

Lincoln University Digital Thesis

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the thesis and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the thesis.

INFLUENCE OF ROOT EXUDATES ON SOIL MICROBIAL DIVERSITY AND ACTIVITY

A thesis submitted in partial fulfilment of the requirements for the
Degree of Doctor of Philosophy

at

Lincoln University

by

Shengjing Shi

Lincoln University

New Zealand

2009

**Abstract of a thesis submitted in partial fulfilment of the requirements for the
Degree of Doctor of Philosophy**

**Influence of Root Exudates on Soil Microbial
Diversity and Activity**

by Shengjing Shi

Interactions between plant roots and soil microorganisms in the rhizosphere are critical for plant growth. However, understanding of precisely how root exudates influence the diversity and activity of rhizosphere microorganisms is limited. The main objective of this study was to investigate the effect of radiata pine (*Pinus radiata*) root exudates on rhizosphere soil microbial communities, with an emphasis on the role of low molecular weight organic anions. The study involved the development and validation of new methods for investigating rhizosphere processes in a purpose-built facility. This included development of an *in situ* sampling technique using an anion exchange membrane strip to collect a range of organic anions exuded from radiata pine roots grown in large-scale rhizotrons. These included tartarate, quinate, formate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate. Soil microbial activity and diversity were determined using dehydrogenase activity and denaturing gradient gel electrophoresis. Links between organic anions in root exudates and rhizosphere soil microbial community structures were investigated by comparing wild type and genetically modified radiata pine trees which were grown in rhizotrons for 10 months. As expected, there was considerable temporal and spatial variability in the amounts and composition of organic anions collected, and there were no consistent or significant differences determined between the two tree lines. Significant differences in rhizosphere microbial communities were detected between wild type and genetically modified pine trees; however, they were inconsistent throughout the experiment. The shifts in microbial communities could have been related to changes in exudate production and composition. Based on results from the main rhizotron experiment, a microcosm study was carried out to investigate the influence of selected pine root exudate sugars (glucose, sucrose and fructose) and organic anions (quinate, lactate and maleate) on soil microbial activity and diversity. Soil microbial activity increased up to 3-fold in all of the sugar and organic anion treatments compared to the control, except for a mixture of sugars and maleate where it decreased. The corresponding impacts on soil microbial diversity were assessed using

denaturing gradient gel electrophoresis and 16S rRNA phylochips. Addition of the exudate compounds had a dramatic impact on the composition and diversity of the soil microbial community. A large number of bacterial taxa (88 to 1043) responded positively to the presence of exudate compounds, although some taxa (12 to 24) responded negatively. Organic anions had a greater impact on microbial communities than sugars, which indicated that they may have important roles in rhizosphere ecology of radiata pine. In addition, a diverse range of potentially beneficial bacterial taxa were detected in soil amended with organic anions, indicating specific regulation of rhizosphere microbial communities by root exudates. This project highlighted the considerable challenges and difficulties involved in detailed investigation of *in situ* rhizosphere processes. Nonetheless, the findings of this study represent a significant contribution to advancing understanding of relationships between root exudates and soil microbial diversity, which will be further enhanced by refinement and application of the specific methodologies and techniques developed.

Key words: rhizosphere, root exudates, organic anions, soil microbial community, diversity, *in situ*, anion exchange membrane, radiata pine (*Pinus radiata*), genetically modified, rhizotron, rRNA, DGGE, phylochip

Acknowledgements

After two and half years' lab work and more than six months writing, this PhD project has finally been finished. This is not the thing that I could have done alone. Instead, many people helped and supported me throughout the whole course.

First and foremost, I would like to thank my supervisors – Prof. Leo Condrón, Dr. Eirian Jones, Prof. Alison Stewart (all Lincoln University), Dr. Maureen O'Callaghan, Dr. Darren Smalley (both AgResearch, Lincoln), Dr. Alan Richardson (CSIRO, Australia) and Dr. Christian Walter (SCION, Rotorua) for taking me as a student for this challenging project, for constant encouragement and guidance, for sharing their expertise and giving me support and advice throughout this project. While having seven supervisors did not seem like the best idea at the beginning, it has worked out beautifully. And, unbelievably, I had every freedom to make decisions regarding to this project. I am especially grateful to Leo for his encouragements, enthusiasms, keeping me well on the track, arranging project meetings and my travels to Australia and USA. I would also like to thank Leo, Eirian, Maureen and Alan for teaching me useful skills for scientific writing, for patiently reading my writings and correcting my spelling and grammar mistakes.

Many thanks go to Stuart Larsen for his excellent ideas and useful creations in rhizotrons. He is a man who can make any thing that I asked for. I also very appreciated his help for soil preparation, packing, tree planting, samplings and all the paper work for transfer permits for GM plants. Without those help, I can never finish this project.

I also want to thank Dr. Jana Lottmann and Emily Gerald from AgResearch, Lincoln, for the useful discussion and advice during these three years and for sharing some reagents. Thanks Hayley Ridgway for her help on fungal PCR and discussion regarding fungal/ectomycorrhizal colonization on pine roots.

Acknowledgments also go to Lincoln analytical groups, especially Joy Jiao for all her efforts to set up HPLC method for separating 12 organic acids, for her trust and allowing me to run the machine over weekends, for her daily life help and interesting chats.

