16, CP-R43, CP-R45, CL-R51 and CL-R53 as bacillomycin D producers.

Results for the PCR screening for *ituD* gene fragments corresponding to the iturin A synthetase gene cluster are shown in Figure (3.5 (B)). Amplification products of the expected size (~1200 bp) were detected in seven of the lanes representing isolate CP-R23 and the six isolates that had been shown previously to be *bmyC* positive. Of these, only CP-R23 produced iturin A when grown in Landy's medium. In addition to these isolates, faint bands of comparable size were also detected for *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7, *B. subtilis* DSM3258 and isolate CL-R59. Sequenced PCR products of strains R16, CP-R23, CP-R43, CL-R51, CL-R53 and CT-R64 all showed high levels of homology (>98%) to a malonyl-CoA transacylase (*ituD*) of the iturin synthetase gene cluster of *B. amyloliquefaciens* PPCB004 (Accession No. FJ815155) as well as malonyl-CoA transacylases (*bmyD*) associated with bacillomycin synthetase gene clusters found in *B. amyloliquefaciens* subsp. *plantarum* FZB42 (Accession no. CP000560) and *B. amyloliquefaciens* subsp. *plantarum* YAU B6901-Y2 (Accession no. HE774679) (Table 3.5). Attempts to sequence the *ituD* amplicon from *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 proved unsuccessful. Instead, the sequence datum for the *ituD* gene of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 (Accession no. 597644) was aligned to the sequences obtained from the ITUD1F/1R amplicons. When a similarity matrix was generated, the DSM7 sequence showed only a 90% similarity to the corresponding sequences

from isolates R16, CP-R23, CP-R43, CL-R51, CL-R53 and CT-R64. The specificity of the ITUD1F/ITUD1R primers to hybridization sites on the DSM7 sequence was assessed with a nucleotide-to-nucleotide BLAST search alignment. Both primers aligned to single target sites but the guanine at position 19 on the forward primer did not match the adenine nucleotide at the corresponding position on the *ituD* gene fragment. This discrepancy in primer specificity could account for the poor amplification of the *ituD* gene from DSM7 (and possibly DSM3258 and CL-R59).

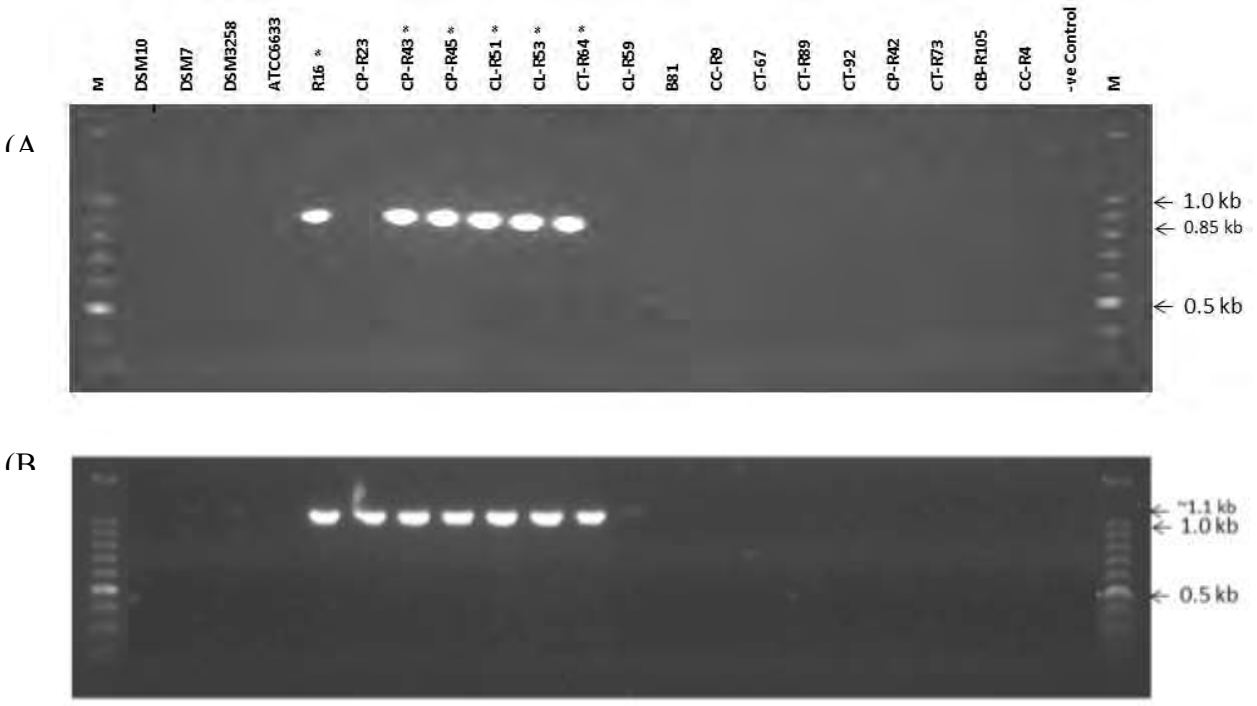


Figure 3.5 Polymerase chain reaction screening for antibiotic biosynthesis genes *bmyC* (A) and *ituD* (B) amongst bacterial isolates antagonistic to *R. solani in vitro*, using primer pairs BACC1-F/BACC1-R (A) and ITUD1-F/ITUD1-R (B). Isolate CP-R23 produced iturin A when grown in Landy's medium, whereas isolates highlighted with * produced bacillomycin D isoforms. Lanes M = 100 bp ladder.

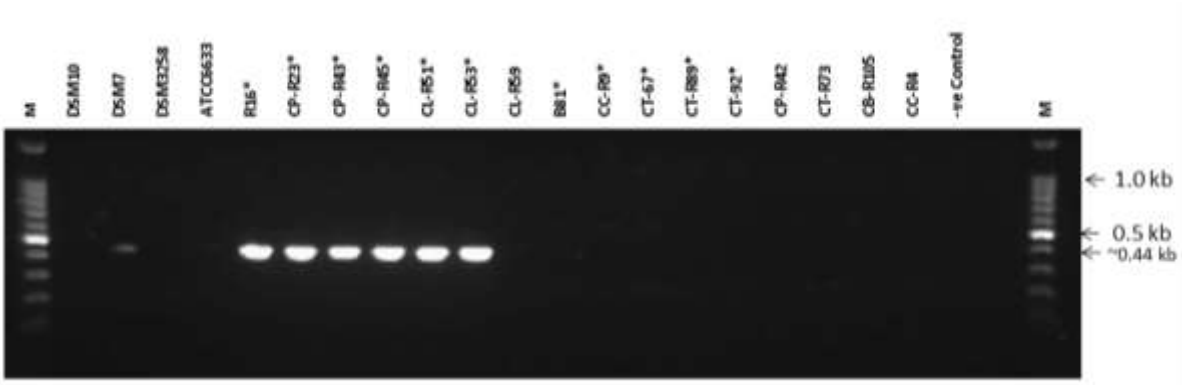


Figure 3.6 Polymerase chain reaction screening for the surfactin synthetase (*srfD*) gene amongst bacterial isolates antagonistic to *R. solani in vitro*, using primer pair SUR3-F/SUR3-R. Isolates highlighted with * produced surfactin isoforms when grown in Landy's medium. Lanes M = 100 bp ladder.

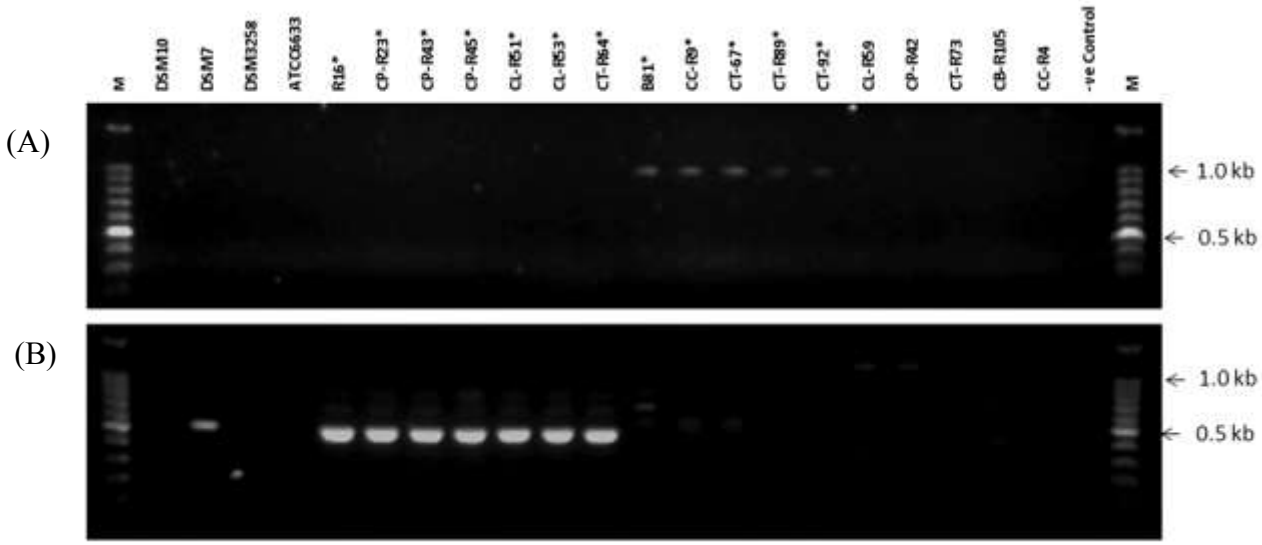


Figure 3.7 Polymerase chain reaction screening for the *fenD* gene of the fengycin biosynthetic cluster amongst bacterial isolates antagonistic to *R. solani in vitro*, using primer pairs FEND1-F/FEND1-R (A) and FENG-F/FENG-R (B). Isolates highlighted with * produced fengycin isoforms when grown in Landy's medium. Lanes M = 100 bp ladder.

Results of the PCR screening for s*rfD* gene fragments corresponding to surfactin sythetase D are shown in Figure 3.6. Isolates R16, CP-R23, CP-R43, CP-R45, CL-R51 and CL-R53 all produced intense PCR bands with the SUR3F/3R primer pair, each with a consistent band size of ~440 bp. *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7, a known surfactin producer, produced a similar PCR product, albeit at lower intensities. This discrepancy was attributed to possible lower primer specificity, stringent PCR reaction conditions and the

relatively low number of reaction cycles used in the PCR protocol. Nucleotide sequences from CP-R23, CP-R43, and CL-R53 amplicons all showed a very high level of homology (≥99%) to the s*rfD* genes of *B.amyloliquefaciens* subsp. *plantarum* FZB42 and YAU B6901-Y2 (Accession nos. NC_009725 and NC_017061 respectively)(Table 3.6). These isolates were all found to produce surfactin when lipopeptide extract fractions were characterized by ESI-TOF MS. However, *srfD* PCR products were not detected for isolates B81, CC-R9, and CT-R67, which were previously shown to produce surfactin in culture. This was attributed to a lack of primer specificity.

To test this assertion, nucleotide sequence data corresponding to the *srfD* gene of 16 strains of *Bacillus* spp. were downloaded from GenBank for comparison to the SUR3F/3R amplicons. Phylogenetic analyses of *srfD* sequence data (360 bp) were conducted using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods (Figure 3.8 A and B). Both trees exhibited very similar topologies. Sequences were found to group into clearly delineated clusters according to species. Within the *B. subtilis* and *B. amyloliquefaciens* groupings, sub-clusters distinguishing sub-species were also evident. Isolates CP-R23, CP-R43, and CL-R53 all grouped within the *B. amyloliquefaciens* subsp. *plantarum* sub-cluster, which supported earlier findings from comparative sequence analysis of partial *gyrA* genes (Section 2.3.4.4, Fig. 2.7 and 2.8). When primer pair specificity of the SUR3F/3R primers was checked against *srfD* sequences belonging to subspecies of *B. subtilis* (primer-BLAST, http://www.ncbi.nlm.nih.gov/genbank/), no significant matches were identified for either of the primers. Similarity matrix comparisons revealed that *srfD* sequences from *B. subtilis* strains exhibited only 69 to 71% similarity to isolates CP-R23, CP-R43, and CL-R53. These findings support the original assertion that the SUR3F/3R primer pair lacked specificity towards isolates B81, CC-R 9, and CT-R67.

When the FenD1F/1R primer pair targeting *fenD* genes of the fengycin biosynthetic cluster was evaluated only B81, CC-R9, CT-R67, CT-R89 and CT-R92 showed faint amplicons (~930 bp) similar to those reported in the literature (Athukorala *et al*., 2009) (Fig. 3.7A). These isolates were amongst those found to produce fengycin in culture when lipopeptide extract fractions were characterized by ESI-TOF MS. However, a number of other fengycin-producing isolates namely, R16, CP-R23, CP-R43, CP-R45, CL-R51, CLR53 and CT-R64, did not yield *fenD* amplicons with these primers. Again, this was possibly due to a lack of primer specificity. *FenD* sequences for B81, CC-R9 and CT-R67 were identical and revealed

a 98 % similarity to the sequence for a plipastatin synthetase found in *Bacillus sp.* JS (Accession no. NC017743)(Table 3.7). The next closest match (90 % similarity) was to a condensation domain protein associated with fengycin synthetase found in *B. subtilis* subsp. *subtilis* strain RO-NN-1 (Accession no. CP002906). A comparison with *B. amyloliquefaciens* subsp. *plantarum* FZB42 sequence data (Accession no. CP000560) revealed a limited similarity match (71%) to a fengycin synthetase B (*fenB*) gene.

Nucleotide sequences homologous to the *fenD* amplicons of B81, CC-R9 and CT-R67 were downloaded from GenBank and their evolutionary relationships were inferred (Figure 3.9 A and B). Once again, the NJ and ML phylogenetic trees exhibited very similar topologies. Strains clustered along similar lines to the *srfD* phylogenetic tree, with sequences grouping according to species. Significantly, isolates B81, CC-R9 and CT-R67 formed a sub-cluster with *Bacillus* sp. JS that was distinct from *B. subtilis* subsp. *subtilis* strains.

Results for the FENG1F/1R primers that were developed to target a 500 bp region of the fengycin synthetase D gene (*fenD*) found in *B. amyloliquefaciens* strains DSM7 and FZB42 are shown in Figure 3.7 B. Positive results were obtained for isolates R16, CP-R23, CP-R43, CP-R45, CL-R51, CL-R53, CT-R64 as well as DSM7. Sequences from R16, CP-R23, CP-R43, and CL-R53 amplicons all showed high levels of homology (>98%) to common regions found in the *fenA, fenC* and *fenD* genes of *B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and YAU B6901-Y2PCR (Table 3.7).

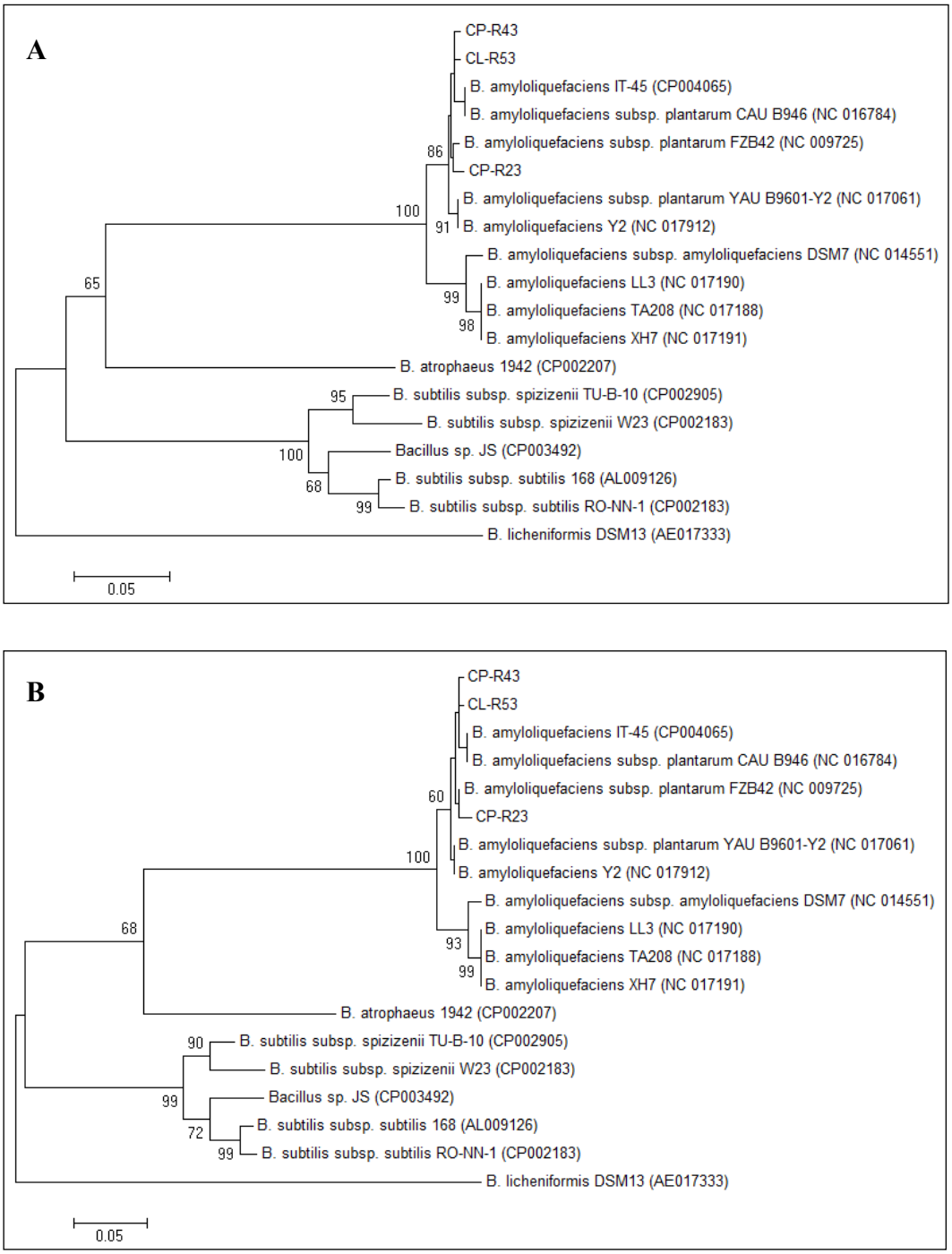


Figure 3.8 Unrooted neighbour-joining (A) and maximum likelihood (B) phylogenetic trees inferring the evolutionary relationship of nucleotide sequences homologous to surfactin synthetase (*srfD*) gene fragments (360 bp) derived from isolates CP-R23, CP-R43 and CL-R53. Evolutionary distances were computed using the Jukes-Cantor and Tamura-Nei models respectively (MEGA6.06). Only bootstrap values from 1000 replicates that were >50% are shown. The scale bar corresponds to 0.05 nucleotide substitutions per sequence position. Genus abbreviation: *B.* = *Bacillus*.

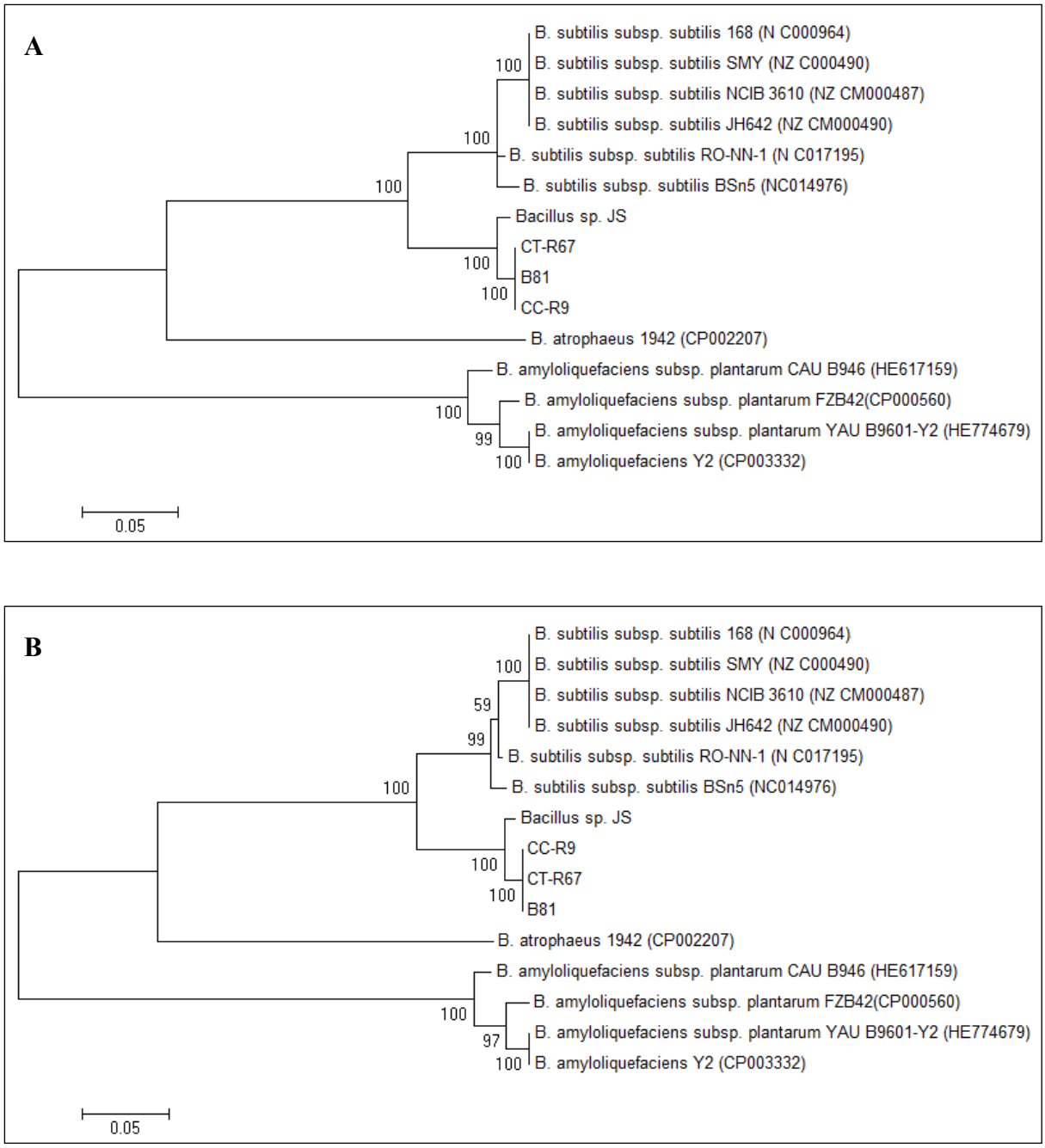


Figure 3.9 Unrooted neighbour-joining (A) and maximum likelihood (B) phylogenetic trees inferring the evolutionary relationship of nucleotide sequences homologous to *fenD* gene fragments (874 bp) of the fengycin biosynthetic cluster derived from isolates B81, CT-R67 and CC-R9. Evolutionary distances were computed using the Jukes-Cantor and Tamura-Nei models respectively (MEGA6.06). Only bootstrap values from 1000 replicates that were >50% are shown. The scale bar corresponds to 0.05 nucleotide substitutions per sequence position. Genus abbreviation: *B.* = *Bacillus*

Table 3.5 Similarity matches of partial lipopeptide biosynthetic gene sequences amplified from *Bacillus* spp. by PCR using primers targeting the *bmyC* and *ituD* genes (Accessed November 2014)

Isolate(s)	Primers	Product size (bp)*	Genbank match	Organism	Accession No.	Identity (%)	S-score	E-value
R16, CP-R43, CL-R64	BACC1F/ BACC1R	762	Bacillomycin D synthetase C (*bmyC*)	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	99	1337	0
				YAU B6901-Y2	HE774679	98	1328	0
CL-R51, CL-R53	BACC1F/ BACC1R	762	Bacillomycin D synthetase C (*bmyC*)	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	98	1615	0
				YAU B6901-Y2	HE774679	99	1543	0
R16, CP-R43, CL-R64	ITUD1F/ ITUD1R	1069	Malonyl-CoA transacylase (*bmyD*), Non-ribosomal synthesis of bacillomycin D	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	99	1865	0
				YAU B6901-Y2	HE774679	98	1865	0
CL-R51, CL-R53	ITUD1F/ ITUD1R	1069	Malonyl-CoA-transacylase-like (*ituD*) gene,	*Bacillus amyloliquefaciens* strain				
				PPCB004	FJ815155	99	1900	0
			Malonyl-CoA transacylase (*bmyD*)	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42	CP000560	99	1895	0
CP-R23	ITUD1F/ ITUD1R	1069	Malonyl-CoA-transacylase-like (*ituD*) gene,	*Bacillus amyloliquefaciens* strain				
				PPCB004	FJ815155	99	1851	0
			Malonyl-CoA transacylase (*bmyD*)	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42	CP000560	98	1847	0
DSM7	ITUD1F/ ITUD1R	1069	Malonyl-CoA-transacylase (*ituD*),	*Bacillus amyloliquefaciens* strains XH7,	CP002927	100	1929	0
				LL3	CP002634	100	1929	0
			Malonyl-CoA transacylase (*bmyD*), Non-ribosomal synthesis of bacillomycin D	*B.amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	90	1467	0
				YAU B6901-Y2	HE774679	90	1467	0

Note: S-Score is a measure of similarity of the BLAST query to the sequence shown. E-value is a measure of the reliability of the S-score
*Product size based on sequence obtained and used for the GenBank search.