I am thankful to Drs. David Baird and Chris Frampton for their statistical analysis and responding to my numerous consultations. Thanks Prof. Alan McKinnon and Dr. Keith Unsworth (Lincoln University) for designing software which can calculate the surface area of roots in my special interesting case. Thanks Dr. Ute Roessner (Australian Centre for Plant Functional Genomics, School of Botany, University of Melbourne) for assistance in

the initial identification of compounds in root exudate samples by GC-MS analysis. Also, I would like to thank Tomoko Pearson (Scion) for measuring NptII protein in my samples.

Thanks Andrew Holyoake, Candice Barclay and Amanda Clifford for their help ordering so many materials for me. Thanks all the people in B612 and B609 for the easier and fun lab working environment.

Thanks also go to all the student and staff in the Soil and Physical Groups, AGLS, Lincoln. It has been great to be in such a group and thanks for all the relaxing and wonderful social activities and dinner parties.

I also thank New Zealand Tertiary Education Commission for the funding of this project and my scholarship over 3 years, and thank National Centre for Advanced Bio-Protection Technologies for the grant for my travel to Berkeley, USA.

Special thanks to Prof. Mary Firestone for inviting me to UC Berkeley for phylochip analysis, for letting me stay at her house while in USA, and for useful discussion about this project. Big thanks to Dr. Kristen DeAngelies (Lawrence Berkeley National Laboratory, CA, USA) for teaching me the phylochip technique, data analysis and R statistic language, for accompanies for delicious dinners, and for the wonderful ski trip.

Last but not least, thanks to my parents for their believe in me and their unconditional love. I really appreciated my husband Bin for his love and moving to NZ with me, for his constant encouragement, patience and understanding throughout these 3 years. Thanks for taking care of all the house work during my writing period and thanks for all the entertainment to make me keeping smile even in those tough times.

Shengjing Shi

Contents

Abstract	i
Acknowledgements.....	iii
Contents	v
List of Tables	x
List of Figures	xiii
Abbreviations.....	xvii
Chapter 1 Introduction	1
1.1 General Introduction.....	1
1.1.1 The Rhizosphere	1
1.1.2 Root Exudates	2
1.1.3 Plant-microbe Interactions Mediated by Root Exudates	7
1.1.4 Technical Challenges in Rhizosphere Studies.....	10
1.1.4.1 Root exudate collection	10
1.1.4.2 Root exudate analysis	12
1.1.4.3 Corresponding samples for exudate and microbial community analysis.....	12
1.1.5 Rhizosphere Study Systems.....	13
1.2 Research Context and Objectives.....	14
1.2.1 Research Context.....	14
1.2.2 Hypothesis.....	15
1.2.3 Objectives.....	15
1.3 Thesis Format	15
Chapter 2 Molecular Methods for Characterisation and Analysis of Rhizosphere and Non-rhizosphere Microbial Communities.....	16
2.1 Introduction.....	16
2.1.1 PCR-DGGE	17
2.1.2 Strengths and Weaknesses of PCR-DGGE.....	17
2.1.3 Analysis of PCR-DGGE Microbial Community Profiles.....	18
2.1.4 DNA vs. RNA-based profiling.....	19
2.1.5 Extraction of RNA	21
2.1.6 Primers for Use in PCR-DGGE.....	21
2.2 Methods	23
2.2.1 Rhizosphere and Non-rhizosphere Soil Preparation.....	23
2.2.2 Extraction of Microbial Community RNA from Soil.....	24
2.2.2.1 Preliminary comparison of extraction methods	24
2.2.2.2 Modified CTAB RNA extraction method.....	25
2.2.3 RNA Samples and Subsequent cDNA Preparation	25

2.2.4	PCR Amplification of Bacterial 16S rRNA Gene Fragment.....	27
2.2.5	PCR Amplification of Taxon-specific 16S rRNA Gene Fragments.....	27
2.2.6	PCR Amplification of Fungal 18S rRNA Gene Fragments.....	28
2.2.7	DGGE.....	28
2.2.8	Analysis of DGGE Gels.....	30
Chapter 3 Rhizotron Sampling Techniques and Spatial Variability in Soil Microbial Communities in a Rhizotron System.....		31
3.1	Introduction.....	31
3.2	Materials and Methods.....	33
3.2.1	Detection and Quantification of OAs by HPLC.....	33
3.2.2	Validation of AEM Collection of OAs.....	34
3.2.2.1	Recovery of OAs by AEM.....	34
3.2.2.2	The effect of elution times on recovery of OAs from AEM.....	35
3.2.2.3	Recovery of low concentration of OAs by AEM.....	35
3.2.2.4	Stability of OAs on AEM in contact with soil.....	35
3.2.2.5	Collection of OAs by AEM from soil.....	36
3.2.3	Rhizotron Experimental Set up.....	36
3.2.4	<i>In situ</i> Collection of OAs in the Rhizotron System.....	38
3.2.5	Analysis of Variability in Soil Microbial Communities.....	40
3.2.6	Statistical Analysis.....	41
3.3	Results.....	42
3.3.1	Detection and Quantification of OAs by HPLC.....	42
3.3.2	Validation of the Use of AEM to Collect OAs.....	44
3.3.2.1	Recovery of OAs by AEM.....	44
3.3.2.2	The effect of elution time on recovery of OAs from AEM.....	45
3.3.2.3	Recovery of low concentrations of OAs by AEM.....	45
3.3.2.4	Stability of OAs on AEM in contact with soil.....	47
3.3.2.5	Collection of OAs from soil by AEM.....	47
3.3.3	<i>In situ</i> Root Exudate Sampling in Rhizotrons.....	49
3.3.3.1	Morphology of <i>Pinus radiata</i> roots in rhizotron units.....	49
3.3.3.2	<i>In situ</i> sampling of exudates in rhizotron units.....	50
3.3.4	Analysis of Variability in Soil Microbial Communities.....	53
3.3.4.1	Variability of microbial communities between rhizotron units.....	53
3.3.4.2	Horizontal variability of microbial communities within rhizotron units	55
3.3.4.3	Vertical variability of microbial communities within rhizotron units..	56
3.4	Discussion.....	58
3.4.1	Development of <i>in situ</i> Method for Collection of OAs.....	58
3.4.1.1	Analysis of OAs by HPLC.....	58
3.4.1.2	Suitability of AEM for collection of OAs.....	59
3.4.1.3	<i>In situ</i> collection of OAs from root-rhizosphere and non-rhizosphere soils.....	61
3.4.2	Variability in Soil Microbial Communities.....	64

3.5	Conclusions.....	69
Chapter 4	Rhizosphere Properties of Genetically Modified and Wild Type Radiata Pine	70
4.1	Introduction.....	70
4.2	Materials and Methods	72
4.2.1	Soil Preparation and Rhizotron Establishment.....	72
4.2.2	Plants and Growing Conditions.....	73
4.2.3	Bulk Soil Bag Preparation and Installation	73
4.2.4	Rhizotron Sampling on Three Occasions.....	74
4.2.4.1	First sampling after three months growth.....	75
4.2.4.2	Second sampling after nine months growth.....	76
4.2.4.3	Third sampling after 10 months growth.....	80
4.2.5	Extraction and Analysis of Water-soluble Exudate Solutions.....	81
4.2.6	Soil Preparation and Microbial Community Analysis.....	82
4.2.7	Statistical Analysis	82
4.3	Results	84
4.3.1	Root Morphology	84
4.3.2	Soil Microbial Community Analyses.....	84
4.3.2.1	Microbial communities in different soil samples.....	84
4.3.2.2	Structure of microbial communities associated with pine tree lines	85
4.3.2.3	Diversity of microbial communities associated with pine tree lines	87
4.3.3	Organic Anions in Root Exudates Collected <i>in situ</i>	88
4.3.3.1	Identification of organic anions	88
4.3.3.2	Organic anion profiles	91
4.3.4	Organic Anions in Water-soluble Exudate Solutions.....	92
4.3.4.1	Identification of organic anions	92
4.3.4.2	Organic anion profiles	98
4.4	Discussion.....	100
4.4.1	Experimental Design.....	100
4.4.2	Soil Microbial Communities.....	101
4.4.2.1	Microbial communities associated with GM and control trees.....	101
4.4.2.2	Variations in DGGE profiles between replicates	105
4.4.3	Organic Anions in Exudates.....	106
4.4.4	Linking the Exudates with Microbial Communities	111
4.4.5	Microbial Communities Associated with GM and WT Pine Trees	112
4.5	Conclusions.....	113
Chapter 5	Influence of Root Exudate Components on Soil Microbial Communities.....	115
5.1	Introduction.....	115
5.2	Materials and Methods	117
5.2.1	Artificial Root Exudate Solution.....	117
5.2.2	Soil Microcosms.....	118
5.2.3	Soil Moisture and pH	119

5.2.4	Soil Dehydrogenase Activity Analysis	119
5.2.5	Molecular Analysis of Soil Microbial Communities	120
5.2.5.1	rRNA-DGGE microbial community analysis	120
5.2.5.2	16S rRNA phylochip microbial community analysis	120
5.2.6	Statistical Analysis	123
5.2.6.1	Phylochip analysis of soil bacterial community structures	123
5.2.6.2	Dynamic taxa in bacterial communities	125
5.2.6.3	Bacterial abundance (HybScore) analysis	125
5.3	Results	125
5.3.1	Soil Moisture and pH	125
5.3.2	Soil Dehydrogenase Activity	126
5.3.3	DGGE Analysis of Soil Microbial Communities	126
5.3.4	Phylochip Analysis of Soil Bacterial Communities	129
5.3.4.1	Bacterial community structures	129
5.3.4.2	Dynamic bacterial taxa response to ARE solutions	134
5.3.4.3	Bacterial abundance (HybScore) response to ARE solutions	139
5.4	Discussion	141
5.4.1	Impact of ARE Solutions on Soil Microbial Activity and Diversity	141
5.4.2	Comparison effects of OAs and Sugars on Bacterial Communities	145
5.4.3	Impact of Exudate Compounds on Specific Bacterial taxa	146
5.5	Conclusions	149
Chapter 6	General Discussion and Future Research	150
6.1	General Discussion	150
6.1.1	Rhizotron System and <i>in situ</i> Sampling Technique	150
6.1.2	Characterisation of Soil Microbial Communities and Root Exudates Associated with GM and WT Radiata Pine	152
6.1.3	Impact of Root exudate Components on Soil Microbial Communities	152
6.1.4	Organic Anions and Microbial Communities	155
6.2	Future Research	155
References	157
Appendix 2.1	<i>Pinus radiata</i> Grown in Pot	179
Appendix 2.2	Selection of Nucleic Acid Extraction Method and Lysing Matrix Tubes	180
Appendix 2.3	Solutions and Reagent Preparation	185
Appendix 2.4	Solution Preparation for DGGE and Silver Staining of Gels	186
Appendix 2.5	rRNA-DGGE Gels	187
Appendix 3.1	Detection and Selection of Compounds for Radiata Pine Root Exudates Analysis	190
Appendix 3.2	Optimization of HPLC Method for Analysing Low Molecular Weight OAs in Root Exudates of Radiata Pine	195
Appendix 3.3	Comparison of Efficiency of OA Recovery Using Filter Paper and Anion Exchange Membrane	199