Table 3.6 Similarity matches of partial lipopeptide biosynthetic gene sequences amplified from *Bacillus* spp. by PCR using primers specific for surfactin synthase (*srfC*) (Accessed November 2014)

Isolate(s)	Primers	Product size (bp)*	Genbank match	Organism	Accession No.	Identity (%)	S-score	E-value
R16, CP-R43	SUR3F/ SUR3R	360	Surfactin synthase	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	NC_009725	99	636	4e-179
				YAU B6901-Y2	NC_017061	99	636	4e-179
CP-R23	SUR3F/ SUR3R	360	Surfactin synthase	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	NC_009725	99	649	0
				YAU B6901-Y2	NC_017061	99	643	0
CL-R53	SUR3F/ SUR3R	360	Surfactin synthase	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	NC_009725	99	636	4e-179
				YAU B6901-Y2	NC_017061	99	632	4e-179
DSM7	SUR3F/ SUR3R	360	Surfactin synthase	*B. amyloliquefaciens* XH7	NC_017191	99	623	3e-175
				and LL3;	NC_017190	99	623	3e-175
				B. amyloliquefaciens subsp. *plantarum* strain FZB42	NC_009725	96	578	9e-162

Note: S-Score is a measure of similarity of the BLAST query to the sequence shown. E-value is a measure of the reliability of the S-score.
*Product size based on sequence obtained and used for the GenBank search.

Table 3.7 BLAST search results of partial lipopeptide biosynthetic gene sequences amplified from *Bacillus* spp. by PCR using two primer pairs FEND1F/1R and FENG1F/1R targeting fengycin synthetase (*fenD*) (Accessed November 2014)

Isolate(s)	Primers	Product size (bp)	Genbank match	Organism	Accession No.	Identity (%)	S-score	E-value
B81, CC-R9, CT-R67	FEND1F/ FEND1R	874	Plipastatin synthetase, Condensation domain protein,	*Bacillus sp.*JS,	CP003492	98	1498	0
				B. subtilis subsp. *subtilis* RO-NN-1,	CP002906	90	1177	0
			Fengycin synthetase *B (fenB)*	*B. amyloliquefaciens* subsp. *plantarum* strain FZB42	CP000560	71	167	1e-37
R16, CP-R43	FENG1F/ FENG1R	431	Fengycin synthetase (*fenA, fenC, fenD*)	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	98	744	0
				YAU B6901-Y2	HE774679	99	753	0
			Non-ribosomal fengycin synthesis	*B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7	FN597644	96	695	0
CP-R23	FENG1F/ FENG1R	431	Fengycin synthetase (*fenA, fenC, fenD*)	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	99	756	0
				YAU B6901-Y2	HE774679	99	756	0
			Non-ribosomal fengycin synthesis	*B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7	FN597644	95	690	0
CL-R53	FENG1F/ FENG1R	431	Fengycin synthetase (*fenA, fenC, fenD*)	*B. amyloliquefaciens* subsp. *plantarum* strains FZB42 and	CP000560	98	744	0
				YAU B6901-Y2	HE774679	99	753	0
			Non-ribosomal fengycin synthesis	*B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7	FN597644	96	695	0

Note: S-Score is a measure of similarity of the BLAST query to the sequence shown. E-value is a measure of the reliability of the S-score.
*Product size based on sequence obtained and used for the GenBank search.

3.4 Discussion

The primary focus of this study was to characterize the antifungal lipopeptide compounds associated with *Bacillus* isolates that showed clear biocontrol activity towards *Rhizoctonia solani* in *in vivo* greenhouse pot trials. A secondary aim was to evaluate the presence of genes linked to lipopeptide biosynthesis by PCR as a means of rapidly screening isolates for their potential to produce antifungal compounds.

Initially, isolates R16 and B81, which both showed broad spectrum antifungal activity *in vitro,* were selected to develop and establish methods for lipopeptide extraction and characterization. Extraction of lipopeptide fractions from the culture broth of both isolates by acidification proved to be a simple but effective isolation step. This method has been used widely as a convenient way of extracting lipopeptides from *Bacillus* spp. cultures (Vater *et al*., 2002; Bais *et al*., 2004; Hsieh *et al*., 2008; Bie *et al*., 2009). Concerns regarding the formation of methyl esters during this process have largely been dispelled by Vater *et al*. (2002), who showed that lipopeptide fractions obtained by precipitation with HCl and extracted with methanol, did not differ from the lipopeptide profile obtained for a crude culture filtrate of *B. subtilis* C-1, when analyzed using MALDI-TOF MS.

Partial purification of the methanol extracts containing lipopeptide mixtures was achieved by TLC. The detection of amphiphilic lipopeptide/biosurfactant compounds by spraying TLC plates with dH_2O also proved to be a simple but effective detection method. The bioautography overlay method was not as sensitive as the filter-disc agar-diffusion bioassay in detecting antifungal activity associated with TLC fractions. This was ascribed to a concentration effect whereby insufficient sample was applied to the bioautogram TLC plate to produce a clear antifungal response when assayed. Using the filter-disc method, three fractions from R16 and two from B81 showed evidence of antifungal activity; whereas, only a single zone on the R16 TLC plate (corresponding to Rf 0.74) showed antifungal activity when the bioutography overlay method was used.

UPLC coupled with ESI-TOF MS analysis revealed that each of these TLC fractions comprised distinct groups of well-separated peaks that could be ascribed to different types of recognized lipopeptides based on the matching mass spectra (Table 3.3). Both isolates were found to produce several isoforms of surfactin and fengycin. In addition, R16 also produced a number of bacillomycin D isoforms (C14-C17). TLC fractions containing either bacillomycin D or fengycin, exhibited antifungal activity when filterpaper-disc agar-diffusion bioassays

were performed, whereas the surfactin fractions did not. It was also apparent that the bacillomycin D fraction exhibited a greater degree of fungal inhibition than either of the fengycin fractions. A similar finding was reported by Koumoutsi *et al*. (2004) for lipopeptides associated with *B. amyloliquefaciens* subsp. *plantarum* FZB42. Examination of a crude preparation of the lipopeptide extract from R16 by UPLC/ESI-TOF MS, showed that bacillomycin D was present at higher relative intensities than fengycin (Fig. 3.3A). Surfactin isoforms were present at the highest relative intensities in both crude samples (Fig. 3.3 A and B).

The overall quality of the mass spectra obtained for the crude lipopeptide extracts of R16 and B81 was similar to those obtained for the TLC fractions. The resolution and sensitivity of the UPLC/ESI-TOF method used was sufficient so that further separation or purification of the crude lipopeptide extracts of the remaining isolates was unnecessary. Results showed that related strains, as previously grouped by RAPD-PCR, produced very similar lipopeptide profiles (Table 3.4). All of the strains screened produced surfactin and fengycin, while those that showed phylogenetic similarities to *B. amyloliquefaciens* R16 also produced bacillomycin D. Isolate *B. amyloliquefaciens* CP-R23 was the exception because it produced iturin A instead of bacillomycin D. Both compounds are closely related, having the same chiral peptide arrangement (LDDLLDL), differing only at positions 4, 5, 6 and 7 of the heptapeptide (Jacques, 2011). Interestingly, CP-R23 showed a close phylogenetic relationship to the bacillomycin producers when 16S rRNA and *gyrA* gene sequence data were compared (Fig.2.5 and Fig.2.7, Chapter 2). RAPD fingerprint profiles obtained with three of the four arbitrary primers evaluated (Fig.2.2, Chapter 2) were also very similar for these organisms. Only the RAPD fingerprint generated using an OPG-5 primer distinguished CP-R23 from the rest of the strains (Fig. 2.2A, lane 10, Chapter 2). This finding highlights the sensitivity of the RAPD-PCR method and confirms the need to use multiple primer sets if subtle strain differences are to be detected.

From the results it was clear that there was a large degree of heterogeneity within each class or type of lipopeptide detected, which could be attributed to the presence of isoforms with fatty acid chains of varying length. This phenomenon is explained by the fact that lipopeptides are synthesized non-ribosomally by multi-enzyme complexes referred to as non-ribosomal peptide synthetases (NRPS) (Finking and Marahiel, 2004). Such systems exhibit a degree of "flexibility" during the assembly of lipoptide compounds which can lead to

variation in amino acid composition or conformation as well as the length and branching of fatty acid side chains. The availability and composition of nutrients present in the culture medium can also influence this outcome. Changes in fatty acid length can affect the physicochemical properties of these compounds, which in turn influences how they interact with cell membranes and impacts on their antimicrobial activity (Bonmatin *et al*., 2003). A general observation is that antimicrobial activity is increased with increasing fatty acid chain length.

Amongst the array of bioactive secondary metabolites produced by *Bacillus* spp., cyclic lipopeptides belonging to the iturin, fengycin and surfactin families of closely related compounds, are widely acknowledged to play important roles in the biocontrol of plant diseases (Magnet-Dana and Peypoux, 1994; Asaka and Shoda, 1996; Moyne *et al*., 2001; Yu *et al*., 2002; Touré *et al*., 2004; Ramarathnam *et al*., 2007; Romero *et al.*, 2007a; Ongena and Jacques, 2008; Arrebola *et al*., 2009; Athukorala *et al*., 2009; Chen *et al*., 2009; Raaijmakers *et al*., 2010; Borriss, 2011; Jacques, 2011; Alvarez *et al*., 2012; Yuang *et al*., 2012; Cawoy *et al.*, 2015). Various studies that have compared the biocontrol efficacy of lipopeptide deficient mutants or transformants with wild-type producers, have revealed the significant role that antifungal compounds, such as iturin A, bacillomycin D and fengycin play in biocontrol (Asaka and Shoda, 1996; Arrebola *et al*., 2009, Liu *et al*., 2011). Consequently, representatives of these lipopeptide compounds have been subject to wide-ranging studies that have focused on their antagonistic activity, structural diversity, as well as their biosynthesis and regulation (For reviews see Stein, 2005; Ongena and Jacques, 2008; Jacques, 2011).

Ecological studies that seek to unravel the roles that these lipopeptides play in the day-to-day functioning of bacteria in their natural habitats have received less attention (Raaijmakers *et al*., 2010; Nihorimbere *et al*., 2012). The ability to produce lipopeptides is not uniform amongst different species and strains of *Bacillus*. Price *et al*. (2007) surveyed the lipopeptide producing capabilities of numerous *Bacillus* strains from different geographic locations and concluded that the ability to produce lipopeptides was linked to the habitat in which they were found. Lipopeptide producing bacteria devote a significant proportion (up to 8.5 %) of their genome to lipopeptide biosynthesis (Chen *et al*., 2007; Borriss, 2011). It has been argued that this investment in the production of secondary metabolites serves a broad ecological purpose, enabling organisms to compete within specific habitats or conditions

(Ongena and Jacques, 2008, Raaijmakers *et al*., 2010). Recent studies suggest that lipopeptides contribute to the ecological fitness of the producer organisms in a number of ways. In addition to their role in antagonism of competing organisms and/or potential predators, lipopeptides have also been linked to the motility and dispersion of bacteria, biofilm formation and the induction of systemic resistance in plants (Raaijmakers *et al*., 2010; Cawoy *et al*., 2014; Chowdhury *et al*., 2015). The upshot of all this is that traits for ecological fitness are as important as criteria for screening and selecting biocontrol agents, as is their antagonistic activity. Screening bacteria for the co-production of the three lipopeptide families is therefore desirable. Further credence is given to this suggestion when one takes into account that commercial biocontrol strains such as *B. subtilis* GB03 (Kodiak®) and *B. amyloliquefaciens* subsp. *amyloliquefaciens* FZB42 (Rhizo-Plus®) both exhibit lipopeptide co-production (Brannen and Kenny, 1997; Joshi and McSpadden-Gardener, 2006; Chen *et al*., 2009).

Surfactins are powerful surfactants and are able to disrupt and solubilize lipid membranes in a dose dependant manner (Jacques, 2011). They are known to be active against bacterial phytopathogens such as *Xanthomonas campestris* pv. *campestris* (Etchegaray *et al*., 2008) and *Pseudomonas syringae* pv. *tomato* (Bais *et al*., 2004) but do not show marked antagonism towards fungi. The presence of ergosterol in the phospholipid bilayer of fungal cell membranes is thought to diminish surfactin's disruptive effect (Jacques, 2011). The bactericidal properties of surfactin are thought to afford the producing organisms a means of eliminating potential competitors within their immediate environment. Indirectly, surfactin production may also prevent the development of biofilms associated with competing microorganisms by inhibiting cell attachment or interfering with the early stages of biofilm formation (Bais *et al*., 2004).

Surfactin has also been found to play a key role in the motility and dispersion of bacilli over surfaces (Kinsinger *et al*., 2003; Koumoutsi *et al*., 2004). *In vitro* studies have shown that surfactin-deficient mutants of *B. amyloliquefaciens* subsp. *amyloliquefaciens* FZB42 lost their swarming capabilities on semi-solid agar plates. By reducing the surface tension of liquids, surfactin facilitates cell spreading over surfaces. This trait is thought to have a significant influence on bacterial distribution within their natural habitats and could be a major determinant of root colonization. Additionally, surfactin production has been linked to biofilm formation. Bais *et al*. (2004) found that the biocontrol efficacy of *B. subtilis* 6051

against *P. syringe* pv. *tomato* on *Arabidopsis* roots were positively linked to its ability to colonize roots through biofilm formation, which in turn was linked to the secretion of surfactin. The ability of *Bacillus* strains to form a pellicle at the air-water interface has also been correlated to surfactin production (Branda *et al*., 2001).

Due to its lack of antifungal activity, surfactin production often appears to be overlooked in biocontrol screening protocols. For isolates that showed significant *in vivo* control of *R. solani,* surfactin isoforms were the predominant lipopeptides found in the lipopeptide extracts. This could be explained by the fact that surfactin production is initiated during exponential growth, peaking during the transition from exponential to stationary phase; whereas bacillomycin/iturin and fengycin production are associated with late stationary phase (Mizumoto and Shoda, 2007). It is likely that surfactin production plays a key role in determining an organism's rhizosphere competency. This trait should therefore also be included in *in vitro* screening protocols. It would be interesting to ascertain whether the level of surfactin production influences biocontrol efficacy of strains. A simple agar-based bioassay based on surfactin's ability to haemolyse red blood cells could be used as a preliminary screening method (Mulligan *et al*., 1984). However, care needs to be exercised when interpreting results since this bioassay does not readily distinguish between surfactin production and haemolysin production found in some *Bacillus* strains.

The production of iturins, such as iturin A and bacillomycin D, appears to be restricted to strains of *B. subtilis* and *B. amyloliquefaciens* and has been linked to the *in vitro* and *in vivo* suppression of a broad range of pathogenic fungi that include *Aspergillus flavus* (Moyne *et al*., 2001), *Candida albicans* (Tabbene *et al*., 2012), *Fusarium oxysporum* (Yuan *et al*., 2012), *Monilinia fructicola* (Liu *et al*., 2011), *Podosphaera fusca* (Romero *et al*., 2007b) and *Rhizoctonia solani* (Asaka and Shoda, 1996; Yu *et al*., 2002; Borriss, 2011). Iturins display strong antifungal activity that is linked to the formation of ion-conducting pores in fungal cytoplasmic membranes, which bring about a disruption of osmotic regulation (Aranda *et al*., 2005). The acyl chain length of the ß-amino acid fatty–acid side chain in iturins is thought to influence the antagonistic activity of these compounds. For example, Tabbene *et al*. (2011) found that C16 isoforms of bacillomycin D displayed greater levels of fungicidal activity towards *C. albicans* than did C14 or C15 isoforms.

Fengycins have also been shown to inhibit filamentous fungi (Jacques, 2011). They interact with lipid membranes and are thought to adversely affect membrane permeability in a dose-

dependent manner; however, their exact mode of action has not been fully elucidated (Bie *et al.,* 2009). There is some evidence that fengycins act synergistically with iturins in inhibiting pathogens such as *P. fusca* (Romero *et al*., 2007a). Significantly, fengycins have been linked to an immune response known as induced systemic resistance (ISR) found in plants (Ongena *et al*., 2007). ISR benefits the plant by potentiating its defense mechanisms to respond timeously to future disease interactions. To date, fengycin has been linked to ISR responses in beans, tomatoes and potatoes (Ongena *et al*., 2007). Surfactin has also been shown to elicit an ISR response in certain plants but this reaction does not appear to be as wide-spread as it is to fengycin (Jourdan *et a*l., 2009).

PCR-based screening of bacterial isolates having lipopeptide biosynthesis related genes.

In recent years the elucidation and sequencing of biosynthetic genes involved in lipopeptide synthesis has enabled the development of specific primers for use in PCR-based detection of lipopeptide-producing candidate bacteria (de Souza and Raaijmakers, 2003). PCR-based screening is attractive because it potentially offers a means of circumventing laborious *in vitro* and *in vivo* screening methods. It provides information regarding the type(s) of antibiotic compound potentially produced without having to resort to biochemical characterization (Hsieh *et al*., 2004; Hsieh *et al*., 2008). It can also be used as a method of targeting specific lipopeptide producers for further evaluation and has been recommended as a means of speeding up the rate of discovery of new biocontrol candidates (de Souza and Raaijmakers, 2003; Joshi and McSpadden-Gardener, 2006).

In this study established primers specific for *bmyC*, *ituD, srfA* and *fenD* genes were evaluated. The results for the BACC1F/1R primers that targeted bacillomycin D synthetase C (*bmyC*) showed good correlation with the ESI-TOF MS findings. The primers targeting a malonyl-CoA-transacylase (*ituD*) gene linked to iturin A biosynthesis, successfully identified isolate CP-R23 as an iturin producer. However, the ITUD1F/1R primer pair was not able to distinguish between CP-R23 and isolates that produced bacillomycin D in culture. These isolates yielded amplicons that were homologous to the *ituD* gene but also closely matched a malonyl-CoA-transacylase (*bmyD*) gene associated with bacillomycin production. The specificity of the ITUD1F/1R primers was further called into question when it was found that the *ituD* gene known to occur in *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 was only poorly amplified. By conducting a primer-BLAST alignment of the ITUD1F/1R primers to the *ituD* gene of DSM7, a mismatch was found, possibly accounting for the result

obtained. BLAST searches of *bymC* and *ituD/bmyD* sequences revealed that those isolates closely related to isolate R16, all showed close matches to these genes found in strains of *B. amyloliquefaciens* subsp. *plantarum.* These results further substantiated earlier findings when the phylogenetic relationships of gyrA gene amplicons were inferred (Fig. 2.7 and 2.8, Chapter 2).

Primer sets SUR3F/3R and FEND1F/1R both showed limited specificity under the selected PCR conditions. SUR3F/3R primers only detected isolates that fell within the R16 phylogenetic grouping whereas FEND1F/1R only detected isolates related to isolate B81, although both groups of organisms were found to be surfactin and fengycin producers. Since both primer sets were designed from *srfD* and *fenD* sequences derived from the original *B. subtilis* strains used to elucidate surfactin and fengycin operons (Ramarathnam *et al*., 2007), these finding could indicate a lack of specificity towards *srfD* and *fenD* genes found in different groups or species of lipopetide producers. Phylogenetic comparisons of *srfD* and *fenD* sequences obtained in this investigation with those available in the GenBank database showed that each gene sequence dataset could be clearly delineated according to species, with significant differences in sequence composition between clusters being evident (Fig. 3.8 and 3.9). This raises interesting questions with regards to the evolution and divergence of surfactin and fengycin gene clusters amongst different species of *Bacillus* and warrants further investigation. Sequence disparities were, therefore, considered the main reason for the limited success obtained with the primers used. Subsequently, using *fenD* sequence data derived from *B. amyloliquefaciens* subsp. *plantarum* FZB42, it was possible to design novel primers that were able to positively detect *fenD* gene fragments within the "R16 group" of related organisms. Given the *gyrA* and *fenD* sequence similarities of "B81 group" organisms to *Bacillus* spp. JS (Accession no. CP003492), it is envisaged that *srfD* sequence data from this organism could be used to develop primers targeting the *srfD* gene of B81.

The findings from this study highlighted the shortcomings of PCR-based screening. Ultimately, this approach is only as good as the primers used. Athukorala *et al*. (2009) recommended that biochemical characterization of lipopeptides should be undertaken subsequent to PCR screening to confirm the initial PCR amplicon findings. However, this approach potentially excludes lipopeptide producing organisms that display marked heterogeneity in the gene regions targeted by specific primers. Interestingly, Athukorala *et al*. (2009) using FEND1F/1R primers found that the *fenD* gene marker was less prevalent than

those of *ituD* or *srfD* found amongst *Bacillus* strains. This finding might reflect the incidence of *fenD* within the sample set or highlight the limitations of the primers used.

Recent advances in sequencing technology, including more rapid through-put and lower costs, have allowed for numerous whole genomes being sequenced. This has resulted in a recent surge in the numbers of whole genome sequences now available in online databases, with currently 114 whole genomes sequenced for *Bacillus* spp. (KEGG database, http://www.genome.jp/kegg/, accessed December 2015). As such, these databases now afford researchers the opportunity to mine sequence data and assess primer specificity amongst closely related organisms. Perhaps more significantly, the opportunity to develop more robust lipopeptide specific primers that target conserved regions within these biosynthetic gene clusters has never been better.

Whilst PCR screening and ESI-TOF MS characterization of lipopeptide fractions offer some significant advantages over the more conventional *in vitro* and *in vivo* screening methods, they still require sample preparation. From a screening perspective, methods that are able to detect different lipopeptides directly from whole cells or culture supernatant preparations potentially offer the greatest opportunity for rapidly selecting *Bacillus* strains for further biocontrol evaluation. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry has shown potential in this regard (Vater *et al*., 2002; Price *et al*., 2007). As such, MALDI-TOF MS screening of isolates obtained in this study will be the focus of the following chapter.

3.5 References

Alvarez, F., Castro1, M., Príncipe, A., Borioli, G., Fischer, S., Mori, G. and Jofré, E. (2012) The plant-associated *Bacillus amyloliquefaciens* strains MEP218 and ARP23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. Journal of Applied Microbiology, 112, 159-174.

Aranda, F.J., Teruel, J.A. and Ortiz, A. (2005) Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. Biochimica et Biophysica Acta Biomembranes, 1713, 51-56.

Arrebola, E., Jacobs, R. and Korsten, L. (2009) Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. Journal of Applied Microbiology, 108, 386-395.

Asaka, O. and Shoda, M. (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Applied Environmental Microbiology, 62, 4081-4085.

Athukorala, S.N.P., Fernando, W.G.D. and Rashid, K.Y. (2009) Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Canadian Journal of Microbiology, 55, 1021-1032.

Bais, H.P., Fall, R. and Vivanco, J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology, 134, 307-319.

Bie, X., Lu, Z. and Lu, F. (2009) Identification of fengycin homologues from *Bacillus subtilis* with ESI-MS/CID. Journal of Microbiological Methods, 73, 272-278.

Bonmatin, J.M., Laprévote, O. and Peypoux, F. (2003) Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Combinatorial Chemistry and High Throughput Screening, 6, 541-556.

Borriss, R. (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In: Maheshwari, D. K. (ed.) Bacteria in Agrobiology: Plant Growth Responses. Springer-Verlag, Berlin Heidelberg, Germany, pp. 41-76.

Borriss, R., Chen, X-H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., Fan, B., Pukall, R., Schumann, P., Sprőer, R., Junge, H., Vater, J., Pűhler, A. and Klenk, H-P. (2011) Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM7^{T} and FZB42^{T}: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. *nov*. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. *nov*. based on complete genome sequence comparisons. International Journal of Systematics and Evolutionary Microbiology, 61, 1786-1801.

Branda, S.S., Gonzalez-Pastor, J.E., Ben-Yehuda, S., Losick, R. and Kolter, R. (2001) Fruiting body formation by *Bacillus subtilis*. Proceedings National Academy of Sciences, USA, 98, 11621-11626.

Brannen, P.M. and Kenney, D.S. (1997) Kodiak – a successful biological-control product for suppression of soil-borne plant pathogens of cotton. Journal Industrial Microbiology and Technology, 19, 169-171.

Carrillo, C., Teruel, J.A., Aranda, F.J. and Ortiz, A. (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochimica et Biophysica Acta, 1611, 91-97.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. Microbial Biotechnology, 8, 281-295. doi: 10.1111/1751-7915.12238.

Cawoy, H., Mariutto, M., Henry, G., Fisher, C., Vasilyeva, N., Thonart, P., Dommes, J. and Ongena, M. (2014) Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. Molecular Plant-Microbe Interactions, 27, 87-100.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut, P.R., Vater, J., Sűssmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G. and Borriss, R. (2007) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nature Biotechnology, 25, 1007-1014.

Chen, X.H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Sussmuth, R., Piel, J. and Borriss,R. (2009) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. Journal of Biotechnology, 140, 27-37.

Chowdhury, S. P., Uhl, J., Grosch, R., Alquéres, S., Pittroff, S., Dietel, K., Schmitt-Kopplin, P., Borriss, R. and Hartmann, A. (2015) Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*. Molecular Plant-Microbe Interactions, 28, 984-95. doi:10.1094/MPMI-03-15-0066-R.

Danhorn, T. and Fuqua, C. (2007) Biofilm formation by plant-associated bacteria. Annual Reviews Microbiology. 61, 401-422.

de Souza, J.T. and Raaijmakers, J.M. (2003) Polymorphisms within the *prnD* and the *pltC* genes from pyrrolnitrin and puoluteorin-producing *Pseudomonas* and *Burkholderia* spp. FEMS Microbiology Ecology, 43, 21-34.

Etchegaray, A., de Castro Bueno, C., de Melo, I.S., Tsai, S.M., Fiore, M.F., Silva-Stenico M.E., de Moraes, L.A. and Teschke, O. (2008) Effect of a highly concentrated lipopeptide extract of *Bacillus subtilis* on fungal and bacterial cells. Archives in Microbiology, 190, 611-622.

Finking, R. and Marahiel, M.A. (2004) Biosynthesis of non-ribosomal peptides. Annual Review of Microbiology, 58, 453-488.

Gond, S.K., Bergena, M.S., Torresa, M.S. and White, J.F. (2015) Endophytic *Bacillus* spp. produce antifungal lipopeptides and induce host defence gene expression in maize. Microbiological Research, 172, 79-87.

Hsieh, F.C., Li, M.C., Lin, T.C. and Kao, S.S. (2004) Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Current Microbiology, 49, 186-191.