Appendix 3.4	Chemical and Properties Analysis of the Soil Used in the Rhizotrons in Chapter 3	202
Appendix 3.5	Creation of the Horizontal Access Portals in a Rhizotron	203
Appendix 3.6	Causes of the Void Peak at the HPLC Chromatogram and Possible Solutions	204
Appendix 3.7	Comparison of Rhizosphere Bacterial Communities Associated with Different Root Types of Radiata Pine	208
Appendix 4.1	Measurement of Npt II protein in root samples	211
Appendix 4.2	Chemical and Properties Analysis of the Soil Used in the Rhizotron Experiments in Chapter 4	212
Appendix 4.3	Radiata Pine Exude Different Amounts of Organic Anions into the Rhizosphere at Various Periods within a Day	213
Appendix 4.4	Causes and Possible Solutions of the Errors in Calculation of Root Area using software	219
Appendix 4.5	Shannon Diversity Indices in Soil Microbial Communities Associated with Pine Trees	223
Appendix 4.6	ANOVA Analysis of Individual OAs between Water-soluble Exudate Solutions from Root-rhizosphere and Non-rhizosphere soils	225
Appendix 4.7	Soil pH and Moisture in Non-rhizosphere Soil from the 1 st and 3 rd Samplings in Chapter 4	226
Appendix 4.8	Representative DGGE Gel of Basidiomycete Communities in the Rhizosphere and Non-rhizosphere Soil Samples Associated with GM and WT Radiata Pines at the 3 rd Sampling	227
Appendix 4.9	Representative DGGE Gel of <i>gacA</i> Gene in <i>Pseudomonas</i> in the Rhizosphere and Non-rhizosphere Soil Samples Associated with GM and WT Radiata Pines at the 1 st Sampling	228
Appendix 5.1	HPLC Analysis of a Root Exudate Sample of Radiata Pine Seedling Collected by CaCl ₂ Solution as Described in Appendix 3.1	229
Appendix 5.2	Archaeal 16S PCR	230

List of Tables

Table 1.1	Organic compounds and enzymes released by plants in root exudates and their function in the rhizosphere. Modified from Faure et al. (2009).	3
Table 1.2	Influence of environmental factors of root exudation. Modified from Koo et al. (2005).	5
Table 1.3	Activities of microorganisms in the rhizosphere. Taken from Kennedy and de Luna (2005).	7
Table 2.1	Sequences of the primers used in this project.	26
Table 2.2	Details of the DGGE gradient and running conditions used for separation of the different ribosomal RNA target.	29
Table 3.1	Calibration standards for 12 OAs, limit of detection and recovery of anions from spiked root exudate samples using the HPLC analysis method. The recovery from spiked soils are expressed as mean percent recovery ($n=3$) \pm 1 standard error.	42
Table 3.2	Quantities of OAs and their frequency of detection from roots and rhizosphere soils collected <i>in situ</i> from <i>Pinus radiata</i> grown in rhizotron units and from adjacent non-rhizosphere soils. Values are presented as mean \pm 1 standard error for samples ($n=24$ for rhizosphere soil, $n=12$ for non-rhizosphere soil) where positive detection (i.e. greater than limit of detection; Table 3.1) occurred.	51
Table 3.3	Hotelling T^2 probabilities of the level of difference between the communities associated with rhizosphere soils from two rhizotron units A and B and non-rhizosphere soils, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) are shown in bold.	53
Table 3.4	Hotelling T^2 probabilities of the level of difference between the general bacterial 16S communities associated with rhizosphere soils from three different sites and non-rhizosphere soils, with each sample under the null hypothesis that all soil samples have the same mean. The probability values with significant difference ($P<0.05$) are shown in bold.	56
Table 3.5	Hotelling T^2 probabilities of the level of difference between various taxonomic communities associated with rhizosphere soils from various depths and non-rhizosphere soils, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) are shown in bold.	57
Table 4.1	Hotelling T^2 probabilities of the level of difference between microbial communities in rhizosphere (RS) and non-rhizosphere (N-RS) or bulk (BS) soil samples collected at various depths on three sampling periods, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two soil categories are shown in bold.	85
Table 4.2	Hotelling T^2 probabilities of the level of difference between microbial communities in non-rhizosphere (N-RS) and bulk (BS) soil samples collected at various depths on the third sampling, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two soil categories are shown in bold.	85
Table 4.3	Hotelling T^2 probabilities of the level of difference between various microbial communities associated with GM and WT tree lines with soil samples collected at various depths on the a). first sampling period ($n=8$), b). second sampling period ($n=8$), c). third sampling period ($n=4$), with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two tree lines are shown in bold.	86
Table 4.4	Shannon diversity indices of soil microbial communities associated with GM and WT pine trees collected at various depths on the first sampling period ($n=8$), and the P -values of ANOVA analysis across treatments. Values are presented as the mean \pm 1 standard error for samples in each treatment. The probability values with significant difference ($P<0.05$) between treatments are shown in bold. For each row, different letters indicate significant difference ($P<0.05$) between the treatment means.	87