Hsieh, F.C., Lin, T.C., Meng, M. and Kao, S.S. (2008) Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide Iturin A. Current Microbiology, 56, 1-5.
Jacques, P. (2011) Surfactin and other lipopeptides from *Bacillus* spp. In: Soberσn-Chàvez, G. (ed.) Biosurfactants. Springer-Verlag, Berlin Heidelberg, Germany, pp. 57-91.

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthélémy, J.P., Thonart, P. and Ongena, M. (2009) Insights into the defence-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Molecular Plant Microbe Interactions, 22, 456–468.

Joshi, R. and McSpadden Gardener, B.B. (2006) Identification and characterization of novel genetic markers associated with biological control activities in *Bacillus subtilis*. Phytopathology, 96, 145–154.

Kim, P.I., Bai, H., Bai, D., Chae, H., Chung, S., Kim, Y., Park, R. and Chi, Y-T (2004) Isolation and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. Journal of Applied Microbiology, 97, 942-949.

Kinsinger, R.F., Shirk, M.C. and Fall, R. (2003) Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. Journal of Bacteriology, 185, 5627–5631.

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. (2004) Structural and functional characterization of gene clusters directing non-ribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology, 186, 1084–1096.

Koumoutsi, A, Chen, X-H, Vater, J. and Borriss, R. (2007) DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillus amyloliquefaciens* Strain FZB42, Applied and Environmental Microbiology, 73, 6953-6964.

Liu, J., Zhou, T., He, D., Wu, H., Liu, W. and Gao, X. (2011) Functions of lipopeptides bacillomycin D and fengycin in antagonism of *Bacillus amyloliquefaciens* CO6 towards *Monilinia fructicola*. Journal of Molecular Microbiology and Biotechnology, 20, 43-52.

Magnet-Dana, R. and Peypoux, F. (1994) Iturins, a special class of poreforming lipopeptides: biological and physicochemical properties. Toxicology, 87, 151-174.

McKeen, C.D., Reilly, C.C. and Pusey, P.L. (1986) Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology, 76, 136-139.

Mizumoto, S., Hirai, M. and Shoda, M. (2007) Enhanced iturin A production by *Bacillus subtilis* and its effect on suppression of the plant pathogen *Rhizoctonia solani*. Applied Microbiology and Biotechnology, 75, 1267-1274.

Moyne, A.L., Shelby, R., Cleveland, T.E., Tuzun, S. (2001) Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. Journal of Applied Microbiology, 90, 622-629.

Mulligan, C.N., Cooper, D.G. and Neufeld, R.J. (1984) Selection of microbes producing biosurfactants in media without hydrocarbons. Journal of Fermentation Technology, 62, 311-314.

Nihorimbere, V., Cawoy, H., Seyer, H., Brunelle, A., Thonart, P. and Ongena, M. (2012) Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. FEMS Microbiology Ecology, 79, 176-191.

Ongena, M., Adam, A., Jourdan, E., Paquot, M., Brans, A., Joris, B., Arpigny, J.L. and Thonart, P. (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environmental Microbiology, 9, 1084-1090.

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J. and Thonart, P. (2005) *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. Applied Microbiology and Biotechnology, 67, 692-698.

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology, 16, 115-125.

Pérez-García, C., Romero, D., Zerioub, H. and de Vicente, S. (2011) Biological control of phytopathogenic fungi by aerobic endospore-formers. In: Logan, N.A. and De Vos, P. (eds) Endospore-forming Soil Bacteria, Soil Biology Volume 27, Springer-Verlag, Berlin Heidelberg, Germany, pp.157-180.

Peypoux, F., Bonmatin, J.M. and Wallach, J. (1999) Recent trends in the biochemistry of surfactin. Applied Microbiology and Biotechnology, 51, 553-563.

Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F. M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil, 340, 505-520.

Price, N. P. J., Rooney, A. P., Swezey, J. L., Perry, E. and Cohan, F. M. (2007) Mass spectroscopic analysis of lipopeptide for *Bacillus strains* isolated from diverse geographical locations. FEMS Microbiology Letters, 271, 83-89.

Raaijmakers, J. M., De Bruijn, I., Nybroe, O. and Ongena, M. (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiology Reviews, 34, 1037-1062.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W.G.D., Xuewen, G. and de Kievit, T. (2007) Molecular and biochemical detection of fengycin- and bacillomycin D- producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Canadian Journal of Microbiology, 53, 901-911.

Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.W., Arrebola, E.,Cazorla, F.M., Kuipers, O.P., Paquot, M. and Perez-Garcia, A. (2007a) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Molecular Plant-Microbe Interactions, 20, 430-440.

Romero, D., de Vicente, A., Zeriouh, H., Cazorla, F.M., Fernandez-Ortuno, D., Tores, J.A. and Perez-Garcia, A. (2007b) Evaluation of biological control agents for managing cucurbit powdery mildew on greenhouse-grown melon. Plant Pathology, 56, 976-986.

Silo-Suh, L.A., Lethbridge, B.J., Raffel, S.J., He, H.Y., Clardy, J. and Handelsman, J. (1994) Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* Uw85. Applied and Environmental Microbiology, 60, 2023-2030.

Stabb, E. V., Jacobson, L. M. and Handelsman, J. (1994) Zwittermicin A-producing strains of *BaciIlus cereus* from diverse soils. Applied Environmental Microbiology, 60, 4404-4412.
Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology, 56, 845-857.

Tabbene, O., Kalai, L., Ben Slimene, I,, Karkouch, I., Elkahoui, S., Gharbi, A., Cosette, P., Mangoni, M.L., Jouenne, T. and Limam, F. (2012) Anti-candida effect of bacillomycin D-like lipopeptides from *Bacillus subtilis* B38. FEMS Microbiology Letters, 316, 108-114.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

Tewelde, T.W. (2004) Characterization of selected *Bacillus* isolates exhibiting broad spectrum antifungal activity. MSc thesis, University of KwaZulu-Natal, South Africa.

Touré, Y., Ongena, M., Jacques, P., Guiro, A. and Thonart, P. (2004) Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. Journal of Applied Microbiology, 96, 1151-1160.

Tsuge, K., Akiyama, T. and Shoda, M. (2001) Cloning, sequencing, and characterization of the iturin A operon. Journal of Bacteriology, 183, 6265–6273.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. (2002) Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Applied and Environmental Microbiology, 68, 6210-6219.

Yu, G.Y., Sinclair, J.B., Hartman, G.L. and Bertagnolli, B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry, 34, 955-963.

Yuan, J., Li, B., Zhang, N., Waseem, R., Shen, Q. and Huang, Q. (2012) Production of bacillomycin- and macrolactin-type antibiotics by *Bacillus amyloliquefaciens* NJN-6 for suppressing soilborne plant pathogens. Journal Agricultural Food Chemistry, 60, 2976-81.

Zeriouh, H., Vicente, A., Pérez-García, A. and Romero, D. (2014) Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. Environmental Microbiology, 16, 2196-2211.

Zhang, S-M., Wang, Y-X., Meng, L-Q., Li, J., Zhao, X-Y., Cao, X., Chen, X-L., Wang, A-X and Li, J-F. (2012) Isolation and characterization of antifungal lipopeptides produced by endophytic *Bacillus amyloliquefaciens* TF28. African Journal of Microbiology Research, 6, 1747-1755.

CHAPTER FOUR

MALDI-TOF MASS SPECTROMETRY: A ROBUST SCREENING METHOD FOR DIFFERENTIATING AND PROFILING AEROBIC ENDOSPORE-FORMING BACTERIA AS CANDIDATE BIOCONTROL AGENTS

4.1 Introduction

Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) is a non-destructive ionization method that provides a fast, sensitive and accurate means of analysing and characterizing biomolecules over a broad *m/z* range (Claydon *et al*., 1996; Maier *et al*., 2006; Freiwald and Sauer, 2009; Welker and Moore, 2011). MALDI-TOF MS has been established as a powerful analytical tool that is especially useful for identifying and characterizing biomarkers (Gebhardt *et al*., 2002; Madonna *et al*., 2003; Price *et al*., 2007) and secondary metabolites (Leenders *et al*., 1999) produced by bacteria. It has also been recommended as a simple and reliable means of screening large numbers of bacterial isolates for bioactive compounds (Vater *et al*., 2002) and as a means of identifying and classifying microorganisms based on protein mass fingerprinting (Freiwald and Sauer, 2009). The application of MALDI-TOF MS to screen environmental isolates, namely aerobic endospore-forming bacteria, for specific biocontrol traits is therefore attractive.

MALDI-TOF MS has an advantage over other forms of mass spectrometry in that it can detect diverse constituents within complex mixtures without the requirement of additional extraction and clean-up steps (Stets *et al*., 2013). This has led to the development of intact cell mass spectrometry (ICMS) which allows "whole" bacterial cells to be analysed directly (Holland *et al*., 1996). Sample molecules are mixed with a photo-absorbing matrix material and are allowed to co-crystalize onto a sample plate. The analyte mixture is irradiated with UV-laser pulses, leading to desorption of high molecular weight molecular ions in the gaseous phase. These ions are accelerated within a vacuum and transferred to a mass analyser, where they are separated and detected based on molecular weight to charge (*m/z*) ratios.

MS analysis of bacterial cells, or cell lysates, gives rise to molecular fingerprint profiles that can be used to differentiate specific bacteria to genus, species and even strain level (Sandrin

et al., 2013). Mass spectra are typically acquired in the mass range 2 to 20 KDa, and detect stable ribosomal proteins and "house-keeping" protein/peptide biomarkers such as DNA binding proteins, ribosome modulation factors, RNA chaperones, and cold shock proteins that are expressed in this mass range within the cell, in most growth phases (Dieckmann *et al*., 2008; Sauer and Kliem, 2010). Microbial identification is achieved by comparing the mass spectra of the unknown isolate with those of known species using pattern matching algorithms (Maier *et al*., 2006). Prior knowledge of biomarker identities is not required and the taxonomic relationship between organisms can also be determined. MALDI-TOF MS based identification of microorganisms' offers a useful and convenient alternative to traditional identification methods; yielding results comparable to 16S rRNA gene sequencing techniques (Mellmann *et al*., 2008; Ghyselinck *et al*., 2011; Böhme *et al*., 2013).

Reference spectra databases are generally biased towards clinical isolate strains; consequently, this shortfall can negatively impact MALDI-TOF MS identification of environmental isolates (Uhlik *et al*., 2011). Inconsistency in the reproducibility of MALDI-TOF MS fingerprints is another limitation of this method (Giebel *et al*., 2010). This variability has been linked to factors such as performance differences between instruments, the influence of media and growth conditions on protein expression, and the effect of sample preparation method and type of matrix solution used on spectrum quality (Williams *et al*., 2003; Wunschel *et al*., 2005). Standardization of sample preparation protocols is therefore central to the successful implementation of MALDI-TOF MS based identification of bacteria.

Cyclic peptides and lipopeptides (mass range 800-5000 Da) are amongst the most commonly described biomarkers that have been used to distinguish different species and strains of *Bacillus* (Leenders *et al.,* 1999). These biomarkers include several families of antimicrobial compounds such as surfactins, iturins, polymyxins, fengycins, kurkstatins and bacitracins (Stein, 2005, Price *et al*., 2007; Béchet *et al*., 2012). A number of these compounds have been positively correlated with biocontrol activity in a number of *Bacillus* spp. strains (Ongena, and Jacques, 2008; Cawoy *et al.*, 2015) and, therefore, offer convenient targets for screening aerobic-endospore forming bacteria for biocontrol traits using MALDI-TOF MS.

MALDI-TOF MS has been used to identify and characterize lipopeptide compounds associated with putative biocontrol strains of several *Bacillus* spp. (Alvarez *et al*., 2012); however, its application as a primary screening tool in the search for potential biocontrol agents appears to have been overlooked in the literature. Athukorala *et al*. (2009) used MALDI-TOF MS to verify the antibiotic producing capabilities of biocontrol *Bacillus* spp. selected for on the basis of PCR-based detection of antibiotic biosynthetic genes, but it was not used in the initial selection phase of the study. The detection of antibiotic biosynthetic genes is dependent on primer specificity and does not confirm the presence of the desired product of biosynthesis; the direct detection of antibiotic production using MALDI-TOF MS is therefore preferable. The application of MALDI-TOF MS to determine lipopeptide profiles has also been used as a rapid means of identifying various strains of *Bacillus* spp. (Leenders *et al.,* 1999). Price *et al*. (2007) showed that lipopeptide profiles are diverse even among narrowly defined taxa within the *B. subtilis-B. licheniformis* clade. They speculated that these differences could account, in part, for the ecological differentiation of strains by contributing to a population's ability to compete against other microorganisms in different microhabitats.

Its ease of use and minimal sample preparation requirements, recommends MALDI-TOF MS as a method suited to the rapid screening of large numbers of bacterial isolates. Thus, a study was undertaken to assess the suitability of MALDI-TOF MS as a rapid means of *de novo* identification and grouping of candidate biocontrol bacterial strains and as a means of screening isolates for lipopeptide biomarkers associated with biocontrol traits.

4.2 Materials and methods

4.2.1 Endospore-forming isolates

A collection of 27 aerobic endospore-forming bacterial isolates, selected previously for their *in vitro* antagonism of *R. solani,* were used in this study (Table 4.1). Several reference strains were also included for comparative purposes: *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7^T; *B. licheniformis* DSM13^T; *B. pumilus* DSM27^T*; B. subtilis* subsp. *subtilis* DSM10^T; *B. subtilis* subsp. *spizizenii* DSM347 (ATCC6633); and, *B. subtilis* DSM3258 (DSMZ, Deutsche, Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Strains were routinely grown on Tryptic Soy agar (TSA) (Merck) media for 24-48 h and incubated at 30^{o}C.

Table 4.1 Bacterial isolates selected for MALDI-TOF MS profiling

Isolate code	ITS-PCR Grouping[a]	RAPD-PCR Grouping[b]	Species assignment[c]	Origin
R 16	I	A	*Bacillus amyloliquefaciens* subsp. *plantarum*	Lettuce roots
CP-R41	I	A	ND	Capsicum pepper roots
CP-R43	I	A	*B. amyloliquefaciens* subsp. *plantarum*	Capsicum pepper roots
CP-R45	I	A	*B. amyloliquefaciens* subsp. *plantarum*	Capsicum pepper roots
CL-R64	I	A	*B. amyloliquefaciens* subsp. *plantarum*	Lettuce roots
CB-R106	I	A	*B. amyloliquefaciens* subsp. *plantarum*	Cabbage roots
CP-R23	I	B	*B. amyloliquefaciens* subsp. *plantarum*	Capsicum pepper roots
CL-R49	I	B	ND	Lettuce roots
CL-R51	I	B	*B. amyloliquefaciens* subsp. *plantarum*	Lettuce roots
CL-R53	I	B	*B. amyloliquefaciens* subsp. *plantarum*	Lettuce roots
B81	I	C	*Bacillus subtilis*	Squash roots
CC-R9	I	C	*B. subtilis*	Cucumber roots
CT-R67	I	C	*B. subtilis*	Tomato roots
CT-R89	I	C	*B. subtilis*	Tomato roots
CT-R90	I	C	ND	Tomato roots
CT-R92	I	C	ND	Tomato roots
CL-R59	I	D	*B. subtilis* subsp. *subtilis*	Lettuce roots
CC-R7	II	-	*Bacillus cereus* group	Cucumber roots
CC-R10	II	-	ND	Cucumber roots
CP-R15	II	-	ND	Capsicum pepper roots
CP-R25	II	-	*B. cereus* group	Capsicum pepper roots
CP-R30	II	-	ND	Capsicum pepper roots
CP-R42	II	-	*B. cereus* group	Capsicum pepper roots
CT-R73	II	-	*B. cereus* group	Tomato roots
CT-R77	II	-	*B. cereus* group	Tomato roots
CB-R105	II	-	*B.cereus* group	Cabbage roots
CC-R4	III	-	*Brevibacillus laterosporus*	Cucumber roots

[a] ITS-PCR fingerprint groupings of isolates achieved with Primers ISR-35/ISR-1494 (Chapter 2, Section 2.3.3a)
[b] RAPD-PCR fingerprint grouping of isolates achieved with Primer OPG-11 (Chapter 2, Section 2.3.3b)
[c] Identification based on 16S rRNA and/or gyrA gene sequence analysis (Chapter 2, Section 2.3.4 and 2.3.5)
ND – Not determined

4.2.2 MALDI-TOF MS

Mass spectra of microbial cells or cell extracts were acquired using a Microflex L20 MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an N_2 laser (337 nm) and run in a linear positive mode at pulse rate frequencies ranging up to 60 Hz. Samples were spotted onto a 96-place stainless steel target plate, air-dried and overlaid with α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution [HCCA (Bruker Daltonics) was dissolved in 50% ($^{v}/_{v}$) acetonitrile (Merck, Darmstadt, Germany) and 2.5% ($^{v}/_{v}$) trifluoroacetic acid (Merck) to give a final concentration of 10 mg HCCA/ml]. Plates were left to dry at room temperature before being placed in the MALDI-TOF reader and analysed.

4.2.2.1 MALDI Biotyper bacterial identification

Spectral processing and analysis was carried out using MALDI FlexControl (Version 2.4), FlexAnalysis (Version 2.4) and Biotyper (Version 3.0) software (Bruker Daltonics) according to the manufacturer's instructions. Profiling of the isolates selected from within the collection was achieved using two methods; one involving the direct transfer of whole cells and the other, application of an ethanol/formic acid extract prepared according to the protocol outlined in the Biotyper (3.0) user manual. In both instances bacterial isolates were first sub-cultured onto TSA and incubated at 30^{o}C for 24 h to ensure that fresh biological material was used for identification purposes. Target plates were cleaned with 80% aqueous trifluoroacetic acid (TFA) (Merck) and rinsed with ultra-pure deionized H_2O (Millipore, Billericia, USA) prior to use.

For the direct transfer method, a thin film of bacterial cells from a single colony was smeared onto the stainless steel target plate using a sterile toothpick. For the ethanol/formic acid extraction procedure one colony of each isolate was transferred separately into a 1.5 ml microfuge tube (Eppendorf) containing 300 µL of ultra-pure deionised H_2O (Millipore) and mixed thoroughly for 1 minute. Nine hundred microlitres of absolute ethanol were added to each tube and mixed for 1 minute. The samples were then centrifuged at 12,000 x g for 2 minutes at ambient temperature, after which the supernatant was removed and the resultant pellet was allowed to dry at room temperature. Formic acid (25 µl, 70 %) was added to the pellet and mixed by vortexing. An equal volume of acetonitrile was then added and mixed briefly before being centrifuged at 12,000 x g for 2 minutes at ambient temperature. The resultant supernatant was then spotted (1 µl) onto the target plate.

Duplicate samples were prepared for both profiling methods. In each instance, sample spots were left to air-dry before being overlaid with 1 µl of HCCA matrix solution (4.2.2), before MALDI-TOF-MS measurements were made. Mass spectra in the 1.9-20.1 kDa range were obtained by measuring 40 laser shots from six different locations per spotted sample. The MALDI system was first calibrated using a Bacterial Test Standard (BTS) (*Escherichia coli* DH5α, Bruker Daltonics) according to Bruker standard operating procedures (SOP).

Spectra were analysed with MALDI Biotyper 3.0 software used in conjunction with the Biotyper BDAL Reference Library (3.1.2) database [Bruker Daltonics database (2011)] to

putatively identify isolates. Identification was achieved using the propriety pattern matching algorithm that compares individual peak lists from unknown isolates to the spectral information of known species recorded in the existing database. The results were then scored and ranked by matching identified peak masses and determining their intensity correlation. Identification scores were presented as log (score) values; scores of 2.300-3.000 indicate highly probable species identification, scores of 2.000–2.299 indicate a secure genus identification with probable species identification, and scores of 1.700–1.999 represent a probable genus identification. Scores less than 1.700 were deemed to be unreliable for identification purposes. The reference library used in this study contained mass spectral data for 3995 reference strains including 67 of *Bacillus* spp..

4.2.2.2 Mass spectra profile (MSP) creation

Mass spectra profiles were generated for selected isolates and processed according to Bruker Biotyper (3.0) SOP protocols in order to create an "in-house" MSP library to supplement the existing Biotyper BDAL library database. MSP creation also served as a means of determining taxonomic relationships between the isolates and reference strains, allowing for cluster analysis and dendrogram construction (MSP dendrogram creation method, Biotyper 3.0, Bruker Daltonics).

To create an MSP, ten replicates of bacterial extract from each isolate (4.2.2) were spotted (1 µl) onto a cleaned target plate and immediately after drying, overlaid with 1 µl of HCCA matrix before measuring mass spectra in the mass range 2-20 KDa. Each sample spot was measured 3 times and the spectral data averaged (240 laser shots per measurement) to give a total of 30 spectra per organism. For calibration purposes a bacterial test standard preparation (*E.coli* DH5α, Bruker Daltonics) was also included with each set of bacterial extracts spotted onto the target plate. MSPs were created from processed raw spectra after smoothing, baseline subtraction, normalization, and peak picking. A minimum of 20 sum spectra per strain was used for MSP calculation. Each MSP contained the average peak position and peak intensities and their frequency in the set of spectra for the most prevalent peaks. Newly created MSPs were added to a MSP library where cross-identification against the existing Bruker BDAL database could be performed. Dendrograms were then generated to establish the level of MSP similarity between isolates and selected reference strains.

4.2.2.3 MALDI-TOF-MS data analysis

Further processing and analysis of MSP spectral data was undertaken to identify common biomarkers shared amongst isolates. mMass (Version 5.4.0) open-source software was used to process mass spectra generated from the MSP study (Strohalm *et al*., 2008). For each isolate ten replicate spectra were used. Averaged spectral data were normalised, smoothed and baseline corrected before peak detection was performed. Mass lists were generated from the mass spectra and comprised signals with relative intensities ≥2% (Fernández-No *et al.*, 2013). The mass lists were then analysed and compared using SPECLUST, an open-source web tool (http://bioinfo.thep.lu.se/speclust.html) designed for the hierarchical clustering of peptide mass spectra (Alm *et al*., 2006). For each set of peak mass lists analysed, the arithmetic means and standard deviation were calculated taking into account a mass tolerance error of ±3 Da (Fernández-No *et al*., 2013). Consensus peaks were identified and an averaged mass list for each isolate was resubmitted to SPECLUST to detect common peak masses and to perform cluster analysis. Peak matching was achieved using the Needleman and Wunsch algorithm (Needleman and Wunsch, 1970).

4.2.2.4 MALDI-TOF-MS detection of lipopeptide compounds

MALDI-TOF-MS analysis was also used to screen for lipopeptide production amongst isolates. Commercial preparations of surfactin and iturin A (Sigma-Aldrich) dissolved in methanol (100 mg/l) were used as reference standards. Strains R16 and B81, which both exhibited antifungal activity towards *Rhizoctonia solani in vitro* and were previously shown to produce an array of lipopeptide isoforms (Chapter Three), were used to develop and evaluate methods for lipopeptide extraction and characterization.

Several different sample preparation methods were evaluated: whole cells from bacterial colonies after 36 h growth; cell-free culture supernatant from isolates grown in Landy's medium (Chapter Three, 3.2.2); methanolic extracts of lipopeptide fractions obtained by HCl precipitation (Chapter three, 3.2.2); and, bacterial extracts obtained using a trifluoroacetic acid (TFA) extraction method (Biotyper 3.0 manual, Bruker Daltonics).

For TFA extraction, a single colony of each isolate was transferred to a separate Eppendorf tube using a sterile toothpick. Fifty microliters of 80% aqueous TFA was pipetted into each tube and the contents mixed by repeated pipetting until the biological material was

completely dissolved. After 15 min incubation at room temperature, 150 µl of ultra-pure deionised H_2O, followed by 200 µl acetonitrile was added to each tube. After vortexing, the mixture was centrifuged at 12,000 x g for 2 minutes at ambient temperature. The resultant supernatant was used for MALDI-TOF-MS analysis. Whole cells and cell free extracts were transferred to target plates as described previously (4.2.2.1). After drying at room temperature all samples were overlaid with 1 µl of HCCA solution and left to air-dry before MALDI-TOF-MS measurements were made. All samples were run in duplicate.

Spectra were generated with the laser in positive linear mode, as the mean of 300 laser shots per sample comprising 50 shots taken from six randomised positions. Laser intensity was set at 12% and peaks in the mass range 750-2500 Da were targetted. Spectral processing was carried out using MALDI FlexAnalysis (2.4) software which made use of a snap-peak detection algorithm with averaging. Tryptic digest of Bovine Serum Albumin (Bruker Daltonics) was used as a reference peptide standard (peak list ranging from 927.493 to 2045.029) for calibration purposes. Peak mass lists generated from FlexAnalysis (2.4) were also exported to mMass software for further processing and visualization. The assignment of *m/z* peaks to specific lipopeptide compounds was done manually taking into account the protonated species as well as the sodium and potassium adducts of lipopeptide biomarkers previously reported for members of the genus *Bacillus* (Leenders *et al*., 1999; Vater *et al*., 2002; Koumoutsi *et al*., 2004; Price *et al*., 2007).

4.3 Results

4.3.1 Biotyper based identification of aerobic endospore-forming bacteria

Results of the MALDI Biotyper-based identification of the aerobic endospore-forming bacterial isolates using the Bruker mass spectra BDal library (Version 3.1.2), before and after the inclusion of an "in-house" MSP database generated in this study, are presented in Table 4.2. Generally, the two sample preparation methods yielded similar results for each isolate, but in some instances their identification scores differed noticeably. In a number of cases, colony pick-off samples yielded no peaks or scores less than 1.700 (no reliable identification) were obtained; whereas ethanol/formic acid extracts from these isolates usually yielded identification scores > 1.700. An exception was isolate R16 which was not reliably identified with either method.

Taking the highest log values for each isolate, in the first round of screening 18 of the 34 isolates (7 reference strains and 27 environmental isolates) were classified to genus level with probable species identification (Scores 2.000-2.299), 3 were identified to species level (scores 2.300-3.000), 12 were accorded probable genus identification (scores 1.700-1.999) and one, R16, was not reliably identified (Score < 1.700). The bacterial test standard (*Escherichia coli* DH5α), which was included in each run, was consistently identified to the species level with scores > 2.300. Interestingly, only three of the six reference strains (viz., *B. subtilis subsp. subtilis* DSM10, *B. licheniformis* DSM13 and *B. pumilis* DSM27) were matched to the correct species, albeit with scores in the range 2.000-2.299. These lower identification scores have been attributed to factors that affect the quality of mass spectra such as differences in the media or incubation conditions compared to those used in establishing the commercial reference database (Wybo *et al*., 2012). The remaining reference strains (*B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7*, B. subtilis* subsp. *spizizenii* ATCC6633 and *B. subtilis* DSM3258) were classified only to the genus level (Scores < 2.000). This was attributed to limitations of the available mass spectra database which contained a single strain of *B.amyloliquefaciens* and six strains of *B. subtilis* which included only one representative of the subspecies *B. subtilis* subsp*. spizizenii.*

The inclusion of the MSP database generated in this study, in a subsequent Biotyper identification run resulted in a greater number of secure identifications being made; with 13 isolates being classified to the genus level with probable species identification (Scores 2.000-2.299) and 21 isolates being identified to the species level (scores 2.300-3.000). In this instance *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 was identified to the species level, however, *B. subtilis* subsp. *spizizenii* ATCC6633 and *B. subtilis* DSM3258 were incorrectly identified as probable strains of *B. amyloliquefaciens* (Scores 2.000-2.299). It was interesting to note that the MSP created for *B. subtilis* subsp. *spizizenii* ATCC6633 was not matched to a freshly prepared culture of ATCC6633 following either preparation method, when the identification run was conducted with the extended MSP database. A possible explanation for this is that if the MSP sample was inadvertently prepared using culture material older than 36 h; the onset of sporulation would likely have influenced the resulting mass spectrum (Wybo *et al*., 2012). This might explain why the MSP for *B.subtilis* subsp. *spizizenii* ATCC6633 showed limited similarity to the *B.subtilis* subsp. *spizizenii* reference strain in the BDal library when the MSP dendrogram was generated (Figure 4.1).

Table 4.2 MALDI-TOF MS identification of bacterial isolates using the Bruker mass spectra BDal database and "in-house" MSP libraries

Isolate	Sample prep	MALDI Biotyper classification	Score Ranking*	MALDI Biotyper + MSP Library classification	Score Ranking*
E.coli DH5 alpha		*E. coli*	+++	*E. coli*	+++
B. amyloliquefaciens subsp. *amyloliquefaciens* DSM7	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	++
B. subtilis subsp. *subtilis* DSM10	Colony Pick-off	*B. subtilis*	++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
B. subtilis subsp. *spizizenii* ATCC6633	Colony Pick-off	No reliable ID	-	*B. amyloliquefaciens*	++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	++
B. subtilis DSM3258	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
	Ethanol/Formic acid extract	*B. subtilis/ B.mojavensis*	+	*B. amyloliquefaciens*	++
B. licheniformis DSM13	Colony Pick-off	*B. licheniformis*	++	*B. licheniformis*	++
	Ethanol/Formic acid extract	*B. licheniformis*	++	*B. licheniformis*	++
B. pumilis DSM27	Colony Pick-off	*B. pumilus*	++	*B. pumilus*	++
	Ethanol/Formic acid extract	*B. pumilus*	++	*B. pumilus*	++
R 16	Colony Pick-off	No reliable ID	-	*B. amyloliquefaciens*	++
	Ethanol/Formic acid extract	No reliable ID	-	*B. amyloliquefaciens*	+++
B81	Colony Pick-off	*B. subtilis*	+++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	++
CC-R4	Colony Pick-off	*Brevibacillus*	+	*Br. laterosporus*	+++
	Ethanol/Formic acid extract	*Br. laterosporus*	++	*Br. laterosporus*	+++
CC-R7	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CC-R9	Colony Pick-off	*B. subtilis*	++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++/++
CC-R10	Colony Pick-off	*B. subtilis*	++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
CP-R15	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CP-R23	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	++

*Score ranking: (+++) indicates highly probable species identification (Log score 2.300-3.000); (++) indicate a secure genus identification with probable species identification (Log score 2.000–2.299); (+) indicates a probable genus identification (Log score 1.700–1.999); (-) indicates an unreliable identification (log score < 1.700).

Table 4.2 continued

Isolate	Sample prep	MALDI Biotyper classification	Score Ranking*	MALDI Biotyper + MSP Library classification	Score Ranking*
CP-R25	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CP-R30	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CP-R41	Colony Pick-off	*B. mojavensis/ B.subtilis*	++	*B. amyloliquefaciens*	++
	Ethanol/Formic acid extract	No peaks detected	-	No peaks detected	-
CP-R42	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*Bacillus sp.*	+
CP-R43	Colony Pick-off	*No reliable ID*	-	*B. amyloliquefaciens*	++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
CP-R45	Colony Pick-off	*No peaks detected*	-	*No peaks detected*	-
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
CL-R49	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
CL-R51	Colony Pick-off	*Bacillus sp.*	+	*B.amyloliquefaciens*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
CL-R53	Colony Pick-off	No peaks detected	-	No peaks detected	-
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
CL-R59	Colony Pick-off	No reliable ID	-	*B. subtilis*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. subtilis*	+++
CL-R64	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
	Ethanol/Formic acid extract	No peaks detected	-	No peaks detected	-
CT-R67	Colony Pick-off	No peaks detected	-	No peaks detected	-
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
CT-R73	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CT-R77	Colony Pick-off	*B. cereus*	+++	*B. cereus*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*Bacillus sp.*	+
CT-R89	Colony Pick-off	*B. subtilis*	+++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
CT-R90	Colony Pick-off	*B. subtilis*	++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
CT-R92	Colony Pick-off	*B. subtilis*	++	*B. subtilis*	+++
	Ethanol/Formic acid extract	*B. subtilis*	++	*B. subtilis*	+++
CB-R105	Colony Pick-off	*B. cereus*	++	*B. cereus*	++
	Ethanol/Formic acid extract	*B. cereus*	++	*B. cereus*	++
CB-R106	Colony Pick-off	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++
	Ethanol/Formic acid extract	*Bacillus sp.*	+	*B. amyloliquefaciens*	+++

*Score ranking: (+++) indicates highly probable species identification (Log score 2.300-3.000); (++) indicate a secure genus identification with probable species identification (Log score 2.000–2.299); (+) indicates a probable genus identification (Log score 1.700–1.999); (-) indicates an unreliable identification (log score < 1.700).

4.3.2 MSP Creation

Of the initial 27 isolates that showed antifungal activity towards *Rhizoctonia solani in vitro* 22 isolates, representative of the major phylogenetic and molecular fingerprint groupings identified previously in this study (Chapter Two), were chosen to develop a mass spectra profile (MSP) library. Included in the library were three reference strains *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7, *B. subtilis* subsp. *spizizenii* ATCC6633 and *B. pumilis* DSM27. The taxonomic relationship of the MSPs created for the isolates, relative to each other and to reference mass spectra of *Bacillus* spp. strains in the Biotyper BDal database, were determined by MSP cluster analysis using proprietary Biotyper software (Bruker) (Figure 4.1).

The MSP spectra for *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 and *B. pumilus* DSM27 both showed high similarity to the MSP spectra of representative strains of these species present in the BDal database. Whereas the MSP spectrum for *B. subtilis* subsp. *spizizenii* ATCC6633 did not; it formed a distinct sub-grouping, along with isolate CP-R41, within the clade that grouped members of the "*Bacillus subtilis"* group of closely related taxa. This grouping was unexpected since CP-R41 had been previously grouped by RAPD-PCR fingerprinting with isolates identified as strains of *B. amyloliquefaciens* subsp. *plantarum.*

Generally, the environmental isolates evaluated clustered into groupings that were consistent with previous molecular fingerprint groupings and/or phylogenetic findings (Chapter Two). Isolates CB-R106, CL-R49, CL-R51, CL-R53, CL-R64, CP-R23, CP-R43, CP-R45 and R16, which had been putatively identified as, or grouped with, strains of *B. amyloliquefaciens* subsp. *plantarum* (16S rRNA gene sequence analysis, RAPD-PCR), formed a distinct sub-grouping within the "*Bacillus subtilis* group" clade. Similarly, isolates B81, CC-R9, CT-R67, CT-R89, CT-R90 and CT-R92, which were previously grouped together according to their RAPD fingerprint clustering and putatively identified as strains of *B. subtilis*, formed a distinct sub-grouping that showed high similarity to several *B. subtilis* strains (and closely related taxa) present in the BDal database. The MSP for isolate CC-R10 also fell within this sub-group, however, this result was unexpected since CC-R10 had previously been grouped based on ITS-PCR, with strains identified as members of the "*B. cereus* group" of related taxa.

Again, the MSP for isolate CL-R59 fell within the "*Bacillus subtilis* group" clade but as a separate sub-group. This isolate was previously identified as a strain of *B. subtilis* subsp. *subtilis* and was distinguishable from the other environmental isolates evaluated based on ITS-PCR and RAPD fingerprint profiles (Fig. 2.1 and 2.2, Chapter Two). Isolates CC-R7, CP-R42 and CB-R105 formed a loose grouping with representatives of the "*Bacillus cereus* group*"* of related taxa in the BDal database, which is consistent with the phylogenetic groupings determined for these isolates previously. Likewise, isolate CC-R4 which had been identified as a strain of *Br. laterosporus* was found to group with strains of this species listed in the BDal database. Examples of representative mass spectra from each of the main groupings distinguished are presented in Figure 4.2. A mass range of 2.0-14.0 kDa is used to depict these spectra since all of the major peaks detected fell within this mass range.

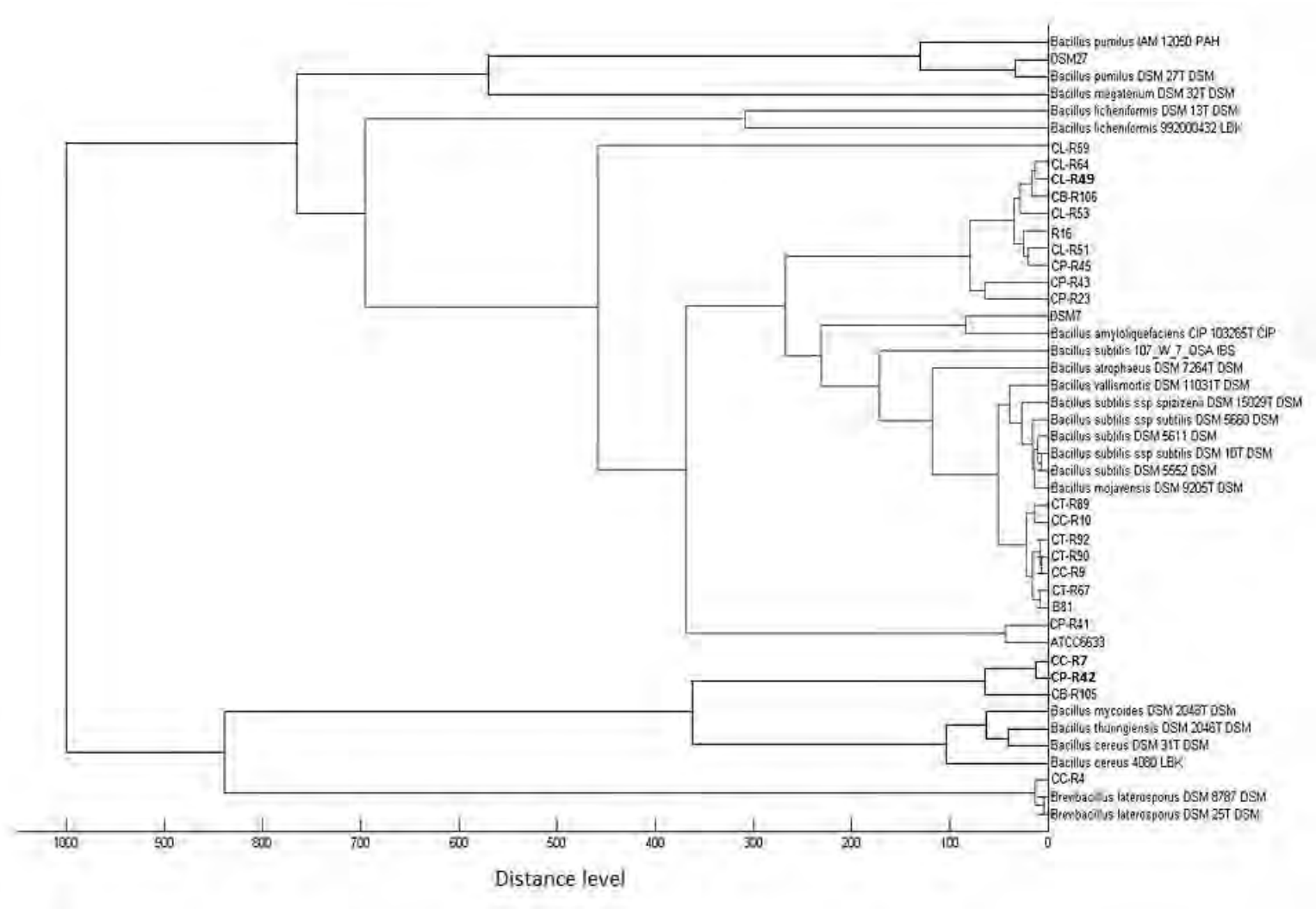


Figure 4.1 Biotyper cluster analysis depicting the possible taxonomic relationship of bacterial isolates chosen for MSP profiling in relation to selected reference spectra in the Biotyper BDal database

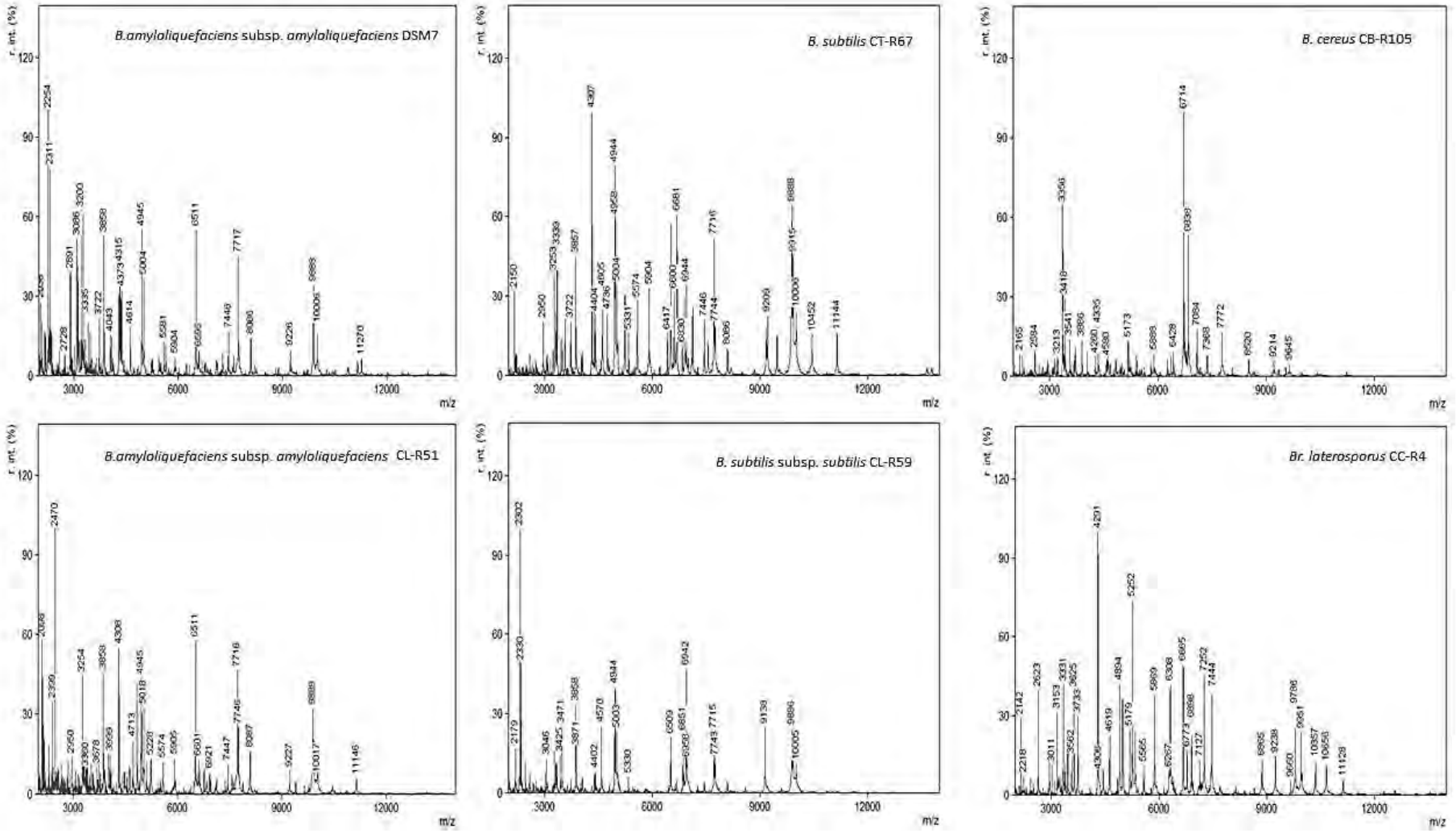


Figure 4.2 Representative MALDI-TOF mass spectra (m/z 2000 to 14000) of bacterial isolates from the main groupings distinguished by Biotyper cluster analysis generated using mMass (Version 5.4.0)

4.3.3 SPECLUST hierarchical cluster analysis

Further to the library based approach used to identify and group isolates, peak mass lists were generated from MSP for hierarchical cluster analysis and the detection of common biomarkers using SPECLUST software (Alm *et al*., 2006). A dendrogram illustrating the grouping of isolates based on SPECLUST hierarchical cluster analysis is shown in Figure 4.3. A compilation of peaklists from averaged spectra highlighting common biomarkers associated with each grouping is included in Appendix B.

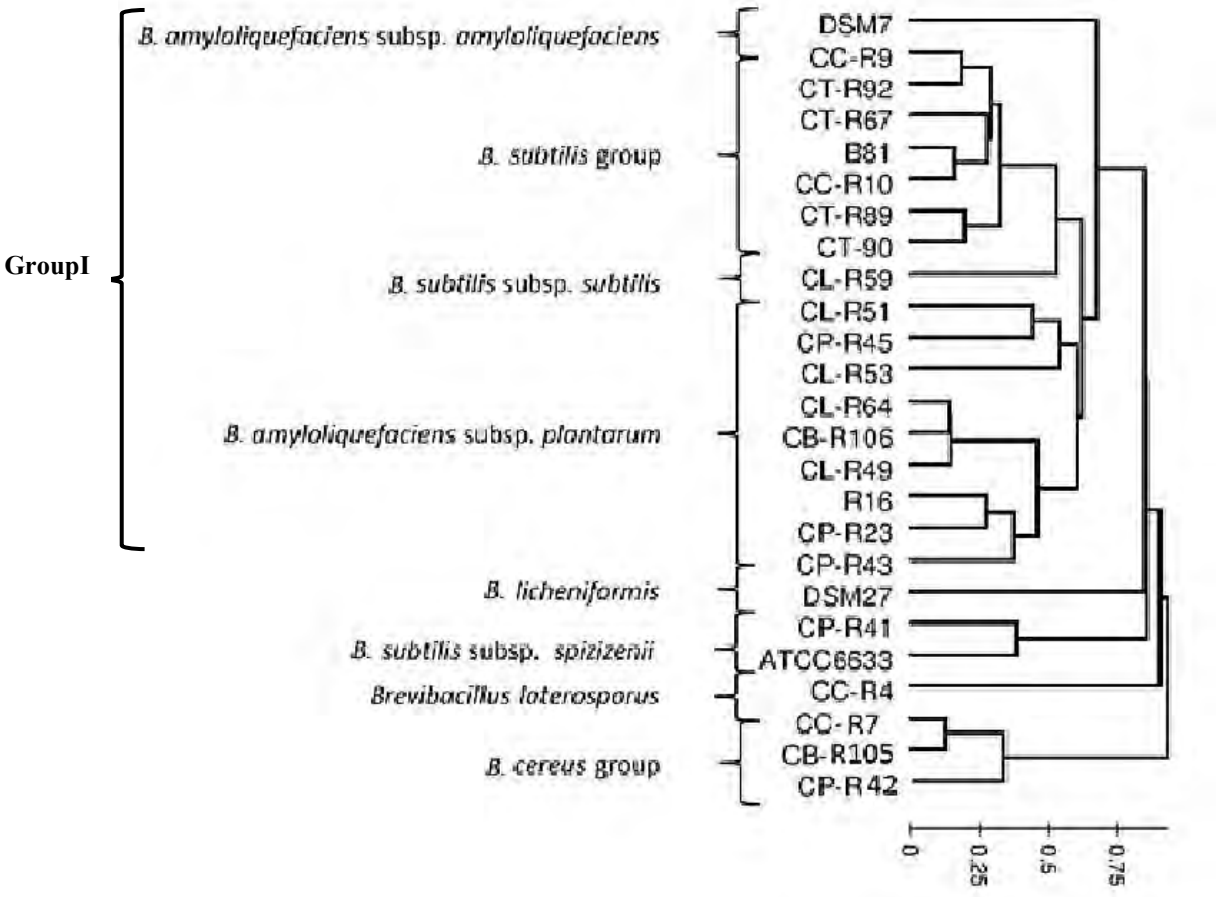


Figure 4.3 Cluster analysis of the peak mass lists of bacterial isolates selected for MSP profiling using the SPECLUST web tool (http://bioinfo.thep.lu.se/speclust.html)

SPECLUST cluster analysis revealed groupings of isolates that were similar to the Biotyper dendogram results (Figure 4.1). In this instance CL-R59 grouped more closely with those isolates identified as representatives of the "*Bacillus subtilis* group". ATCC6633 and CP-R41

still formed a distinct sub-group but was clearly distinguished from the rest of the "*Bacillus subtilis* group" isolates.

Several common mass fragments could be distinguished within and between the major groupings identified (Appendix B). Peaks with *m/z* values of 3253, 3297, 3857, 3871, 4944, 4957, 6510, 7716, 7744 and 9887 were common to isolates identified as representatives of the "*Bacillus subtilis* group" of closely related taxa (Group1, Fig 4.3). Isolates B81, CC-R9, CC-R10, CT-R67, CT-R89, CT-R90 and CT-R92 yielded several biomarkers that were group specific namely 3339, 4736, 6681, 6830, 7545 and 9470 *m/z* whereas isolates identified as strains of *B. amyloliquefaciens* subsp. *plantarum* yielded only two group specific mass fragments of 4473 and 4613 *m/z*. Group specific mass fragments for isolates CC-R7, CP-R42 and CB-R105 (*Bacillus cereus* group) included 3418, 3541, 4030, 5173, 5887, 6713, 6729, 6838 and 7772 *m/z*. Isolate CP-R41 produced a mass spectrum similar to *B. subtilis* subsp. *spizizenii* ATCC6633 and six group specific mass fragments were distinguished viz., 2484, 2958, 3728, 5920, 6159 and 6180 *m/z*. No strain specific mass fragments were determined for isolates CC-R4 (*Br. laterosporus*) and CL-R59 (*B. subtilis* subsp. *subtilis*).

4.3.4 MALDI-TOF MS based screening and detection of lipopeptides produced by isolated bacteria exhibiting *in vitro* antagonism of *R. solani*

Surfactin and iturin A standards were used to assess the accuracy and precision of the MALDI-TOF MS system used. Duplicate samples of each standard were analysed on three separate occasions and average peak *m/z* values and levels of standard deviation were determined (Table 4.3). Standard deviation values of 0.52 Da or less were recorded. Several isoforms were identified for each standard (Figure 4.4); the *m/z* values obtained were consistent with results determined previously using ESI-MS (Table 3.2, Chapter Three). Major peaks could be assigned to protonated species, sodium adducts and/or potassium adducts of lipopeptide isoforms based on *m/z* values specified in the literature (Vater *et al*., 2002; Koumoutsi *et al*., 2004; Price *et al*., 2007). Sodium and potassium adducts were distinguished on the basis that they form peaks 22 and 38 mass units higher than the protonated species respectively (Price *et al*., 2007).