Table 4.5	The number of exudate samples collected by AEM where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table 3.1) in four treatments (GM 10 cm, WT 10 cm, GM 20 cm, WT 20 cm) ($n=8$) and in total ($n=32$).	89
Table 4.6	Total C of OAs in exudate samples of GM and WT pine trees collected by AEM ($\mu\text{g C cm}^{-2}$ root) during the second sampling, and the probability values across the treatments analysed by ANOVA. The amounts are presented as the mean \pm 1 standard error for samples from different treatments ($n=8$).	91
Table 4.7	Hotelling T^2 probabilities of the level of difference between the structure of OA profiles in the exudate samples associated with GM and WT tree lines collected by AEM at the second sampling ($n=8$), with each sample under the null hypothesis that all the samples have the same mean.	91
Table 4.8	Shannon diversity indices of OA profiles in the exudate samples collected by AEM during the second sampling, and the probability values across the treatments analysed by ANOVA. Diversity indices are presented as the mean \pm 1 standard error for samples across the treatments ($n=8$).	92
Table 4.9	The number of water-soluble root-rhizosphere exudate solutions collected at three sampling periods where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table 3.1) in each treatment ($n=8$ for the first and second samplings, $n=4$ for the third sampling) and in total ($n=32$ for the first and second samplings, $n=24$ for the third sampling).	93
Table 4.10	The number of water-soluble exudate solutions from non-rhizosphere soils collected at the second sampling where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table 3.1) in each treatment ($n=8$) and in total ($n=32$).	97
Table 4.11	Total C of OAs in water-soluble root-rhizosphere exudate solutions ($\mu\text{g C g}^{-1}$ root-soil) collected at all three samplings, and the probability values across the treatments in each sampling period analysed by ANOVA. The amounts are presented as the mean \pm 1 standard error for samples at different treatments ($n=8$ for the first and second samplings, $n=4$ for the third sampling).	99
Table 4.12	Organic anions selected for the analysis of structure of OA profiles in water-soluble root exudate solutions at three samplings.	99
Table 4.13	Hotelling T^2 probabilities of the level of difference between the structure of OA profiles in the water-soluble root-rhizosphere exudate samples from GM and WT pine trees at all three sampling periods ($n=8$ for the first and second sampling, $n=4$ for the third sampling), with each sample under the null hypothesis that all the samples have the same mean.	99
Table 4.14	Shannon diversity indices of OA profiles in the water-soluble root-rhizosphere exudate samples at all three samplings, and the probability values across the treatments analysed by ANOVA. Diversity indices are presented as the mean \pm 1 standard error ($n=8$ for the first and second samplings, $n=4$ for the third sampling). The probability values with significant difference ($P<0.05$) across the treatments are shown in bold. Different letters indicate significant difference ($P<0.05$) between the treatment means.	99
Table 5.1	Composition and concentration of sugars and organic anions in ARE solutions.	117
Table 5.2	List of all the bacterial phyla and the number of taxa in each bacterial phylum available for hybridization in 16S rRNA Genephip (Affymetrix).	122
Table 5.3	Soil moisture and pH in microcosms amended daily with either DI water (Con) or various ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. Values are presented as the mean \pm 1 standard error ($n=4$). Different letters indicated significant difference ($P<0.05$) between treatments.	126
Table 5.4	Hotelling T^2 probabilities of the level of difference between microbial communities in soils amended with either DI water or ARE solutions, with each sample under the null hypothesis that all soil samples have the same mean ($n=4$ for bacterial 16S, $n=3$ for fungal 18S). The probability values where means were significantly different ($P<0.05$) are shown in bold.	127

Table 5.5 Numbers of bacterial taxa in each of the phylogenetic groups found in the dynamic communities regulated by ARE solutions or compounds (S = sugars, Q = quinate, L= lactate, M= maleate). The influence of different ARE solutions were determined by comparing individual treatments to treatment Con, and the impact of OAs (individual and a mixture) were determined by comparing treatment SQ, SL, SM and SQLM to treatment S. Values indicate the number of taxa that responded positively to the presence of ARE solutions, whilst these in brackets indicate the number of taxa which responded negatively to the presence of ARE solutions or compounds. Total detected taxa indicate the number of the total detected taxa in each phylogenetic group across all six treatments. Taxa are shown mainly to phylum level, but where appropriate, some of the phyla are divided into class (indicated by *** and highlighted in light blue) or order (indicated by ## and highlighted in yellow) level. 136

Table 5.6 Relative increases (fold) in the abundance of main phylogenetic groups detected by phylochips. The significant changes ($P < 0.05$) in the abundance of phylogenetic group taxa between treatments are shown in bold. + indicates the phylogenetic groups which became detectable in the treatments but not in the control (either treatment Con or S); - indicate the phylogenetic groups that became undetectable in the treatments while detected in the control (either treatment Con or treatment S). nc indicates no significant change ($P < 0.05$) in the presence or absence of a phylogenetic group. Proteobacteria phylum is divided into class (indicated by *** and highlighted). 141