Table 4.3 MALDI-TOF MS detection of surfactin and iturin A standards

Peak Assignment	m/z	std dev
Iturin		
C14 $[M+H]^+$	1043.44	0.22
C15 $[M+H]^+$	1057.47	0.24
C14 $[M+Na]^+$	1065.47	0.23
C16 $[M+H]^+$	1071.52	0.24
C15 $[M+Na]^+$	1079.48	0.24
C16 $[M+Na]^+$	1093.54	0.23
Surfactin		
C12 $[M+H]^+$	994.36	0.29
C13 $[M+H]^+$	1008.30	0.26
C12 $[M+Na]^+$	1016.41	0.33
C14 $[M+H]^+$	1022.39	0.32
C13 $[M+Na]^+$	1030.39	0.31
C15 $[M+H]^+$	1036.44	0.26
C14 $[M+Na]^+$	1044.51	0.52
C13 $[M+K]^+$	1046.47	0.36
C15 $[M+Na]^+$	1058.41	0.30
C14 $[M+K]^+$	1060.55	0.27
C15 $[M+K]^+$	1074.38	0.33

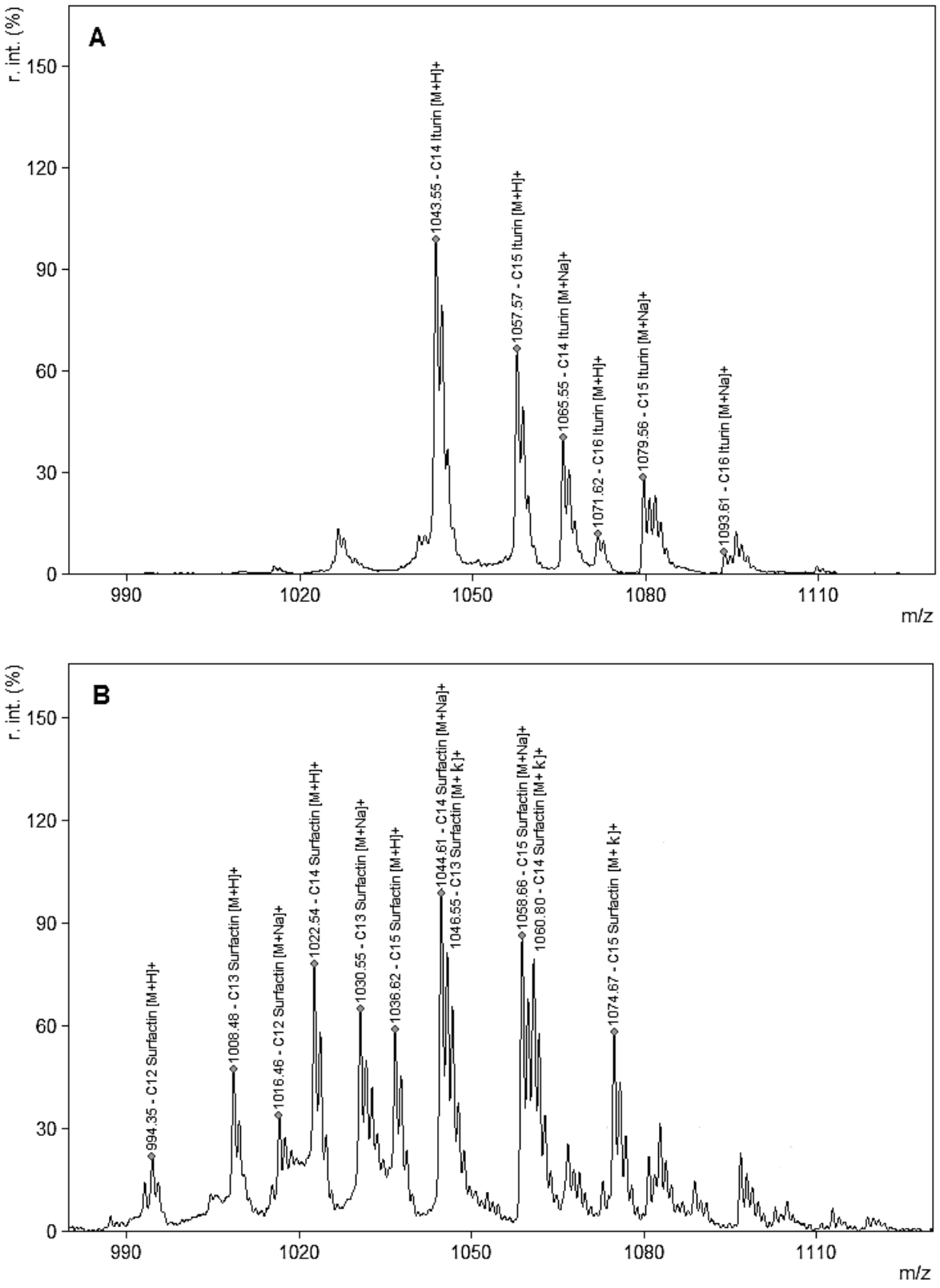


Figure 4.4 MALDI-TOF MS mass spectral profiles of iturin A (A) and surfactin (B) standards generated using mMass (Version 5.4.0)

Mass spectra of methanolic extracts of bioactive lipopeptide fractions obtained from reference isolates B81 and R16 are presented in Figures 4.5 and 4.6 respectively. In the *m/z* range 980-1130 prominent peaks (*m/z* 1030.33, 1044.38, 1058.37, 1074.36) corresponding to sodium and potassium adducts of surfactin C13-15 isoforms were evident for isolate B81 (Fig. 4.5A). Peaks corresponding to various surfactin and bacillomycin isoforms were distinguished for R16 (Fig. 4.6A). In some instances peaks were difficult to assign due to the close proximity of different isoform species and the partial masking of some peaks by others that were present at higher relative intensities. The positive identification of the surfactin and bacillomycin isoforms was substantiated by the detection of peaks that closely matched the *m/z* values of sodium and potassium adducts previously reported for these isoforms (Koumoutsi *et al*., 2004). In the *m/z* range 1400-1590 both isolates yielded peaks that were consistent with *m/z* values reported in the literature for various fengycin isoforms (Fig. 4.5B and 4.6B) (Vater *et al*., 2002; Koumoutsi *et al*., 2004; Price *et al*., 2007).

The major lipopeptide mass fragments distinguished in the methanol extract of R16 were also detected when whole cells and TFA extracts were analysed with MALDI-TOF-MS (Figure 4.7). Similar findings were obtained with isolate B81 (Results not shown). The mass spectra generated for R16 are presented in Figure 4.7. To ensure that secondary metabolites were present, colonies from 36 h plate cultures were used. Peak intensities varied somewhat, depending on the sample preparation method used. The TFA extraction method produced more consistent results than the whole cell analysis approach yielding peaks with greater intensities, as well as additional peaks not present in the whole cell preparations. This was attributed to TFA's solvent action which causes protein precipitation thereby concentrating cellular proteins. Consequently, the TFA extraction method was used for all isolates screening.

The detection of lipopeptide compounds directly from cell-free culture medium (centrifuged at 12000 x g for 10 min, ambient temperature) was also demonstrated using MALDI-TOF MS. Figure 4.8 (A and B) shows the mass spectral evidence of lipopeptides present in the culture supernatant of B81 grown in Landy medium at 30^{o}C (150 rpm) after 8 h and 16 h respectively. Surfactin isoforms were the predominant lipopeptides detected after 8 h, which corresponded to the exponential phase of growth. Fengycin isoforms were also prominent when the culture was sampled during the stationary phase (16h).

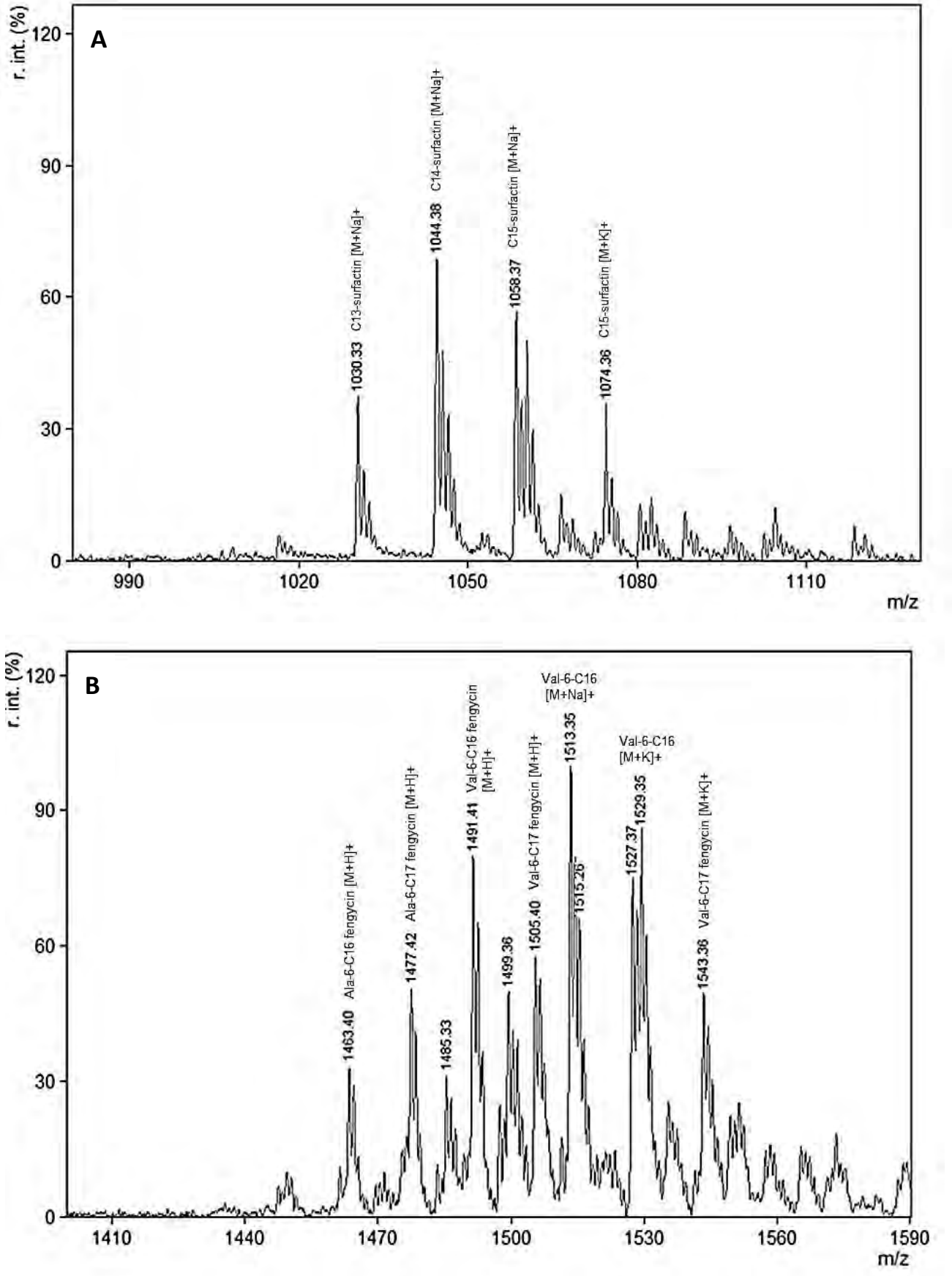


Figure 4.5 MALDI-TOF MS mass spectra of lipopeptides present in methanol extracts of isolate B81 in the *m/z* ranges 980-1130 (A) and 1400 to 1590 (B)

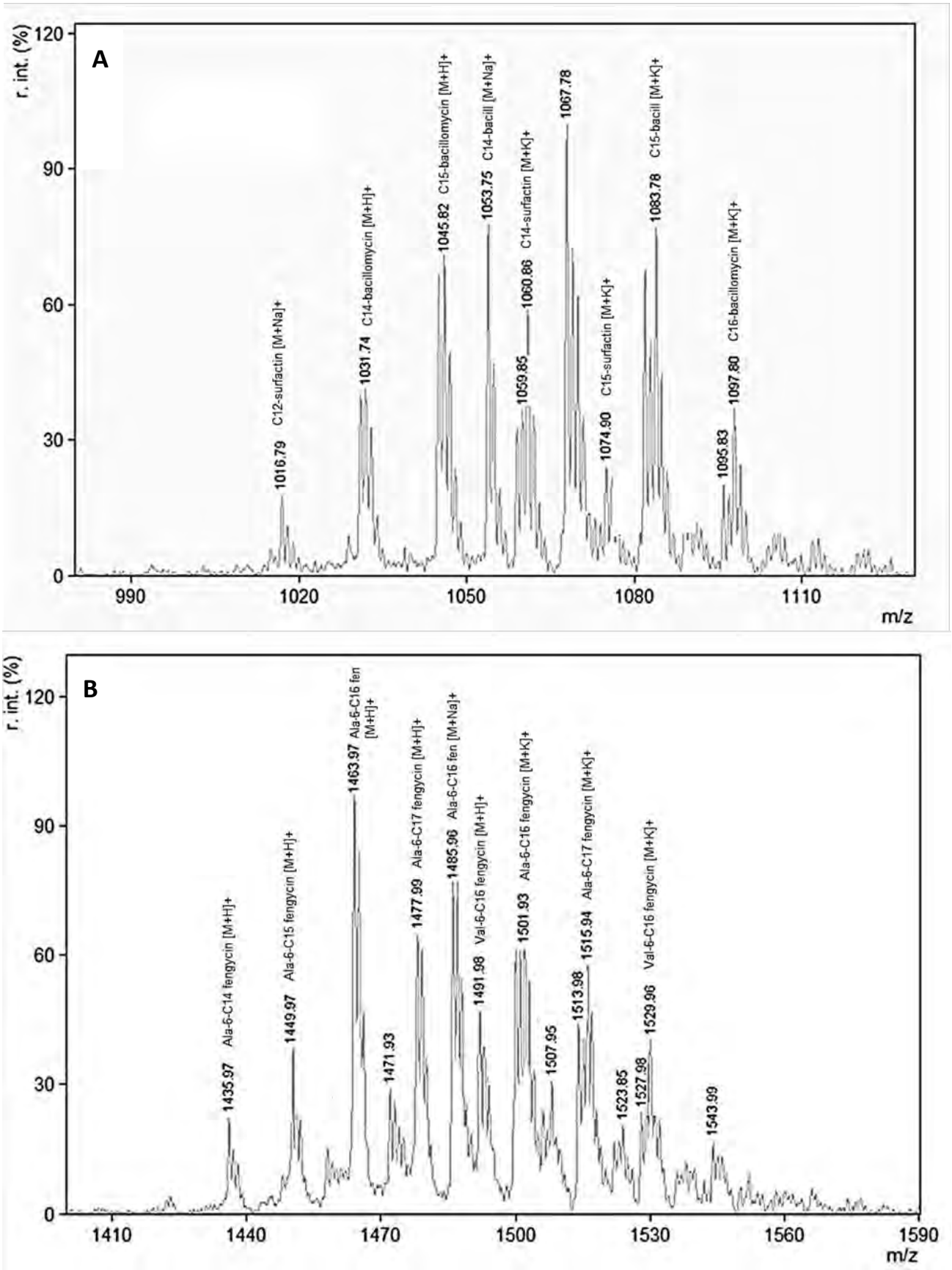


Figure 4.6 MALDI-TOF MS mass spectra of lipopeptides present in methanol extracts of isolates R16 in the *m/z* ranges 980-1130 (A) and 1400 to 1590 (B)

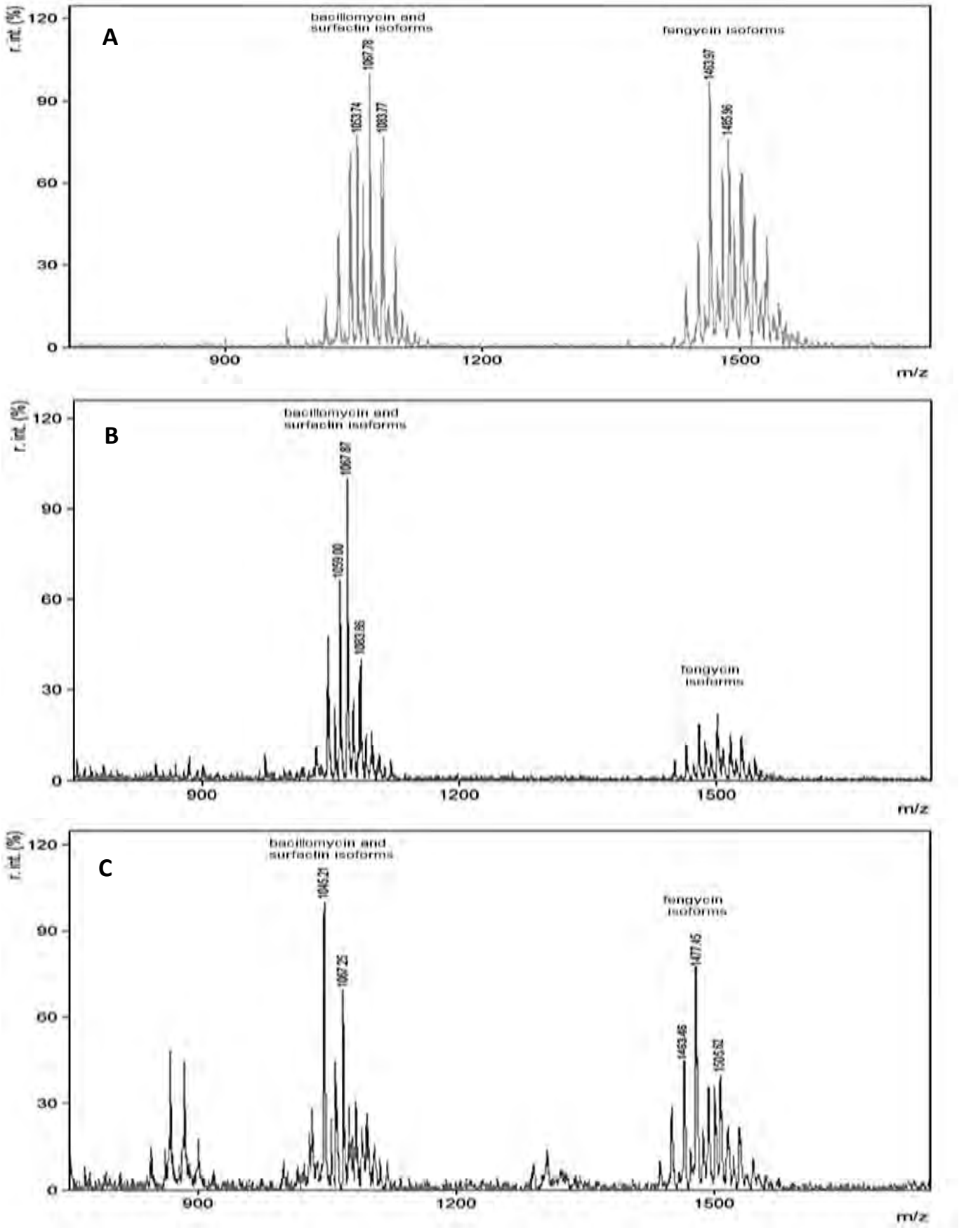


Figure 4.7 Comparison of MALDI-TOF mass spectra obtained from isolate R16: methanolic extract (A); whole cells (B) and TFA extract (C)

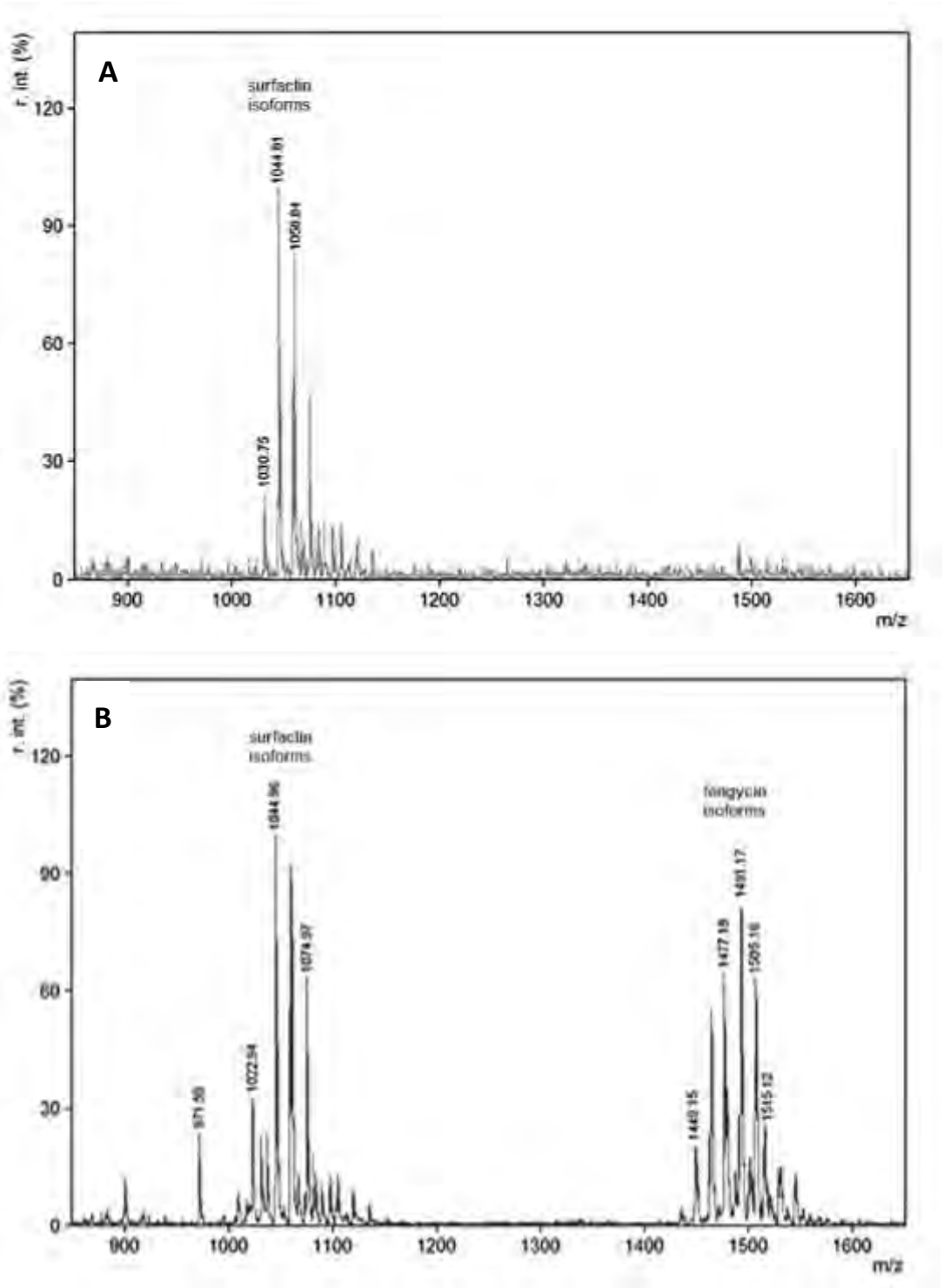


Figure 4.8 MALDI-TOF mass spectra of lipopeptides present in cell-free supernatant from a culture of isolate B81 grown in Landy medium (30^{o}C at 150 rpm) during exponential (A) and stationary phases of growth (B). Surfactin isoforms were the predominant lipopeptides detected after 8 h (exponential growth), whereas fengycin isoforms were also prominent in the mass spectrogram after 16 h (stationary phase)

Results of the MALDI-TOF MS based lipopeptide screening of *Bacillus* spp. is presented in Table 4.5. For comparative purposes gel-view representations of each mass spectrum were generated using mMass software (Version 5.4.0) (Fig 4.9.). Peak intensities were first converted to grey-scale and then plotted as a function of the *m/z* values; each row represents a specific isolate spectrum with the vertical lines indicating the mass peaks present. Lipopeptides belonging to the surfactin, iturin and fengycin families were distinguished by their characteristic signal fragments. As previously experienced, peaks in the *m/z* range 1000-1150 Da were sometimes difficult to distinguish and assign to either surfactin isoforms or lipopeptides belonging to the iturin family, due to the close proximity of their protonated forms and respective Na^+ and K^+ adducts. This problem was further compounded by low levels of instrumental drift (Std $\leq$ 0.52 Da) and the masking effect of adjacent lipopeptide peaks with different intensities. Lipopetides were therefore identified based on evidence of protonated species and their Na^+ and/or K^+ adducts as well as the presence of related isoforms. The *B. cereus* group of isolates was found to produce very consistent spectral profiles that were distinct from the other isolates screened (Fig. 4.9). Several prominent peaks were evident with masses of 878.51, 893.59, 906.6 and 944.67 *m/z* that were consistent with mass spectra previously ascribed to isoforms of the kurstakin class of lipoheptapeptides (Hathout *et al*., 2000). For the purpose of this study, these isolates were recorded as being presumptive kurstakin producers. *Brevibacillus laterosporus* CC-R4 also displayed a unique mass spectrum with several prominent peaks being distinguished (viz., 1605.20, 1627.21, 1643.15 m/z). Examples of representative mass spectra for each of the main groupings distinguished are presented in Figure 4.10.

Table 4.5 Lipopeptides detected by MALDI-TOF MS produced by and putatively assigned to, bacterial isolates exhibiting *in vitro* antagonism of *R. solani*

Strains	Kurstakins	Surfactins	Iturins	Bacillomycins	Fengycins*
B. amyloliquefaciens subsp. *amyloliquefaciens* DSM7	-	+	-	+	+
B. subtilis subsp. *subtilis* DSM10	-	+	-	-	-
B. subtilis subsp. *spizizenii* ATCC6633	-	+	-	-	+
B. subtilis DSM3258	-	+	-	-	+
B. amyloliquefaciens subsp. *plantarum* isolates					
R 16	-	+	-	+	+
CP-R23	-	+	+	-	+
CP-R43	-	+	-	+	+
CP-R45	-	+	-	+	+
CL-R49	-	+	-	+	+
CL-R51	-	+	-	+	+
CL-R53	-	+	-	+	+
CL-R64	-	+	-	+	+
CB-R106	-	+	-	+	+
B. subtilis group isolates					
B81	-	+	-	-	+
CC-R9	-	+	-	-	+
CC-R10	-	+	+	+	+
CT-R67	-	+	+	+	+
CT-R89	-	+	+	+	+
CT-R90	-	+	-	-	+
CT-R92	-	+	-	-	+
CL-R59	-	+	-	-	+
Bacillus cereus group isolates					
CC-R7	+	-	-	-	-
CP-R15	+	-	-	-	-
CP-R25	+	-	-	-	-
CP-R30	+	-	-	-	-
CP-R42	+	-	-	-	-
CT-R73	+	-	-	-	-
CT-R77	+	-	-	-	-
CB-R105	+	-	-	-	-
Br. laterosporus CC-R4	-	-	-	-	-

*Lipopetides were identified on the basis of mass data that matched protonated species and Na^+/K^+ adducts of isoforms, derived either from standards (surfactin and iturin) or reported in the literature (Vater *et al*., 2002; Koumoutsi *et al*., Price *et al*., 2007; Béchet *et al*., 2012).

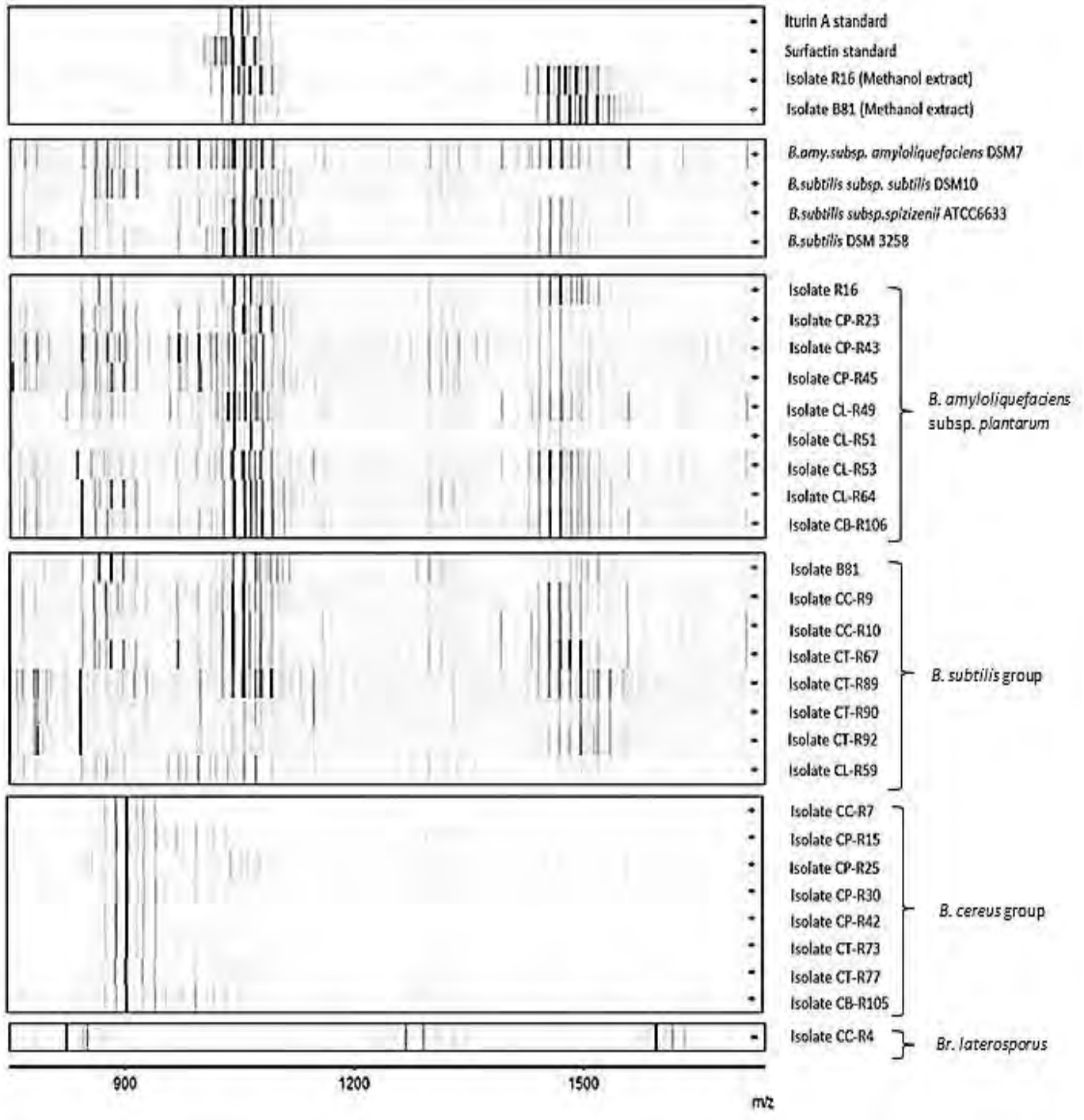


Figure 4.9 Gel-view representation of MALDI-TOF mass spectra (*m/z* range 750-1750) of selected *Bacillus* spp. strains

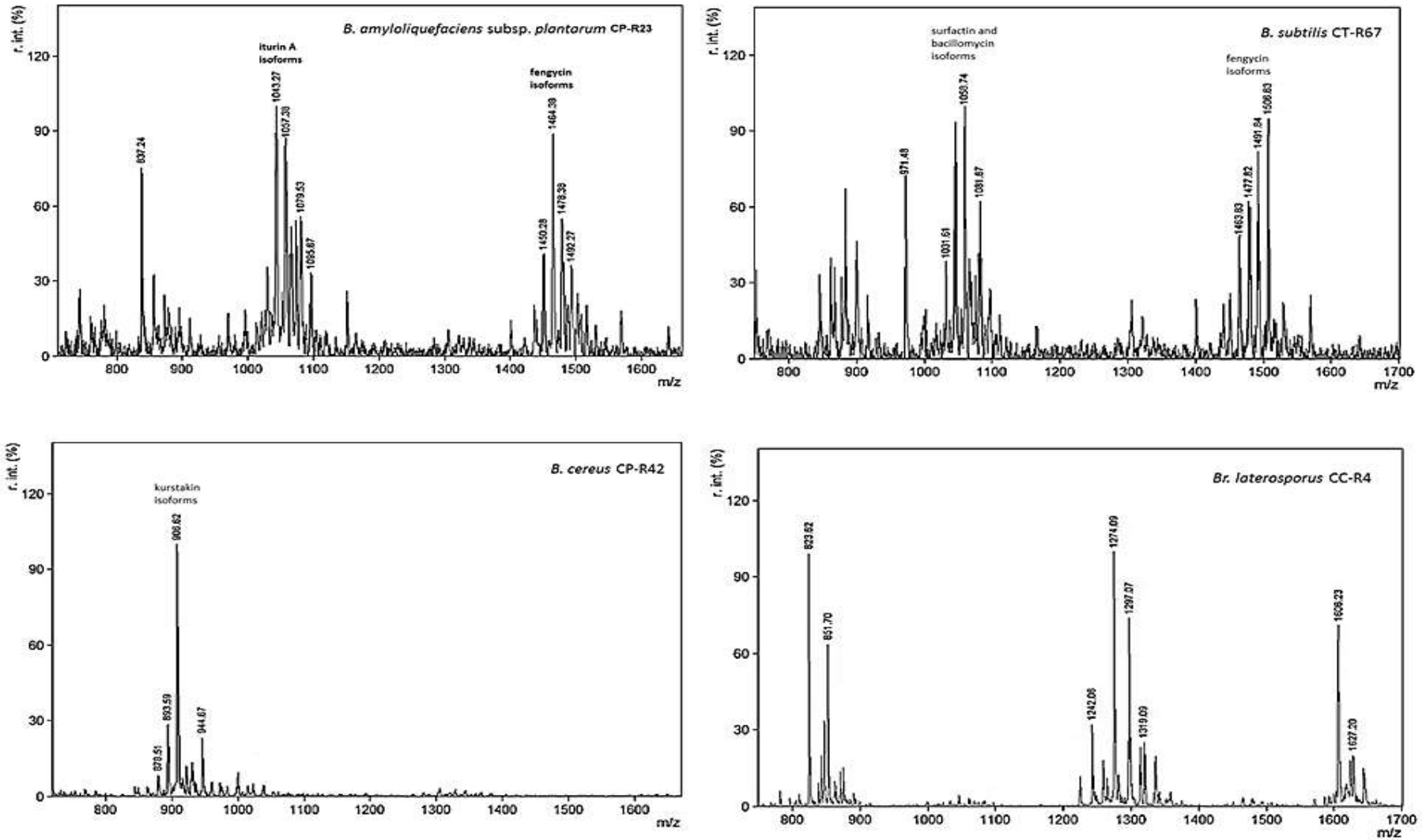


Figure 4.10 Examples of mass spectra (m/z 750 - 1750 Da) obtained for a representative bacterial isolate from each of the main taxonomic groupings distinguished by Biotyper cluster analysis.

4.4 Discussion

This study was undertaken to assess MALDI-TOF MS as a rapid means of identifying and grouping potential bacterial biocontrol agents, and determining their associated lipopeptide profiles. Since the late 1990's, MALDI-TOF MS has been developed as an alternative means of rapidly identifying bacteria (Claydon *et al*., 1996). Much of the research in this regard has focused on identifying and characterizing clinical isolates significant to mankind (Murray, 2010; Benagli *et al*., 2011). Interest in aerobic endospore-forming bacteria, to a large extent, has focused on using MALDI-TOF MS to identify and classify members of the genus *Bacillus*. Rapid detection of the potential biological warfare agent *B. anthracis,* and its differentiation from closely related taxa such as *B. cereus* and *B. thuringiensis,* has been the focus of many of these studies (Elhanay *et al*., 2001; Hathout *et al*., 2003; Krishnamurthy *et al*., 2007; Lasch *et al*., 2009). In other applications, MALDI-TOF MS has been used to characterize and classify *Bacillus* spp. associated with the contamination of food (Fernàndez-No *et al*., 2013) and spacecraft assembly facilities (Dickinson *et al.*, 2004).

Here, MALDI-TOF MS was used in conjunction with commercially available identification software to identify and group isolates. This library-based approach to bacterial identification involves the comparison of mass spectra from unknown organisms to reference spectra deposited within an existing database. Identification to the species- or even subspecies-level can be achieved to a degree comparable to that of 16S rRNA sequence analysis (Ghyselinck *et al.,* 2011). An advantage of this spectral identification method is that prior knowledge of specific biomarkers is not required. However, the successful application of library-based identification of bacterial isolates is dependent on the comprehensiveness of the available reference spectra databases which are usually biased towards clinical strains (Uhlik *et al*., 2011).

Three reference strains *B. subtilis* subsp. *subtilis* DSM10, *B. licheniformis* DSM13 and *B. pumilis* DSM27, which are represented in the Biotyper BDal-library database, were included in the initial identification run to assess the accuracy of the Biotyper identification system. These strains were correctly identified to the species level, although the identity scores achieved (2.000-2.299) did not provide a secure species identification rating. These identification scores have been attributed to a number of variables that can affect the quality

of mass spectra such as culture medium, culture conditions, growth stage and level of sporulation (Valentine *et al*., 2005; Lasch *et al*., 2009; Wybo *et al*., 2012). These findings reflect the level of taxonomic resolution achievable using Biotyper identification software under the prevailing conditions of this study.

Initial results using the BDal database were disappointing. Although all isolates were successfully identified to genus level, 56 % were only classified to probable species level, with only 9 % reliably identified to species level (Table 4.2). Isolate R16 could not be classified using the database. In particular, isolates previously identified as strains of *B. amyloliquefaciens* were poorly identified. Examination of the BDal database revealed that species relevant to this study were poorly represented, which could account for the limited success of the identification process. This drawback has been highlighted in several studies showing that where limited spectra were available within a database the accuracy and reliability of the identifications made were negatively impacted (Christensen *et al*., 2012; Wybo *et al.,* 2012).

Biotyper software provides a user-friendly platform that allows for the extension of the existing spectrum database using "in-house" libraries generated from mass spectra profiles of environmental isolates and additional reference strains. Inclusion of an "in-house" MSP library created in this study significantly improved the level of isolate identification in subsequent identification runs. Sixty-two percent of isolates were confidently identified to the species level and 38% were classified to the genus level with only probable species identification. With the exception of the reference strains *B.subtilis* subsp. *spizizenii* ATCC6633 and *B.subtilis* DSM3258 each of the isolates screened was identified to species level consistent with results obtained previously from 16S rRNA and *gyr*A gene sequence analysis.

Viewed in isolation, the taxonomic resolution of the Biotyper identification method appeared to be limited to the species level. However, when MSPs were compared using Biotyper cluster analysis further levels of intra-species variation could be distinguished. The phyloproteomic relationship of isolates based on MSPs, relative to each other and reference strains in the Biotyper BDal database are presented in Figure 4.1. It was noted that the major groupings allocated to each of the isolates screened were very similar to those obtained with

RAPD-PCR fingerprinting and phylogenetic analysis of *gyr*A genes. Isolates that had been previously identified as closely related strains of *B. amyloliquefaciens* subsp. *plantarum* formed a sub-cluster that was distinct from type strains *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7 and *B. amyloliquefaciens* CIP103265. Similarly, isolates that had been grouped as closely related strains of *B. subtilis* also formed a distinct sub-cluster that grouped in close proximity to other representatives of the *B. subtilis* group of closely related taxa. Isolate CL-R59, previously identified as a strain of *B. subtilis* subsp. *subtilis*, was also clearly distinguished during MSP cluster analysis. The MSP results for isolate CP-R41 and CC-R10 proved to be inconsistent with previous RAPD and ITS fingerprinting findings; however, these discrepancies were attributed to processing errors rather than strain differences.

These findings are comparable to those of Fernàndez-No *et al*. (2013), who showed that MALDI-TOF MS provided valuable information at both inter- and intra-species levels with regards *Bacillus* strain identification. MALDI-TOF mass fingerprinting was able to distinguish between *B. subtilis* and *B. amyloliquefaciens* strains*;* something that is difficult to do using the 16S rRNA sequencing approach alone (Wang *et al*., 2007). In addition to these findings, the present data also suggest that strain differentiation at the sub-species level is possible using Biotyper cluster analysis. However, further work involving a larger number of reference strains is required to validate this assertion. Various studies have shown the potential of MALDI-TOF MS to differentiate bacterial isolates at the strain level (Siegrist *et al*., 2007; Ghyselinck *et al*., 2011; Stets *et al*., 2013). In terms of taxon resolution MALDI-TOF has been found to be comparable to REP-PCR fingerprinting techniques (Ghyselinck *et al*., 2011). However, the extent to which genera can be differentiated at the strain level appears to be taxon dependant Ghyselinck *et al*. (2013).

Analysis of MSP peak lists using the SPECLUST software also proved valuable for comparing isolates, but more importantly was useful for identifying potential biomarkers. Using hierarchical cluster analysis, isolates were separated into groupings that corroborated the Biotyper cluster analysis (Fig 4.3). Within each of the major groups distinguished several common mass fragments were identified (Appendix B). A number of these could be matched to biomarkers previously described in the literature. Examples include the biomarker *m/z* 7716 which was reported to be common to members of the *B. subtilis* group (Fernàndez-No *et al*.,

2013); and, the mass fragments *m/z* 5173 and 5887 which were very similar to group-specific biomarkers previously described for members of the *B. cereus* group of related taxa (viz. *m/z* 5171 and 5886) (Lasch *et al*., 2009). In addition to these, isolates CC-R7, CP-R42 and CB-R105 (*B. cereus* group) also displayed a common fragment (*m/z* 6713) which closely matched a species-specific biomarker (*m/z* 6711) previously assigned to *B. cereus* (Elhanany *et al*., 2001; Hathout *et al*., 2003; Castanha *et al*., 2006). Interestingly, this biomarker has been identified as an endospore-specific protein fragment of small acid soluble proteins (SASPs) present in the endospore core. These SASP proteins are commonly detected in cell extracts prepared from samples where vegetative cells are the predominant cell forms present (Lasch *et al*. 2009). It has been speculated that these SASPs may be preferentially detected due to their ready ionization and high concentration in dormant endospores.

In some cases the data did not match common biomarkers described for the *B. subtilis* and *B. cereus* groups reported by Fernàdez-No *et al*. (2013); these inconsistencies were attributed to strain differences and the limited number of reference strains used to identify biomarkers in the present study. Notwithstanding this shortcoming, the information obtained serves as a useful starting point to further distinguish species, or even strain specific biomarkers that could be used in the identification and grouping of environmental isolates in subsequent studies.

The ability to group closely related strains of the order *Bacillales*, as shown in this study, demonstrates the potential for using MALDI-TOF MS for dereplication purposes. Dereplication involves the grouping of isolates to a specified taxonomic level and plays an important role in screening programmes and diversity studies (Dieckman *et al*., 2005; Stets *et al*., 2013; Ghyselinck *et al*., 2013). It allows for the rationalisation of large collections of bacterial isolates by reducing labour inputs, the time involved and the overall costs associated with large-scale screening and subsequent downstream analyses (Ghyselinck *et al*., 2011). Compared to molecular fingerprinting techniques such as REP-PCR and RAPD-PCR, MALDI-TOF MS has simpler sample preparation requirements, lower consumable costs, a somewhat faster turnaround time for analysis and the potential for a higher throughput of samples. However, these advantages must be weighed-up against the high initial capital outlay associated with purchasing a MALDI-TOF MS instrument.

MALDI-TOF MS has been recommended as a robust but sensitive method capable of detecting a range of secondary metabolites produced by members of the genus *Bacillus* (Leenders *et al*., 1999; Vater *et al.,* 2002). Compounds can be detected at the upper femto- to picomolar range directly from whole cell preparations, cellular extracts and even crude culture filtrates. The characterization of lipopeptide profiles using MALDI-TOF MS has previously been used to type strains of *B. subtilis* (Leenders *et al*., 1999) and to analyse the distribution of lipopeptide biomarkers amongst *Bacillus* spp. strains from diverse geographical locations (Price *et al*., 2007). The application of MALDI-TOF MS to screen bacterial isolates for bioactive compounds linked to biological control activity has therefore, enormous potential.

Preliminary experiments confirmed that MALDI-TOF MS was a convenient method of rapidly detecting bioactive lipopeptides within the surfactin, iturin and fengycin families of biosurfactants. Characteristic mass fragment clusters were evident which allowed isoforms for each lipopeptide group to be distinguished. Biomarkers for surfactin and iturin/bacillomycin sometimes proved difficult to differentiate due to the close proximity of peaks in the mass spectra. The assignment of peaks was, therefore, based on evidence of protonated species, their Na^+ and/or K^+ adducts as well as the presence of related isoforms and isotope patterns.

These lipopeptide compounds could be detected in samples prepared by each of the methods evaluated (Fig 4.6); thereby illustrating the ease with which compounds could be detected without prior fractionation or purification steps being required. The ability to detect lipopeptides directly from crude culture filtrates proved useful in correlating lipopeptide production with culture growth phases (Fig 4.8). For example, when isolate B81was cultured in Landy medium, surfactin was detected during late exponential growth whereas fengycin isoforms were detected only during stationary phase. These findings were consistent with previous studies that have shown that lipopeptide compounds are produced as secondary metabolites during the latter stages of bacterial growth (Vater *et al*., 2002). Further, these findings illustrated the impact that the growth stage of a culture has on spectral profiles at low *m/z* ranges, both qualitatively and quantitatively. Therefore, for screening purposes it is important to standardize MALDI-TOF MS sample preparation protocols and ensure that isolates are sampled after the onset of stationary phase.

MALDI-TOF MS screening of the isolates revealed that distinct spectral profiles could be distinguished between each of the taxonomic groupings identified (Fig 4.9). Biomarkers for surfactin and fengycin were detected for all isolates within the *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* taxonomic groupings (Table 4.5). In addition to these, biomarkers for bacillomycin or iturin A were also detected for the *B. amyloliquefaciens* subsp. *plantarum* strains, which was consistent with previous ESI-MS results obtained from the analysis of methanolic extracts of lipopeptide fractions derived from these isolates (Table 3.4, Chapter Three). Significantly, isolate CP-R23 was once again distinguished from the other strains within the *B.amyloliquefaciens* subsp. *plantarum* group based on its unique ability to produce iturin A instead of bacillomycin D. This result illustrated the potential for using MALDI-TOF MS to detect strain variation amongst closely related endospore-formers.

Within the *B. subtilis* taxonomic grouping three strains (viz., CC-R10, CT-R67, CT-R89) were found to co-produce iturin and bacillomycin; this result was unexpected since isolates within this grouping had previously been shown to produce only surfactin and fengycin isoforms when lipopeptide fractions were analysed using ESI-MS. Further to this, PCR screening for bacillomycin (*bmy*C) and iturin (*itu*D) biosynthesis genes was negative for these organisms (Fig. 3.4, Chapter Three). Price *et al*. (2007) reported that for certain strains of *B. subtilis,* iturin compounds could be detected from whole cell preparations using MALDI-TOF MS but not from crude culture filtrates; suggesting that these compounds were not released from the cell into the culture medium. This observation may account for the absence of compounds in lipopeptide fractions analysed in earlier studies (Chapter Three). For screening purposes MALDI-TOF analysis of whole cell extracts and culture filtrates is warranted to establish that lipopeptides of interest are released from the cell.

The *B. cereus* group isolates all produced very consistent spectral profiles that were distinct from the other isolates screened (Fig. 4.8). Several prominent peaks (viz., 878.51, 893.59, 906.6, 944.67 *m/z*) were evident (Fig 4.9); these were consistent with mass spectra previously ascribed to isoforms of the kurstakin class of lipoheptapeptides (Hathout *et al*., 2000). The structure and biosynthesis of kurstakins has recently been reviewed (Béchet *et al*., 2012).

These compounds are typically associated with strains of *B. cereus* and *B. thuringiensis* and show antifungal activity (Hathout *et al*., 2000; Béchet *et al*., 2012). From an ecological perspective it is interesting to note that kurstakin deficient mutants have been shown to lose their ability to swarm on agar media and form biofilms (Du Bois *et al*., 2012). Additionally, Price *et al*. (2007) noted that kurstakins are generally not detected in cell-free culture filtrates and are associated with intact bacterial cells or endospores. This observation provides a possible explanation why cell-free extracts obtained from *B. cereus* group isolates grown in Landy medium failed to show any antifungal activity when assayed for antifungal activity.

Brevibacillus laterosporus CC-R4 also displayed a unique mass spectrum with several prominent peaks being distinguished (Fig. 4.10). Relatively little is known about the antibiotics produced by this species. Recently however, Zhao *et al.* (2012) purified and characterized a novel peptide antibiotic from a strain of *Br. laterosporus* showing broad spectrum antifungal activity. This peptide contained eleven amino acid residues and was found to have a molecular mass of 1602.05 Da. It was interesting to note that the *Br. laterosporus* CC-R4 mass spectrum displayed a series of peaks (viz., 1605.20, 1627.21, 1643.15 *m/z*) that corresponded to the protonated species, sodium adduct and potassium adduct of a compound with a similar molecular mass. This finding could be useful in further characterization of the active compound(s) produced by *Br. laterosporus* CC-R4 and illustrates the potential for using MALDI-TOF MS to screen for novel compounds.

MALDI-TOF MS was demonstrated to be a rapid, sensitive and robust method for profiling aerobic endospore-forming bacteria for biocontrol screening purposes. It proved useful for discriminating between closely related *Bacillus* species and showed potential for differentiating strains at the sub-species level. Cluster analysis of mass spectra of isolates allowed for their relationships to be assessed at various taxonomic levels and provided a convenient means for grouping isolates for dereplication purposes. MALDI-TOF MS also provided information regarding the lipopeptide profiles of different strains which is invaluable for biocontrol screening purposes. The ease with which lipopeptide profiles were obtained with minimal sample preparation also recommends this method. Overall, MALDI-TOF MS was found to complement existing methodologies of bacterial identification, fingerprinting

and characterization and fulfils the need for a practical yet robust screening technique suitable for processing large numbers of environmental isolates.

4.5 References

Alm, R., Johansson, P., Hjerno, K., Emanuelsson, C., Ringner, M. and Hakkinen, J. (2006) Detection and identification of protein isoforms using cluster analysis of MALDI-MS mass spectra. Journal of Proteome Research, 5, 785-792.

Alvarez, F., Castro, M., Príncipe, A., Borioli, G., Fischer, S., Mori, G. and Jofré, E. (2012) The plant-associated *Bacillus amyloliquefaciens* strains MEP218 and ARP23 capable of producing the cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. Journal of Applied Microbiology, 112, 15-174.

Athukorala, S.N.P., Fernando, W.G.D. and Rashid, K.Y. (2009) Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Canadian Journal of Microbiology, 55, 1021-1032.

Béchet, M., Caradeca, T., Husseina, W., Abderrahmanib, A., Cholleta, M., Leclèreb, M., Dubois, T., Lereclusc, D., Pupind, M. and Jacques, P. (2012) Structure, biosynthesis and properties of Kurstakins, nonribosomal lipopeptides from *Bacillus* spp. Applied Microbiology and Biotechnology, 95, 593-600.

Benagli, C., Rossi, V., Dolina, M., Tonolla, M. and Petrini, O. (2011) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for the identification of clinically relevant bacteria. PLoS One, 6, e16424.

Böhme, K., Fernández-No, I. C., Pazos, M., Gallardo, J. M., Barros-Velázquez, J., Cañas, B. and Calo-Mata, P. (2013) Identification and classification of seafood-borne pathogenic and spoilage bacteria: 16S rRNA sequencing versus MALDI-TOF MS fingerprinting. Electrophoresis, 34, 877-887.

Castanha, E.R., Fox, A. and Fox, K.F. (2006) Rapid discrimination of *Bacillus anthracis* from other members of the *B. cereus* group by mass and sequence of "intact" small acid soluble proteins (SASPs) using mass spectrometry. Journal of Microbiological Methods, 67, 230-240.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. Microbial Biotechnology, 8, 281-295. doi: 10.1111/1751-7915.12238.

Christensen, J.J., Dargisa, R., Hammerb, M., Justesenc, U.S., Nielsena, X.C. and Kemp, M. (2012) Matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis of Gram-positive, catalase-negative cocci not belonging to the *Streptococcus* or *Enterococcus* genus and benefits of database extension. Journal of Clinical Microbiology, 50, 1787-1791.

Claydon, M.A., Davey, S.N., Edwards-Jones, V. and Gordon, D.B. (1996) The rapid identification of intact microorganisms using mass spectrometry. Nature Biotechnology, 14, 1584-1586.

Dickinson, D.N., La Duc, M.T., Haskins, W.E., Gornushkin, I., Winefordner, J.D., Powell, D.H. and Venkateswaran, K. (2004) Species differentiation of a diverse suite of *Bacillus* spores by mass spectrometry-based protein profiling. Applied and Environmental Microbiology, 70, 475-482.

Dieckmann, R., Graeber, I., Kaesler, I., Szewzyk, U. and Von Döhren, H. (2005) Rapid screening and dereplication of bacterial isolates from marine sponges of the Sula Ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS). Applied Microbiology and Biotechnology 67, 539-548.

Dieckmann, R., Helmuth, R., Erhard, M. and Malorny, B. (2008) Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Applied and Environmental Microbiology, 74, 7767-7778.

Dubois, T., Faegri, K., Perchat, S., Lemy, C., Buisson, C., Nielsen-LeRoux, C., Gohar, M., Jacques, P., Ramarao, N., Kolsto, A.B. and Lereclus, D. (2012) Necrotrophism is a quorum-regulated lifestyle in *Bacillus thuringiensis*. PloS Pathogens, 8, e1002629.