List of Figures

Figure 1.1	The rhizosphere trinity. Taken from Lynch (1990).....	1
Figure 2.1	The processing procedure of rhizosphere soil preparation from root samples.	24
Figure 3.1	a). Rhizotron system in the New Zealand Biotron facility. Left: cross section view of a growth room positioned above a rhizotron room. Right: front view of a growth room positioned above a rhizotron room. b). Stainless steel rhizotron cylinder in the downstairs rhizotron room. c). <i>Pinus radiata</i> in the upstairs growth room after one year growth. d). Packing the soil into the rhizotron which has been divided into two halves.....	37
Figure 3.2	a). The soil corer used for creating access portals in the rhizotron. b). The physical integrity of the access portal is maintained using a removable inflatable tube between samplings. c). The sealed motorised camera system for image and position recording, with a close up of a motorised camera inside the perspex tube. d). The access portal after removing the inflatable tube for root monitoring or sampling. e). The camera system recording the root image in the access portal. f). AEM strips backed with Whatman 3MM (indicated by blue arrows) fixed on an inflatable tube for root exudates collection in the access portal.....	39
Figure 3.3	Vertical sampling in rhizotron units A and B and their position inside the growth room. Green circle represents radiata pine tree. Yellow circle represents sampling sites. Black line indicates the side of the growth room (5 x 2.4 m). Site L was about 25 cm away from the tree base, site M was about 16 cm away from the tree base, and site R was about 28 cm away from the tree base. Three depths (0-15 cm, 16-30 cm and 31-45 cm) were sampled at each site. .41	
Figure 3.4	Separation of 12 OA standards by isocratic HPLC with 25 mM KH ₂ PO ₄ (pH 2.5) at 0.6 ml min ⁻¹ at 40°C. The concentration of anions shown in this chromatogram were: 30 mg l ⁻¹ for tartarate, quinate, formate and succinate, 20 mg l ⁻¹ for acetate, 10 mg l ⁻¹ for malate, malonate, shikimate, lactate and citrate, 0.5 mg l ⁻¹ for fumarate and 0.3 mg l ⁻¹ for maleate.....	43
Figure 3.5	Recovery of different OAs from AEM. Shown is the percent recovery (%) of individual anions and the average total amount of anions recovered after loading AEM strips with 2 rates (250 µl and 500 µl) of an OA mixture (500 mg l ⁻¹ of each anion). Error bars show 1 standard error and for each OA, columns denoted with different letters are significantly different (P<0.05) between the two loadings (n=3).	44
Figure 3.6	Effect of elution time on recovery of OAs from AEM. Shown is the percent recovery (%) of individual OAs and the average total amount of anions recovered after eluting membranes for 1 to 4 h that were loaded with 500 µl of an OA mixture (500 mg l ⁻¹ of each anion). Error bars show 1 standard error and for each organic anion, columns denoted with different letters are significantly different (P<0.05) across elution times (n=3).	45
Figure 3.7	Capture of OAs from solution by AEM. Shown are the amounts of OAs collected (µg cm ²) on AEM strips (24 cm ²) after 1 or 3 h from 20 ml of an OA mixture containing each anion at a concentration of 30 mg l ⁻¹ . Error bars show 1 standard error and for each OA, columns denoted with a different letter are significantly different (P<0.05) between the 2 collection periods (n=3).	46
Figure 3.8	The stability and recovery of OAs loaded on AEM when incubated in soil. Shown is the percent recovery (%) of individual anions and the average total amounts of anions recovered after loading membranes with 500 µl of an OA mixture (500 mg l ⁻¹ of each anion). AEM were incubated for 3 h with or without soil either a). alone or backed with moist b). Whatman 3MM or c). GB/F filter papers. Error bars show 1 standard error and for each anion in each panel, columns denoted with different letters are significantly different (P<0.05) between the soil (n=3) and no-soil incubations (n=2).	48
Figure 3.9	Capture of OAs from soil by AEM. Shown is the amount of each anion (µg cm ²) collected on AEM strips (24 cm ²) over 3 h of incubation in soil (60 g) containing a mixture of OAs supplied at a concentration of 67.1 µg of each anion per g soil. Anion exchange membrane strips were incubated with the soil alone or were backed with either moist Whatman 3MM or GB/F filter papers. Error bars show 1 standard error and for each organic anion, columns denoted with a different letter are significantly different (P<0.05) between collection methods (n=3).	49

Figure 3.10 Representative images of *Pinus radiata* root morphology observed using the camera system in the access portals in the rhizotron units. a). A root colonized by ECM with the mantle of ECM clearly seen. b). A lateral root colonized by ECM with a different morphology from that in a. Mycelia of ECM spread around the root. c). Fungal hypha, near an ECM colonized root, was observed to connect two roots. d). Roots colonized by ECM with mycelia spreading into the surrounding soil. e). Root partly covered with fungal spores which is indicated by green arrow. f). Young roots with abundant root hairs and presence of several lateral roots. g). Mature root with several lateral roots covered with root hairs. h). An example of a relatively thick and old root in radiata pine root systems. i). Rhizotron wall which is 5 mm thick (as marked by blue line with arrows). The red arrows indicate the ECM in images; yellow arrows indicate the mycelia of ECM or fungi; and pink arrows indicate the root hairs on the roots.....50

Figure 3.11 a). An example of a root exudate sample which was collected *in situ* by AEM from pine roots and analysed by HPLC. Peaks 1 to 5 and peak 9 were identified by comparing to known standards; peaks 6 to 8 were unidentified. b). An example of an exudate sample which was collected *in situ* by AEM from the non-rhizosphere soil where radiata pine were grown and analysed by HPLC.....52

Figure 3.12 An example of rRNA-DGGE gel with samples collected from two rhizotron units A and B, with each rhizotron including samples from the three sites and three depths and composite non-rhizosphere soil. Samples were loaded on DGGE in a random order. Only one replicate from each sampling site were included in this gel. St: bacterial DGGE marker consisted of bulked bacterial 16S PCR products from strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp.. The red letters indicate the samples origin: A: rhizotron unit A; B: rhizotron unit B; nR: non-rhizosphere soil. The black letters indicate the sites where rhizosphere soils were collected from: L: site L; M: site M; R: site R. The pink letters indicate the depth where rhizosphere soils from: d1: 0-15 cm, d2: 16-30 cm, d3: 31-45 cm.....54

Figure 3.13 Principal coordinates analysis of active bacterial communities from the rhizosphere soil from two rhizotron units A and B and non-rhizosphere soil (labelled as "Bulk" in graphs) amplified with general bacterial and taxon-specific primers. Samples from different depths and different sites but the same rhizotrons were composited for analysis. Open circles represent 95% confidence regions for means of samples ($n=18$ for rhizosphere soil for bacterial community, $n=6$ for rhizosphere soil for taxon-specific communities, $n=2$ for non-rhizosphere soil) which are shown by the solid circles. a). general bacterial 16S; b). α -proteobacterial 16S; c). β -proteobacterial 16S; d). pseudomonad 16S. Significance of differences between treatments are indicated in Table 3.3.....55

Figure 3.14 Principal coordinates analysis of active bacterial 16S communities from rhizosphere soil associated with roots sampled from three different sites (L, M and R) and non-rhizosphere soil (labelled as "Bulk" in the graph) within the rhizotron. Open circles represent 95% confidence regions for means of samples ($n=12$ for rhizosphere soil, $n=2$ for non-rhizosphere soil) which are shown by the solid circles. Significance of differences between treatments are indicated in Table 3.4.....56

Figure 3.15 Principal coordinate analysis of active bacterial communities from the rhizosphere soil from three different depths (0-15 cm, 16-30 cm and 31-45 cm) and non-rhizosphere soil (labelled as "Bulk" in graphs) amplified with general bacterial and taxon-specific primers. Open circles represent 95% confidence regions for means of samples ($n=12$ for rhizosphere soil for general bacterial community, $n=4$ for rhizosphere soil for taxon-specific communities, $n=2$ for non-rhizosphere soil) which are shown by solid circles. a). general bacterial 16S; b). α -proteobacterial 16S; c). β -proteobacterial 16S; d). pseudomonad 16S. Significance of differences between treatments are indicated in Table 3.5.58

Figure 4.1 a). Sketch of one rhizotron cylinder which was split into two independent rhizotron units. Brown cycles represent the bulk soil bags filled with horizon A soil and located at two corners in each rhizotron unit. Green circles represent radiata pine trees planted in the middle of each rhizotron unit. One GM and one WT tree seedlings were planted adjacent to each other in separated rhizotron units. b). Pine trees after one month growth in a rhizotron. The tree on the left was a GM pine and the one on the right was a WT pine. Red arrows indicate tags of the buried bulk soil bags at corners in the rhizotron unit. c). Bulk soil bag filled with horizon A soil core which was sampled from a corner of a rhizotron unit.....74

Figure 4.2 a). The endoscope system. b). The camera lens (45 cm long, 1 cm diameter). c). Long armed scissors and forceps (approximate 45 cm long) used during the second sampling. d). Specially designed endoscope lens-rhizotron adapter that can hold the lens in the access portal and allow the lens be able to rotate 360° and move along the access portal.77

Figure 4.3 a). Placing the AEM-Whatman 3MM strip on top of roots for OA collection using long armed forceps under the endoscope light source. b). Checking the AEM-Whatman 3MM strip after being placed on top of roots for OA collection using the endoscope camera system. c). Removing AEM-Whatman 3MM from the root/soil samples and sampling root samples with long arm scissors. d). A specially designed soil sampler for non-rhizosphere soil sampling in access portals.....78

Figure 4.4 a). *In situ* collection of OAs using AEM-Whatman 3MM from roots of radiata pine in the access portal. b). The root area where exudates was sampled from after removal of the AEM-Whatman 3MM strip. c). Roots from where exudates were collected were sampled from the access portal and used for further analyses. d). Calculation of the root area directly in contact with AEM strip using digital imagery designed software. The green area show the roots covered by the AEM strip in figure a. by overlaying images a. and b.79

Figure 4.5 a). Sketch of the third sampling strategy. The green circle represents a radiata pine tree. The light brown circle represents a sampling site which was about 20 cm away from the tree base. Three depths (D1=0-14 cm, D2=14-28 cm and D3=28-45 cm) were sampled at each site. The dark brown circles represent the bulk soil bags at two corners in each rhizotron unit. Soil in the bag was split representing two depths (D1=0-14 cm, D2=14-28 cm). b). Removal of the bulk soil bag from each corner of the rhizotron unit.81