Elhanany, E., Barak, R.,Fisher, M., Kobiler, D. and Altboum, Z. (2001) Detection of specific *Bacillus anthracis* spore biomarkers by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry, 15, 2110-2116.

Fernández-No, I.C., Böhme, K., Díaz-Bao, M., Cepeda, A., Barros-Velázquez, J. and Calo-Mata, P. (2013) Characterization and profiling of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus licheniformis* by MALDI-TOF mass fingerprinting, Food Microbiology, 33, 235-242.

Freiwald, A. and Sauer, S. (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols, 4, 732-742.

Gebhardt, K., Schirnana, J., Müller, J., Fiedler, H.P., Kallenborn, H.G., Holzenkämpfer, M., Krastel, P., Zeeck, A., Vater, J., Höltzel, A., Schmid, D.G., Rheinheimer, J. and Dettner, K. (2002) Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. FEMS Microbiology Letters, 217, 199-205.

Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K. and De Vos, P. (2011) Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. Journal of Microbiological Methods, 86, 327-336.

Ghyselinck, J., Velivelli, S.L., Heylen, K., O'Herlihy, E., Franco, J., Rojas, M., De Vos, P. and Prestwich, B.D. (2013) Bioprospecting in potato fields in the central Andean highlands: screening of rhizobacteria for plant growth-promoting properties. Systematic and Applied Microbiology, 36, 116-127.

Giebel, R., Worden, C., Rust, S. M., Kleinheinz, G. T., Robbins, M. and Sandrin, T. R. (2010) Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): applications and challenges. Advances in Applied Microbiology, 71, 149-184.

Hathout, Y., Ho, Y.P., Ryzhov, V., Demirev, P. and Fenselau, C. (2000) Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. Journal of Natural Products, 63, 1492-1496.

Hathout, Y., Setlow, B., Cabrera-Martinez, R.M. and Fenselau, C. and Setlow, P. (2003) Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores. Applied and Environmental Microbiology, 69, 1100-1107.

Holland, R.D., Wilkes, J.G., Rafii, F., Sutherland, J.B., Persons, C.C., Voorhees, K.J. and Lay, J.O. (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Communications Mass Spectrometry, 10, 1227-1232.

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. (2004) Structural and functional characterization of gene clusters directing non-ribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology, 186, 1084-1096.

Krishnamurthy, T., Deshpande, S., Hewel, J., Liu, H., Wick, C. and Yatesiii, J. (2007) Specific identification of *Bacillus anthracis* strains. International Journal of Mass Spectrometry, 259, 140-146.

Lasch, P., Beyer, W., Nattermann, H., Staemmler, M. and Siegbrecht, E. (2009) Identification of *Bacillus anthracis* by using matrix-assisted laser desorption Ionization time of flight mass spectrometry and artificial neural networks. Applied and Environmental Microbiology, 75, 7229-7242.

Leenders, F., Stein, T.H., Kablitz, B., Franke, P. and Vater, J. (1999) Rapid typing of *Bacillus subtilis* strains by their secondary metabolites using matrix assisted laser desorption/ionization mass spectrometry of intact cells. Rapid Communication Mass Spectrometry, 13, 943-949.

Madonna, A.J., Voorhees, K.J., Taranenko, L., Laiko, V.V. and Doroshenko, V.M. (2003) Detection of cyclic lipopeptide biomarkers from *Bacillus* species using atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry, 75, 1628-1637.

Maier, T., Klepel, S., Renner, U. and Kostrzewa, M. (2006) Fast and reliable MALDI-TOF MS-based microorganism identification. Nature Methods, 3, doi:10.1038/nmeth870.

Mellmann. A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., Dunn, J., Hall, G., Wilson, D., LaSala, P., Kostrzewa, M. and Harmsen, D. (2008) Evaluation of matrix-assisted laser desorption ionization-time-offlight mass spectrometry in comparison to 16S

rRNA gene sequencing for species identification of nonfermenting bacteria. Journal of Clinical Microbiology, 46, 1946-1954.

Murray, P.R. (2010) Matrix-assisted laser desorption ionization time-offlight mass spectrometry: usefulness for taxonomy and epidemiology. Clinical Microbiology and Infection, 16, 1626-1630.

Needleman, S.B. and Wunsch, C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. Journal of Molecular Biology, 48, 443-53.

Ongena, M. and Jacques, P. (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends in Microbiology, 16, 115-125.

Price, N.P.J., Rooney, A.P., Swezey, J.L., Perry, E. and Cohan, F.M. (2007) Mass spectrometric analysis of lipopeptides from *Bacillus strains* isolated from diverse geographical locations. FEMS Microbiological Letters, 271, 83-89.

Sandrin, T. R., Goldstein, J. E. and Schumaker, S. (2013). MALDI TOF MS profiling of bacteria at the strain level: a review. Mass spectrometry reviews, 32, 188-217.

Sauer, S. and Kliem, M. (2010) Mass spectrometry tools for the classification and identification of bacteria. Nature Reviews Microbiology 8, 74-82.

Siegrist, T.J., Anderson, P.D., Huen, W.H., Kleinheinz, G.T., McDermott, C.M. and Sandrin, T.R. (2007) Discrimination and characterization of environmental strains of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Journal of Microbiological Methods, 68, 554-562.

Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology,56, 845-857.

Stets, M.I., Pinto, A.S., Huergo, L.F., de Souza, E.M., Guimarães, V.F., Alves, A.C., Steffens, M.B., Monteiro, R.A., Pedrosa, F.O. and Cruz, L.M. (2013) Rapid identification of bacterial isolates from wheat roots by high resolution whole cell MALDI-TOF MS analysis. Journal of Biotechnology, 165,167-74.

Strohalm, M., Hassman, M., Košata, B. and Kodíček, M. (2008) mMass data miner: an open source alternative for mass spectrometric data analysis. Rapid Communications in Mass Spectrometry, 22, 905-908.

Uhlik, O., Strejcek, M., Junkova, P., Sanda, M., Hroudova, M., Vlcek, C., Mackova, M. and Macek, T. (2011) Matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometry- and MALDI Biotyper-based identification of cultured biphenyl-metabolizing bacteria from contaminated horseradish rhizosphere soil. Applied and Environmental Microbiology, 77, 6858-6866.

Valentine, N., Wunschel, S., Wunschel, D., Petersen, C. and Wahl, K. (2005) Effect of

culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. Applied and Environment Microbiology, 71, 58-64.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. (2002) Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Applied and Environmental Microbiology, 68, 6210-6219.

Wang, L., Lee, F., Tai, C. and Kasai, H., (2007) Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. International Journal of Systematic and Evolutionary Microbiology, 57, 1846-1850.

Welker, M. and Moore, E.R.B. (2011) Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. Systematic and Applied Microbiology, 34, 2-11.

Williams, T.L., Andrzejewski, D., Lay, J.O. and Musser, S.M. (2003) Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. Journal of the American Society for Mass Spectrometry. 14, 342-351.

Wunschel, S.C., Jarman, K.H., Petersen, C.E., Valentine, N.B., Wahl, K.L., Schauki, D., Jackman, J., Nelson, C.P. and White, E.T. (2005) Bacterial analysis by MALDI-TOF mass spectrometry: an inter-laboratory comparison. Journal of the American Society for Mass Spectrometry. 16, 456-462.

Wybo, I., Soetens, O., De Bel, A., Echahidi, F., Vancutsem, E., Vandoorslaer, K. and Piérard, D. (2012) Species identification of clinical *Prevotella* isolates by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. Journal of Clinical Microbiology, 50, 1415-1418.

Zhao, J., Guo, L., Zeng, H., Yang, X., Yuan, J., Shi, H., Xiong, Y., Chen, M., Han, L. and Qiu, D. (2012) Purification and characterization of a novel antimicrobial peptide from *Brevibacillus laterosporus* strain A60. Peptides, 33, 206-211.

CHAPTER FIVE

GENERAL OVERVIEW AND CONCLUSIONS

5.1 Summary of findings

Bacterial-based biocontrol of soil-borne phytopathogens has gained traction in recent years as a promising technology for developing sustainable and economically feasible agricultural pest control practices. However, widespread acceptance of this technology is tempered by the inconsistent performance of biocontrol agents when applied under varying field conditions. The screening procedures and selection criteria used in choosing candidate biocontrol agents are critical to the success of developing biocontrol agents and have a major influence on their subsequent performance *in situ* (Pliego *et al.*, 2011).

This study was undertaken with the intention of establishing screening methods that facilitate the selection of aerobic endospore-forming bacteria as candidate biocontrol agents against *Rhizoctonia solani.* The aim was to develop screening approaches that provide ecologically relevant information which would assist with the selection of promising isolates for further evaluation *in vivo*. A major objective was to establish screening methods that are suited to high throughput evaluation of isolates.

Aerobic endospore-forming bacteria were isolated from seedling roots of five crop types grown in composted pine bark medium and screened for *R. solani* antagonism using traditional *in vitro* dual-culture bioassays. Isolates exhibiting antifungal activity were then evaluated *in vivo* for biocontrol activity against *R. solani* in cucumber seedling trials. From these two screening approaches a pool of isolates was selected from which further screening methods could be applied and evaluated. These approaches included: the use of genomic fingerprinting to compare and group isolates that antagonized *R. solani in vitro*; the characterization of, and screening for, bioactive lipopeptide compounds involved in fungal antagonism; and, the use of matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) as a means of rapidly screening bacterial isolates.

It was established that:

- Approximately 6% of the aerobic endospore-forming bacteria isolated from the roots of crop seedlings grown in composted pine bark showed antagonism towards *R. solani in vitro*. From the assays it was possible to rank isolates based on the extent and stability of the inhibitory response *in vitro* as well as by the spectrum of antifungal activity observed against different test organisms evaluated.

- From *in vivo* seedling trials using a *Rhizoctonia* spp. susceptible cultivar of cucumber, several potential biocontrol candidates were identified. A general observation was that most isolates that achieved high rankings *in vitro* performed better in the *in vivo* trial than those with lesser rankings; although some exceptions were noted. It was also noted that a number of strains isolated from different plant species performed as well as, or better than, those isolated from cucumber roots. The *in vivo* screening approach used in this study proved to be laborious and resource intensive which did not lend itself to the screening of large numbers of isolates. The empirical nature of the *in vivo* test system proved awkward to standardise and variables such as pathogen loading and the sporulation level of the microbial inoculant were identified as potential limiting factors.

- Genomic fingerprinting using ITS-PCR allowed for a preliminary differentiation between isolates to be made with three major groupings being distinguished. Based on 16S rRNA gene sequence analysis two of the groups were identified as members of the "*Bacillus subtilis*" and "*Bacillus cereus*" groups of related taxa respectively; whereas, the third group comprising a single isolate, was identified as a strain of *Brevibacillus laterosporus*. However, ITS-PCR proved to be limited in its ability to detect inter- and intra-specific variation amongst closely related species/organisms.

- RAPD-PCR was found to be better suited for differentiating closely related isolates and additional levels of genetic diversity within each ITS grouping were distinguished. Within the "*Bacillus subtilis*" group four distinct groups were distinguished. Clonal similarities were evident for a number of strains isolated from different plant species and it is possible that these may reflect populations of rhizosphere competent strains and/or plant adapted ecotypes. Isolates belonging to the "*Bacillus cereus*" group exhibited greater levels of

genetic heterogeneity and the groupings discerned were not consistent for each primer set evaluated.

• 16S rRNA gene sequence analysis revealed that members within each respective "*Bacillus subtilis*" and "*Bacillus cereus*" grouping displayed high levels of sequence similarity and the taxonomic assignment of strains at the species level was not always possible. Sequence analysis of *gyrA* gene fragments revealed greater levels of genetic variation; this allowed members of the "*Bacillus subtilis*" group to be further differentiated and identified as strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* respectively. Sequence matches were consistent with the RAPD-PCR groupings determined previously, indicating that RAPD-PCR based fingerprinting was appropriate for differentiating related isolates at the strain and possibly the sub-species level. Strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* were amongst the best performers in the *in vivo* biocontrol seedling trial. In general, members of this group performed better than the "*Bacillus cereus*" group of isolates although several exceptions to this rule were observed namely, *B. amyloliquefaciens* subsp. *plantarum* CL-R53 and *B. subtilis* CT-R89 which both performed poorly in the *in vivo* biocontrol trial.

• ESI-TOF MS was used to detect and characterize lipopeptides purified from isolates that ranked highly in the *in vitro* assays and/or performed well in the *in vivo* seedling trial. Isoforms associated with several lipopeptide classes (viz., surfactins, iturins and and fengycins) were putatively identified. Bacillomycin/iturin and fengycin fractions exhibited antifungal activity *in vitro*, whereas surfactin fractions did not. Isolates that were ranked the highest in terms of fungal antagonism *in vitro* were all found to produce either bacillomycin D or iturin A isoforms. Phylogenetically related strains produced very similar lipopeptide profiles.

• PCR screening for bacillomycin D synthetase C (*bmyC*) amongst isolates correlated well with the ESI-TOF MS findings. However, primers targeting the malonyl-CoA-transacylase (*ituD*) gene linked to iturin A biosynthesis failed distinguish between isolates that produced iturin or bacillomycin in culture. Disparities between some of the PCR and ESI-TOF MS results suggested that primers targeting *srfA* and *fenD* biosynthetic genes showed limited specificity amongst the strains screened. Phylogenetic comparisons of *srfD* and *fenD* sequences revealed that these genes clustered according to species and was attributed to

distinct sequence heterogeneity. These findings highlighted the limitation of this approach to screening for potential biocontrol agents.

• MALDI-TOF MS was assessed as a means of identifying isolates antagonistic to *R. solani in vitro* and determining their associated lipopeptide profiles. The available spectral library database allowed for the identification of isolates to the genus level but proved to be limited for identifying environmental isolates to the species level. Extension of the database using "in-house" mass spectra profiles generated from isolates that were identified during the course of this study significantly improved the level of isolate identification in subsequent identification runs. Cluster analysis of mass spectra allowed for the relationships between isolates to be distinguished and provided a means of grouping closely related isolates. It was possible to discriminate between closely related *B. subtilis* and *B. amyloliquefaciens* species and the potential for differentiating strains at the sub-species level was also shown. MALDI-TOF MS also provided a convenient means of detecting bioactive lipopeptides (viz., surfactins, iturins and fengycins) directly from whole cell preparations, cell extracts and crude culture filtrates. Lipopeptide profiles varied depending on taxonomic groupings. Results for isolates within the "*Bacillus subtilis*" group corroborated the earlier ESI-TOF MS findings and were found to be more reliable then PCR screening for lipopeptide synthesis genes. "*Bacillus cereus*" group isolates produced distinct spectral profiles with fragments that were consistent with biomarkers previously described in the literature as isoforms of the kurstakin class of lipoheptapeptides. *Brevibacillus laterosporus* CC-R4 yielded a unique spectral profile in the *m/z* 750-2000 range with mass fragments which were similar to antimicrobial compounds recently reported in the literature. Overall, MALDI-TOF MS was found to fulfil the requirement for a practical yet robust technique suitable for processing large numbers of aerobic endospore-forming bacteria for biocontrol screening.

5.2 Conclusions

Aerobic endospore-forming bacteria are attractive candidates for biocontrol applications. The positive role that *Bacillus* spp. strains play in disease antagonism, plant growth promotion and induced resistance in plants is increasingly being recognized. Their ability to produce heat and desiccation resistant endospores, in particular, favours the development of stable spore formulations which are amenable to commercial production and applications (Pérez-García *et al*., 2011). Screening strategies that have focused on aerobic endospore-forming bacteria,

namely strains of *Bacillus* spp. as candidate biocontrol agents are therefore, justified. Biocontrol screening programmes typically involve the processing and screening of large numbers of isolates which impacts heavily on space, labour, resources and costs. The adoption of a particular screening method invariably involves a trade-off between ease of use, suitability for processing large numbers and the ecological relevance of the information gained. Screening methods are inherently selective and always run the risk of excluding promising candidates. To improve the chances of discovering effective biocontrol strains the isolation of bacteria from environments consistent with their intended use is advocated (Pliego *et al*., 2011).

The dual-culture bioassay used in this study proved to be a convenient starting point for initiating biocontrol screening of endospore-forming bacteria. The assay met the requirement of a relatively simple and rapid method suitable for screening large numbers of isolates. Isolates could be selected on the basis of their ability to antagonize test fungi and ranked according to the spectrum, extent and stability of the fungal antagonism evidenced. Subsequent *in vivo* screening revealed a number of potential biocontrol candidates that when applied as microbial inoculants in biocontrol seedling trials, performed significantly better than the diseased control. These findings support the idea that fungal antagonism can be used as an important selection criterion in preliminary biocontrol screening programmes for certain plant/pathogen systems (Pliego *et al*., 2011).

The rating of dual-culture bioassays over an extended incubation period was found to be useful; isolates that demonstrated stable inhibition rankings over this period were also amongst the best performing isolates *in vivo;* however, some notable exceptions to this rule were encountered. These findings suggested that the type of compound(s) produced or possibly, the amounts produced *in vitro,* might be indicators of an organism's subsequent performance *in vivo*. However, the fact that some isolates that performed well in the *in vitro* assay did not feature in the *in vivo* biocontrol trial, highlights the point that factors other than antifungal activity are also important determinants of biocontrol potential and need to be considered when selecting isolates for further testing. Whilst being relatively simple to perform and interpret the dual-culture assay provided very limited information of ecological relevance with which to choose isolates for further testing.

Molecular fingerprinting can play a useful role in differentiating bacterial isolates and establishing diversity levels amongst environmental isolates. Used in conjunction with *in vitro* screening, fingerprinting provides a convenient method of distinguishing between bacteria exhibiting antifungal activity and grouping related strains; thereby providing a means of rationalizing isolate numbers and selecting representative organisms for further evaluation. This approach can also provide information relating to the prevalence and distribution of related organisms isolated from different locations, soils, plant types or even cropping systems. From this study it was evident that several strain groupings could be identified with representative isolates being sourced from several different plant types. It is speculated that these findings may reflect the incidence of rhizosphere competent strains present within the experimental system used. Findings from the sequence analysis of 16s rRNA and *gyrA* genes supported this assertion with a number of these isolate groups being closely matched to GenBank sequences of plant associated bacteria. Further to this, representatives of these groups were amongst the best performing isolates in the *in vivo* biocontrol trials suggesting that these isolates were able to colonize seedling roots under the prevailing test conditions. To confirm this theory a root colonization assay, such as the one described by Kamilova *et al.* (2005), could be used to establish rhizosphere competence amongst strains in future studies. The results of genomic fingerprinting suggest that the specific targeting of putative plant adapted strains or ecotypes is feasible and could prove to be advantageous since these organisms represent a valuable pool of plant adapted strains from which biocontrol traits can be identified.

The findings from this study identified lipopeptide producing stains of *B. amyloliquefaciens* subsp. *plantarum* as the most promising candidates for biocontrol applications against *R. solani*. These findings are consistent with numerous reports in the literature that link strains of *B. amyloliquefaciens* with biocontrol of soil-borne fungal pathogens (Boriss, 2011; Cawoy *et al*., 2015; Chowdhury *et al*., 2015a). This species therefore, is an important target for screening purposes.

Screening strategies that target bacteria producing specific antibiotic compounds can be used as a means to select biocontrol candidates. The characterization of bioactive compounds associated with biocontrol candidates is commonly undertaken after the preliminary screening, once candidate biocontrol agents have been selected for further evaluation.

Whereas, the advent of PCR and MALDI-TOF MS screening methods makes the rapid detection of antifungal compound production capability feasible during the preliminary screening phases.

Lipopeptides belonging to the surfactin, fengycin and iturin families of biosurfactants, are amongst the most frequently reported bioactive compounds that are linked to biocontrol in plant-*Bacillus* associations. These compounds were identified from isolates that performed well *in vivo* and these findings are consistent with those reported in the literature for strains of *B. amyloliquefaciens* exhibiting biocontrol potential (Koumoutsi *et al*., 2004; Liu *et al*., 2011). Specifically, isolates that produced bacillomycin D isoforms were amongst the best performing isolates *in vivo*. Surfactin and fengycin are known elicitors of systemic resistance in plants (Cawoy *et al.*, 2014; Chowdhury *et al*., 2015b; Debois *et al*., 2015). In addition, surfactin is linked to the dispersion of bacteria within the rhizosphere and biofilm formation (Zeriouh *et al*., 2014). Screening strategies that select for these classes of compounds are, therefore, potentially useful determinants of prospective biocontrol candidates.

Whilst valuable for characterizing lipopeptide production amongst selected isolates, ESI-TOF MS was not suited to routine high throughput screening approaches. PCR screening of lipopeptide biosynthesis genes showed potential as a screening method but revealed limitations in terms of primer specificity and coverage amongst lipopeptide producing strains. The moderate levels of sample preparation required of this approach also detract from its suitability as a high throughput screening method. The practicality and feasibility of applying large scale screening following this approach is therefore questionable.

MALDI-TOF MS was found to offer the greatest opportunity for high throughput screening of aerobic endospore-forming bacteria. This method is easy to use, cost effective, and highly sensitive with good reproducibility. MALDI-TOF MS is suitable for analyzing compounds over a wide *m/z* range and can detect diverse constituents within complex mixtures without the requirement of additional extraction and clean-up steps (Stets *et al*., 2013).

The *in vivo* evaluation of candidate antagonists is a crucial component of any biocontrol screening programme since it provides information pertaining to the biocontrol potential of an isolate under conditions where plant-pathogen-antagonist interactions are incorporated into the study. *In planta* biocontrol assays select for isolates that are compatible with the test plant

under the prevailing experimental conditions. Traits that contribute to rhizosphere competence and/or biocontrol mechanisms are also selected for, which will contribute to the overall performance of isolates. *In vivo* screening, therefore, provides a basis for evaluating isolates obtained from preliminary stages of screening and a means of rationalizing the selection of isolates for further evaluation. Such approaches are generally not suited to preliminary screening protocols since they can rapidly become unwieldy.

In vivo biocontrol screening using a non-sterile potting medium is recommended since it accounts for the mitigating effect of naturally occurring microflora on the performance of the bacterial inoculant. However the presence of extant microorganisms may result in variability due to microbial competition, nutrient availability, pH and oxygen levels; this in turn may influence the performance of the inoculum. The amount of pathogen inoculum used in the *in vivo* trial was found to have a significant bearing on the outcome of the biocontrol trials; it is therefore essential that loading rates are reflective of the natural situation. Given that *R. solani* does not readily produce reproductive structures, the pathogen was applied as a colonised agar plug on an empirical basis, which could influence variability in subsequent trials. Nonetheless, even with these possible variabilities, positive biocontrol interactions were successfully detected allowing for promising biocontrol agents to be identified.

5.3 Contribution of work undertaken and recommendations for future screening practices

The study established that potential biocontrol isolates, namely strains of *B. amyloliquefaciens* subsp. *plantarum* and *B. subtilis* are antagonistic to *R. solani in vitro* and *in vivo*. The incorporation of selected biocontrol agents into greenhouse and nursery growing media would be advantageous in reducing the existing reliance on fungicides. Many fungicides pose a risk to farm workers during application and fungicide residues influence the time between fungicide application and crop harvest. The use of safe biocontrol agents has the potential to eliminate or mitigate these issues. The application of biocontrol agents to plants cultivated under greenhouse/nursery conditions is therefore seen as a promising solution for controlling soil-borne pathogens and maintaining “healthy” growing media.

This study has shown that genomic fingerprinting and MALDI-TOF MS characterization of bacterial isolates are worthwhile additions to preliminary *in vitro* screening practices. They provide a level of isolate differentiation and characterization that is beneficial for selecting

candidate biocontrol agents, which is not possible with traditional screening practices. Effectively, they allow traditional biocontrol screening to move away from empirically based approaches to ones which are knowledge based; allowing for representative groups of bacteria with specific traits to be selected for further evaluation.

MALDI-TOF MS has the capacity to quickly identify large numbers of isolates to the species level and provides concomitant information regarding the bioactive compounds produced. It has the ability to detect specific compounds of interest or discover novel compounds. This allows isolates to be selected for further testing based on a specific set of criteria and evaluated *in vivo*.

Genomic fingerprinting is suitable for differentiating bacterial isolates at the strain level. This is important for grouping closely related isolates thereby avoiding unnecessary replication which will save time, money and resources. This approach is also useful for determining diversity amongst isolates from various sources/environments thereby enhancing our understanding of the distribution and occurrence of specific strains of bacteria. This approach provides a means of discovering plant- or environment-adapted populations and offers a basis for assessing, evaluating and comparing ecological fitness traits.

In order to implement these screening methods into biocontrol screening practices the following screening strategy is proposed:

- Step 1 – Endospore-forming bacteria are isolated from environments consistent with their intended use. Typically, root material from healthy plants of the targeted crop type(s) grown in soil/media relevant to the biocontrol application should be used to increase the likelihood of discovering rhizosphere competent candidates.
- Step 2 – *In vitro* dual-culture bioassays are performed against the targeted pathogen(s) taking into account the extent and stability of antagonism. Additional pathogens can be included to determine the spectrum of fungal antagonism. Preliminary ranking of isolates can be made on the basis of these findings.
- Step 3 – MALDI-TOF MS screening of isolates is then performed to identify isolates and determine their associated lipopeptide/bioactive compound profile. Isolates can be eliminated or selected on the basis of their identity and/or lipopeptide profiles.

Bacteria that are considered to be potentially pathogenic or harmful (e.g. *B. cereus*) can be excluded whereas those regarded as being novel or benign can be tested further.

- Step 4 – Genomic fingerprinting using RAPD-PCR (or equivalent) is used to further differentiate selected isolates and group them at the strain level. Representatives of these groups can then be selected for *in vivo* screening. To account for possible strain variation those isolates within the group that ranked the highest in terms of *in vitro* antagonism bioassays or those that produced the highest relative intensities of lipopeptide during MALDI-TOF MS analysis can then be selected for *in vivo* screening.
- Step 5 – The biocontrol efficacy of selected isolates is tested *in vivo* in greenhouse experiments. From this step candidate biocontrol agents can be selected for further evaluation.

5.4 The way forward?

"For biocontrol to be implemented on a practical level, the antagonists must be ecologically fit to survive, become established, and function within the particular conditions of the ecosystem" (Pliego *et al*., 2011).

Our current understanding of the ecology of plant associated bacteria is limited and remains a major obstacle to the development of successful biological control technologies targeting soil-borne phytopathogens. The unravelling of mechanisms that determine rhizosphere competence, root colonisation and the regulation of biocontrol traits within the rhizosphere are key factors that will benefit the development of biocontrol technologies. Recent studies focusing on the genomic analysis of promising biocontrol agents such as *B. amyloliquefaciens* FZB42 has yielded information pertaining to the genes associated with root colonisation (Fan *et al*., 2011), plant growth promotion (Budiharjo *et al*., 2014) and antibiotic production (Chen *et al*., 2009); such information will offer new possibilities for the characterization and selection of biocontrol organisms.

The development of screening assays that select for rhizosphere competence on the basis of traits such as swarming motility (Kearns, 2010), chemotaxis and biofilm formation (Zhang *et al*., 2014) would be useful for advancing the research presented in this thesis. Further, an investigation to quantify biosurfactant production of isolates, or expression of genes involved,

would show whether *in vitro* biosurfactant concentration and biocontrol efficacy *in vivo* is correlated.

Quorum sensing (QS) is another area of research that might improve our understanding of biocontrol mechanisms. In *B. subtilis* QS systems are known to regulate production of anti-pathogenic and biofilm-inducing compounds such as surfactins (Oslizlo *et al*., 2015). The ecological significance of QS with regards to biocontrol still needs to be explored. The existence of distinct communication groups (pherotypes) that can efficiently communicate within a group, but not between different groups could have a significant impact on how inoculants behave when introduced into the rhizosphere environment.

There is also a need to expand our understanding of the diversity, distribution and prevalence of aerobic endospore-forming bacteria within different rhizosphere environments in order to assist in selecting appropriate biocontrol candidates. The identification of plant adapted ecotypes associated with specific plant types or cropping systems holds promise as a basis from which to identify traits that are associated with rhizosphere competence. This could lead to improved screening methods that enable to select candidates for biocontrol screening based on ecologically relevant criteria. The advent of metagenomics has played an important role in developing our understanding of the rhizosphere microbiome and is useful for determining the effects biocontrol bacteria have on indigenous microbial communities (Erlacher *et al.*, 2014). However, the adoption of typing methods that distinguish isolates at the strain level are still important for identifying ecotypes adapted to specific environments.

Further developments of fingerprinting methods that meet the practical requirements of a high throughput screening method are also desirable. The application of genomic typing methods such as REP-PCR fingerprinting which can be performed directly from colony pick-offs (Ishii and Sadowsky, 2009) or the application of real-time PCR as a rapid means of comparing and grouping organisms based on high resolution melt analysis of amplified gene fragments (Dhakal *et al*., 2013) hold promise and would be worth exploring from a biocontrol screening perspective.

The capacity of MALDI-TOF MS to identify environmental isolates can be improved by establishing appropriate databases and making these available as open source resources. Not only is MALDI-TOF MS suitable for directed screening and selection for specific lipopeptide

profiles, it also provides a convenient method for the discovery of novel antimicrobial compounds. Its potential for screening for compounds associated with growth promotion and biocontrol traits such as plant growth regulators, siderophores and various antimicrobial secondary metabolites are areas that could be explored in future work.

Ultimately, the success of *Bacillus*-based biocontrol will depend on its acceptance and adoption by agricultural practitioners (farmers) as a feasible alternative to chemical pesticides. Although biocontrol products seldom achieve the consistent levels of control that can be achieved using chemical products, various opportunities for using biocontrol technologies are increasingly being recognized. Instances include: when or where resistance to chemicals occurs, where chemicals are banned, are too costly, or too hazardous to use (Jacobsen *et al*., 2004; Pérez-García *et al.*, 2011). There is also a growing demand for "environmentally friendly" biological pesticides that are compatible with organic farming practices.

The influence of combining or co-inoculating several biocontrol agents is another area worth investigating further. It would be interesting to test this hypothesis using strains selected on the basis of ecotype compatibility. From this it may be possible to achieve a synergistic effect whereby a biocontrol effect may be enhanced for a longer period.

Other important areas of research include the practical aspects of formulating and applying biocontrol products ensuring that the timing, frequency and rate of application are optimised for biocontrol. A better understanding of biocontrol mechanisms will provide a basis from which to develop further inoculation strategies and to incorporate biocontrol practices into existing integrated pest management systems.

5.5 References

Borriss, R. (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In: Maheshwari, D.K. (ed.), Bacteria in Agrobiology: Plant Growth Responses, Springer-Verlag, Berlin, Germany, pp. 41-76.

Budiharjo, A., Chowdhury, S. P., Dietel, K., Beator, B., Dolgova, O., Fan, B., Bleiss, W., Ziegler, J., Schmid, M., Hartmann, A. and Borriss, R. (2014) Transposon mutagenesis of the plant-associated *Bacillus amyloliquefaciens* ssp. *plantarum* FZB42 revealed that the nfrA and RBAM17410 genes are involved in plant-microbe-interactions. PLoS ONE 9(5): e98267. doi:10.1371/journal.pone.0098267.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. Microbial Biotechnology, 8, 281-295. doi: 10.1111/1751-7915.12238.

Cawoy, H., Mariutto, M., Henry, G., Fisher, C., Vasilyeva, N., Thonart, P., Dommes, J. and Ongena, M. (2014) Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. Molecular Plant-Microbe Interactions, 27, 87-100.

Chen, X.H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Süssmuth, R., Piel, J. and Borriss,R. (2009) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. Journal of Biotechnology, 140, 27-37.

Chowdhury, S.P., Hartmann, A., Gao, X. and Borriss, R. (2015a) Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42: a review. Frontiers in Microbiology, 6, 780. doi:10.3389/fmicb.2015.00780.

Chowdhury, S. P., Uhl, J., Grosch, R., Alquéres, S., Pittroff, S., Dietel, K., Schmitt-Kopplin, P., Borriss, R. and Hartmann, A. (2015b) Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*. Molecular Plant-Microbe Interactions, 28, 984-95. doi:10.1094/MPMI-03-15-0066-R.

Debois, D., Fernandez, O., Franzil, L., Jourdan, E., Brogniez, A., Willems, L., Clément, C., Dorey, S., De Pauw, E. and Ongena, M. (2015) Plant polysaccharides initiate underground crosstalk with bacilli by inducing synthesis of the immunogenic lipopeptide surfactin. Environmental Microbiology Reports, 7, 570-582.

Dhakal, R., Chauhan, K., Seale, R. B., Deeth, H. C., Pillidge, C. J., Powell, I. B., Craven, H. and Turner, M. S. (2013) Genotyping of dairy *Bacillus licheniformis* isolates by high resolution melt analysis of multiple variable number tandem repeat loci. Food Microbiology, 34, 344-351.

Erlacher, A., Cardinale, M., Grosch, R., Grube, M. and Berg, G. (2014) The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. Frontiers in Microbiology, 5 (175), 1-8. doi: 10.3389/fmicb.2014.00175.

Fan, B., Chen, X. H., Budiharjo, A., Bleiss, W., Vater, J. and Borriss, R. (2011) Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. Journal of Biotechnology, 151, 303-311.

Ishii, S. and Sadowsky, M. J. (2009) Applications of the rep-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution. Environmental Microbiology, 11, 733-740.

Jacobsen, B. J., Zidack, N. K. and Larson, B. J. (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: Plant diseases. Phytopathology, 94, 1272-1275.

Kamilova, F., Validov, S., Azarova, T., Mulders, I. and Lugtenberg, B. (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environmental Microbiology, 7, 1809-1817.

Kearns, D. B. (2010) A field guide to bacterial swarming motility. Nature Reviews Microbiology, 8, 634-644.

Koumoutsi, A., Chen, X.H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J. and Borriss, R. (2004) Structural and functional characterization of gene clusters directing non-ribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology, 186, 1084-1096.

Liu, J., Zhou, T., He, D., Wu, H., Liu, W. and Gao, X. (2011) Functions of lipopeptides bacillomycin D and fengycin in antagonism of *Bacillus amyloliquefaciens* CO6 towards *Monilinia fructicola*. Journal of Molecular Microbiology and Biotechnology, 20, 43-52.

Oslizlo, A., Stefanic, P., Vatovec, S., Beigot Glaser, S., Rupnik, M. and Mandic-Mulec, I. (2015) Exploring ComQXPA quorum-sensing diversity and biocontrol potential of *Bacillus* spp. isolates from tomato rhizoplane. Microbial Biotechnology, 8, 527-540.

Pérez-García, A., Romero, D., Zeriouh, H. and de Vicente, A. (2011) Biological control of phytopathogenic fungi by aerobic endospore-formers. In: Logan, N.A. and De Vos, P. (eds) Endospore-forming Soil Bacteria, Springer Berlin Heidelberg, Germany. pp. 157-180.

Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F. M. (2011) Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. Plant and Soil, 340, 505-520.

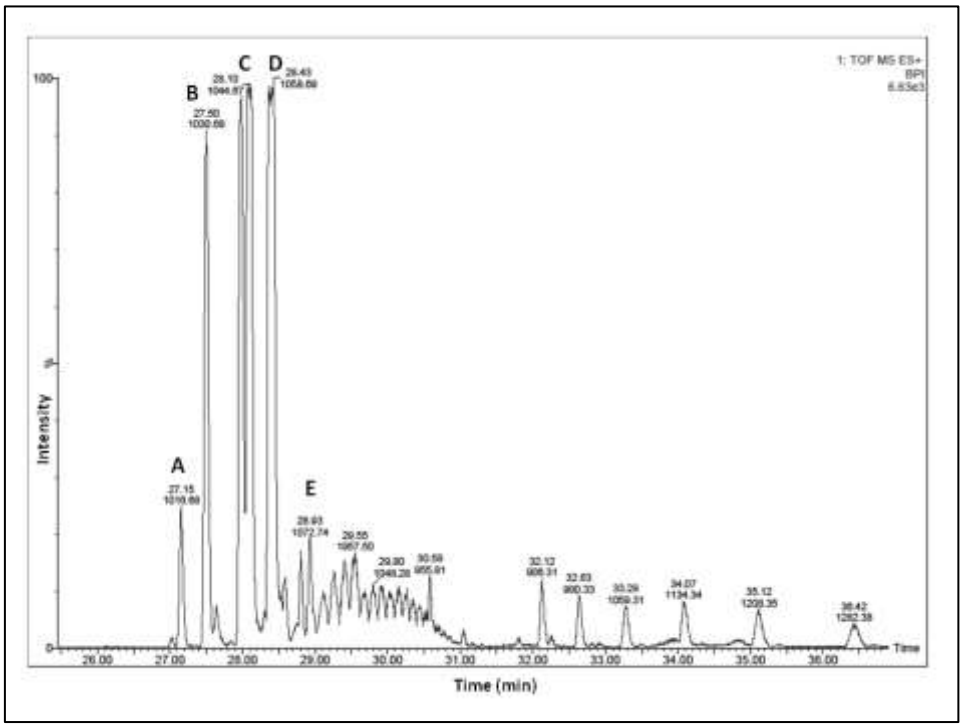
Stets, M.I., Pinto, A.S., Huergo, L.F., de Souza, E.M., Guimarães, V.F., Alves, A.C., Steffens, M.B., Monteiro, R.A., Pedrosa, F.O. and Cruz, L.M. (2013) Rapid identification of bacterial isolates from wheat roots by high resolution whole cell MALDI-TOF MS analysis. Journal of Biotechnology, 165, 167-74.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q. and Zhang, R. (2014) Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. Plant and Soil, 374, 689-700.

Zeriouh, H., Vicente, A., Pérez-García, A. and Romero, D. (2014) Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. Environmental Microbiology, 16, 2196-2211.

APPENDIX A: UPLC CHROMATOGRAMS AND MASS SPECTRA OF COMMERCIAL SURFACTIN AND ITURIN A STANDARDS

(A)



(B)

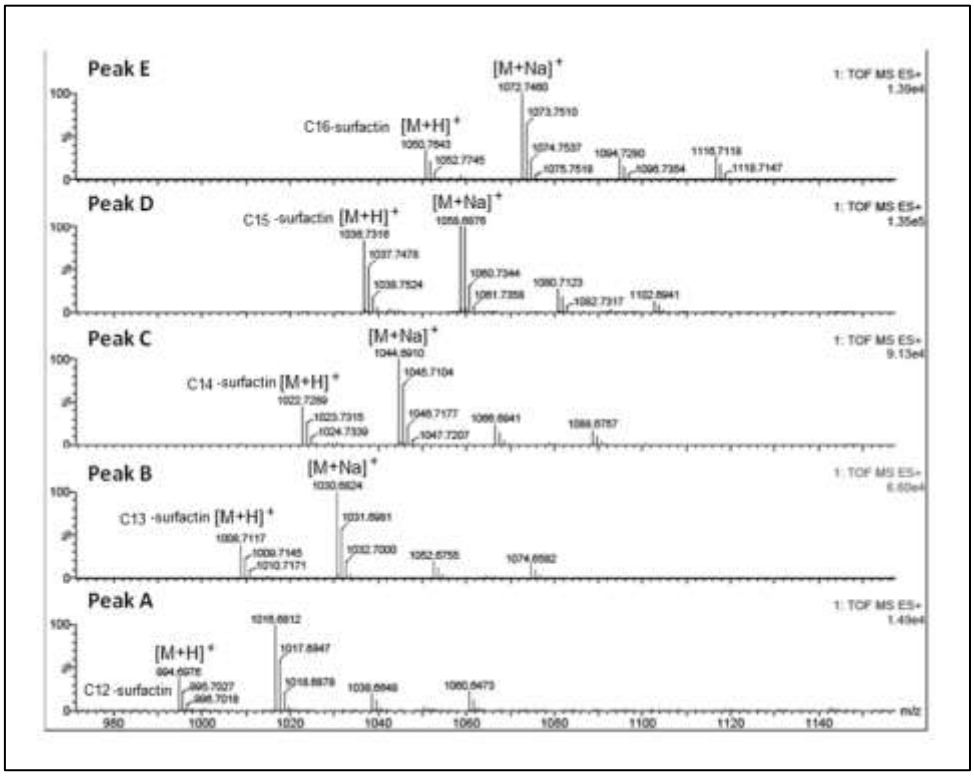
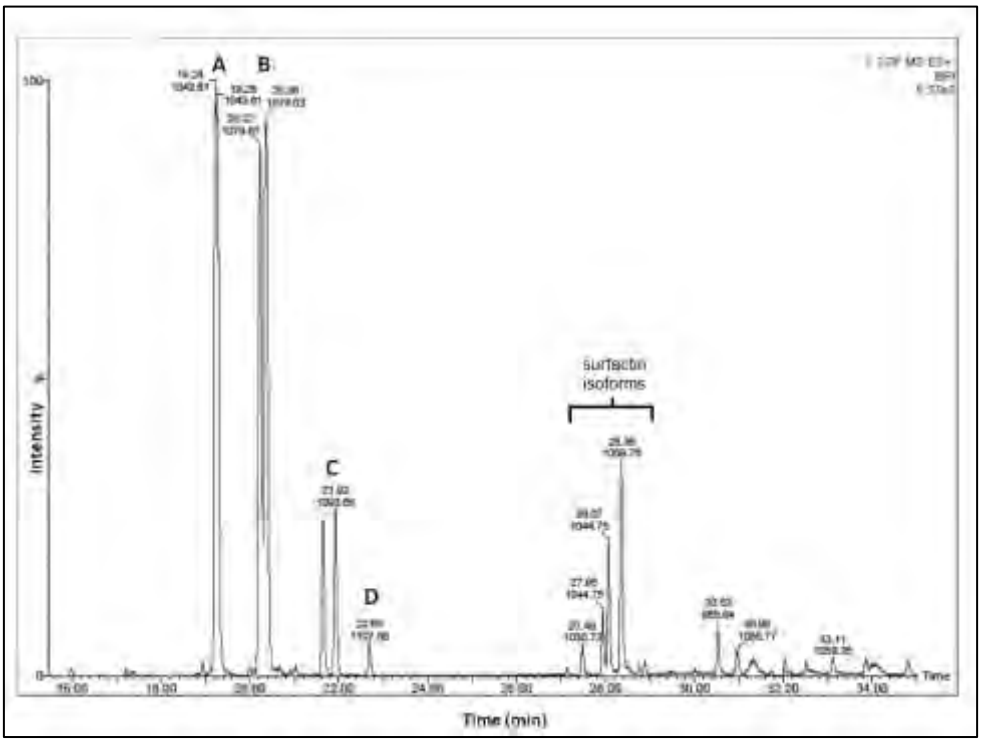


Figure 1: UPLC chromatogram (A) and related mass spectra (B) of surfactin isoforms present in a commercial surfactin standard (Sigma-Aldrich)

APPENDIX A contd.

(A)



(B)

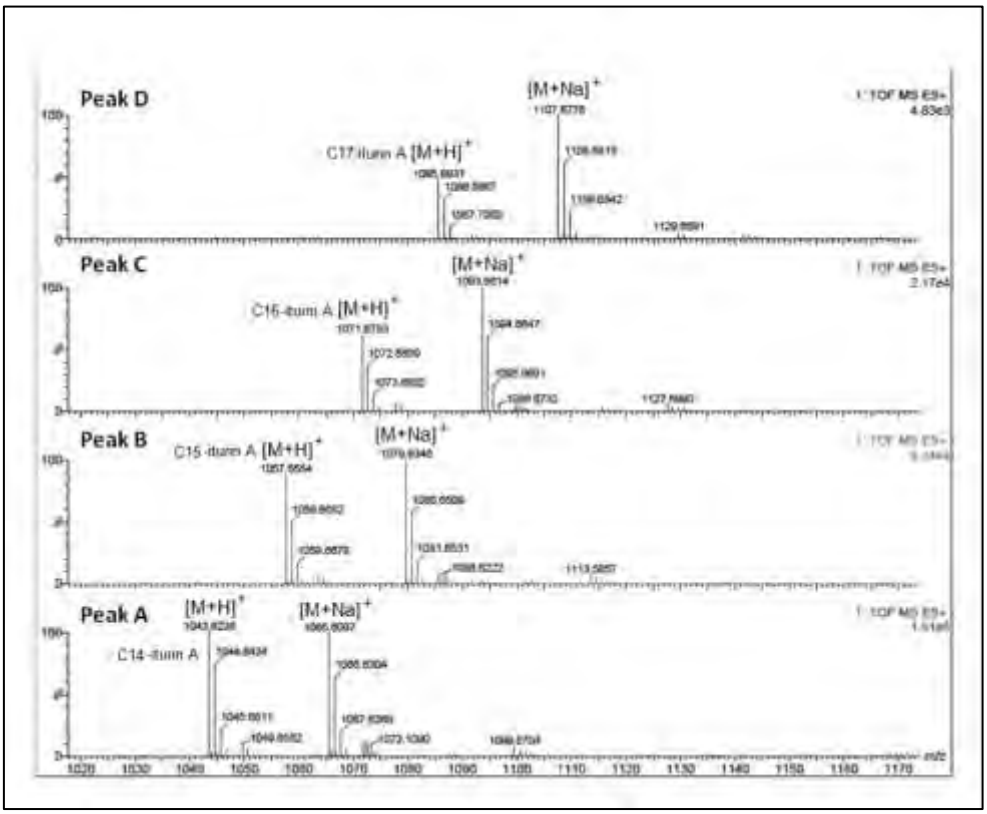


Figure 2: UPLC chromatogram (A) and related mass spectra (B) of iturin A isoforms present in a commercial iturin A standard (Sigma-Aldrich)

APPENDIX B: PEAK MASS LISTS FROM AVERAGED MASS SPECTRA POFILES (MSP) OF *BACILLUS* SPP. ISOLATES HIGHLIGHTING COMMON BIOMARKERS*

		Isolate																								
peak list (m/z)	standard deviation	DSM7	CC-R9	CT-R92	CT-R67	B81	CC-R10	CT-R89	CT-90	CL-R59	CL-R51	CP-R45	CL-R53	CL-R64	CB-R106	CL-R49	R 16	CP-R23	CP-R43	DSM27	CP-R41	ATCC6633	CC-R4	CB-R105	CC-R15	CP-R30
2149.55	0.34	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0
2166.02	0.56	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	0	1	0	0
2196.06	0.41	0	1	1	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
2206.02	1.27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
2223.2	0.22	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2292.63	0.92	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
2329.7	0.82	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2475.9	1.90	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2483.97	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
2569.35	0.34	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0
2625.25	0.65	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	1	0	0	0
2633.14	0.76	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0
2744.15	0.33	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2759.92	0.13	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	1	0	0	0	0	0	0
2949.43	0.52	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
2958.25	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
3045.8	0.22	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3062.97	1.09	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3071.02	2.84	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1	1	0	0	0	0
3155.18	0.99	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
3206.11	0.73	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
3231.22	1.35	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
3253.4	0.47	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
3260.98	0.39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
3275.99	1.23	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3296.74	1.36	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
3303.82	0.55	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0
3318.18	0.76	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	0	0	1	0	0	0
3335.21	0.26	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3339.11	0.29	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*The presence/absence of a mass fragment in a spectral profile is represented by 1/0 respectively.

APPENDIX B contd.

peak list (m/z)	standard deviation	DSM7	CC-R9	CT-R92	CT-R67	B81	CC-R10	CT-R89	CT-90	CL-R59	CL-R51	CP-R45	CL-R53	CL-R64	CB-R106	CL-R49	R 16	CP-R23	CP-R43	DSM27	CP-R41	ATCC6633	CC-R4	CB-R105	CC-R15	CP-R30
3342.65	0.36	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	0	0	0	0	1	0	0
3349.81	0.93	0	0	1	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	0	0	0
3360.97	0.86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
3376.32	1.24	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1
3417.75	0.49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
3425.95	0.68	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
3470.9	0.41	0	0	0	1	1	1	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
3482.64	0.86	0	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0
3540.96	0.50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
3556.92	0.65	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0
3705.39	2.35	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0	1	1	1
3719.11	3.43	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	0	1	0	0	0
3728.17	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
3761.23	0.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0
3857.49	0.54	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
3871.26	0.52	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0
3887.81	1.96	0	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	1	1
4021.24	0.40	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
4030.41	0.49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
4042.33	0.45	1	1	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
4306.45	0.87	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
4322.02	1.42	0	1	1	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0	0
4343.58	1.23	1	1	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
4374.64	1.22	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4403.2	0.88	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4421.08	0.76		1	1	1	1	1	1	1	0	0	0	0	0	0	0		0	0	1	0	0	0	0	0	0
4443.53	0.54	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	1	1	1	0	0	0	0	0	0	0
4472.5	0.89	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
4552.68	0.83	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
4605.39	0.87	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

*The presence/absence of a mass fragment in a spectral profile is represented by 1/0 respectively.

APPENDIX B contd.

peak list (m/z)	standard deviation	DSM7	CC-R9	CT-R92	CT-R67	B81	CC-R10	CT-R89	CT-90	CL-R59	CL-R51	CP-R45	CL-R53	CL-R64	CB-R106	CL-R49	R 16	CP-R23	CP-R43	DSM27	CP-R41	ATCC6633	CC-R4	CB-R105	CC-R15	CP-R30
4613.17	0.80	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
4699.91	1.27	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0
4735.89	0.40	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4823.22	2.41	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0
4944.16	0.83	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	1
4957.97	0.71	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0
5003.75	0.43	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
5016.56	0.74	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0
5029.84	0.47	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
5150.74	1.46	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
5172.53	0.71	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
5210.01	1.79	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	1	0	0	0	0
5226.44	1.53	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0
5255.52	1.30	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0
5330.03	0.61	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
5573.63	0.87	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
5886.91	0.72	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
5902.95	1.06	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0
5920.18	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
5929.1	1.66	0	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
6120.74	1.06	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	0	0
6145.58	0.82	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0
6159.15	0.58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
6180.36	0.81	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
6490.06	1.65	0	1	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6509.61	0.99	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
6597.96	1.60	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
6640.45	1.00	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
6681.12	0.55	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6701.24	1.26	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0

*The presence/absence of a mass fragment in a spectral profile is represented by 1/0 respectively.

APPENDIX B contd.

peak list (m/z)	standard deviation	DSM7	CC-R9	CT-R92	CT-R67	B81	CC-R10	CT-R89	CT-90	CL-R59	CL-R51	CP-R45	CL-R53	CL-R64	CB-R106	CL-R49	R 16	CP-R23	CP-R43	DSM27	CP-R41	ATCC6633	CC-R4	CB-R105	CC-R15	CP-R30
6713.41	0.79	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
6728.88	0.86	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
6741.49	0.38	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6755.46	2.39	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1
6774.58	1.18	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0
6792.84	1.28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1
6829.98	0.46	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6837.9	0.80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
6853.4	1.53	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
6918.92	0.97	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
6943.15	0.51	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7088.1	3.12	0	1	1	1	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1
7115.44	1.59	0	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0
7429.34	0.66	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7444.82	1.34	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0
7523.99	0.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	0
7544.8	0.60	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7654.38	1.44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0
7715.7	1.08	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
7743.66	1.06	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
7771.87	0.91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
8085.25	0.92	1	1	1	1	1	0	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0
8840.89	0.75	0	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9209.29	0.70	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9225.27	1.49	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
9470.24	0.68	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9542.7	1.25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
9886.68	1.25	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
9914.35	1.24	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
10005.5	0.66	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10031.1	1.24	0	0	0	0	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
10449.3	3.03	0	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	0	0	1	0	0	0	0	0	0
11143.9	1.22	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0

*The presence/absence of a mass fragment in a spectral profile is represented by 1/0 respectively.