Figure 4.6 Images of radiata pine roots observed using the endoscope camera system in access portals in rhizotrons. a). A root with its root tip (indicated by an arrow). b). A root with several branching lateral root tips (indicated by arrows). c). A root colonized by ECM (indicated by arrows) with the ECM mantle clearly seen. d). Several roots were colonized by ECM (indicated by black arrows) and the mycelia of ECM or saprophytic fungi (indicated by a pink arrow) were seen to proliferate in the soil. e). Numerous springtails (indicated by a black arrow) were seen near two roots with some unidentified white matter (indicated by an orange arrow). f). Large amount of fungal hypha (indicated by the pink arrow) spread around the wall of the access portal with several roots colonized by ECM (indicated by black arrows).84

Figure 4.7 The concentrations of OAs collected *in situ* by AEM from root-rhizosphere soils during the second sampling, and respective probability values analysed by ANOVA across the four treatments. Error bars show 1 standard error ($n=8$), and the P -values with significant difference across the treatments ($P<0.05$) are shown in bold. Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph.89

Figure 4.8 The concentrations of OAs collected *in situ* by AEM from non-rhizosphere soils during the second sampling, and respective probability values analysed by ANOVA across the four treatments. Error bars show 1 standard error ($n=8$), and the P -values with significant difference across the treatments ($P<0.05$) are shown in bold. "-" indicates that no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates collected by AEM. Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph.90

Figure 4.9 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the first sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph. "-" indicates no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.94

Figure 4.10 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the second sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown within the graph. The P -values with significant difference across treatments ($P<0.05$) are shown as an insert in bold. "-" indicates no P -value was applicable for

some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.	95
Figure 4.11 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the third sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=4$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph. D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm. "-" indicates no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.	96
Figure 4.12 The concentrations of OAs detected in water-soluble exudate solutions extracted from non-rhizosphere soil collected at the second sampling. The levels of those OAs under detection limits were specified as half the limits of detection of the HPLC method (Chapter 3, Table 3.1). Error bars show one standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the figure. ...	97
Figure 5.1 Soil microcosms used to study the impact of ARE solutions on soil microbial community.	119
Figure 5.2 Dehydrogenase activity in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days in microcosms. Error bars show 1 standard error in each treatment, columns denoted with a different letter are significantly different ($P<0.05$) between treatments ($n=4$).	127
Figure 5.3 rRNA-DGGE profiles of bacteria in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. St: bacterial 16S DGGE marker consisted of bulked bacterial 16S PCR products from strains <i>Pectobacterium carotovorum</i> , <i>Variovorax paradoxus</i> and <i>Arthrobacter</i> sp.	128
Figure 5.4 Fungal 18S rRNA-DGGE profiles in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. St: Fungal 18S DGGE marker consisted of bulked fungal 18S PCR products from strains <i>Umbelopsis ramanniana</i> , <i>Trichoderma reesei</i> , <i>Mortierella chlamyospora</i> , <i>Phoma cucurbitacearum</i> , <i>Clonostachys rosea</i> . Two bands that appeared in treatment SQ but not in treatment S are indicated by blue arrows. One band that appeared only in treatment SQLM and not in treatment SQ is indicated by a red arrow.	129
Figure 5.5 Number of bacterial taxa from various phyla detected in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochip analysis. Taxa from different phyla are represented by different colours. A taxon was considered to be present if it occurred in two out of three replicates for each treatment. The number above each bar indicates the number of phyla detected in each treatment.	130
Figure 5.6 Bacterial rank abundance curves of communities in soil amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochips analysis.	132
Figure 5.7 Hierarchical cluster dendrogram generated based on rank similarity of bacterial communities in soils amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochip analysis.	132
Figure 5.8 Non-metric MDS ordination plots of bacterial communities in soils amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data obtained from phylochip analysis ($n=3$). a). Ordination of samples from all six treatments. b). Ordination of samples from four treatments SQLM, SQ, SL, SM.	134

Abbreviations

AEM	anion exchange membrane
Al	aluminium
ANOVA	analysis of variance
ANOSIM	analysis of similarity
ARE	artificial root exudates
BSA	bovine serum albumin
Bt	<i>Bacillus thuringiensis</i>
C	carbon
cDNA	complementary DNA
CTAB	hexadecyltrimethylammonium bromide
DGGE	denaturing gradient gel electrophoresis
DI water	deionised water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	ectomycorrhizae
GC-MS	gas chromatography-mass spectrometry
GM	genetically modified
HybScore	hybridization intensity score
ITS	internal transcribed spacer
LSD	least significant differences
MDS	multi-dimensional scaling
MM	mismatch
neMDH	nodule enhanced malate dehydrogenase
NptII	neomycin phosphotransferase II
OA	organic anion
OAs	organic anions

P	phosphorus
PCR	polymerase chain reaction
PCoA	principal coordinates analysis
PEG	polyethylene glycol
pf	positive fraction
PM	perfect match
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SOM	soil organic matter
TAE	Tris-acetate-ethylenediamine tetraacetic acid
T-RFLP	terminal restriction fragment length polymorphism
TTC	triphenyltetrazolium
WT	wild type

Chapter 1 Introduction

1.1 General Introduction

1.1.1 The Rhizosphere

The term “rhizosphere” was first introduced by Hiltner in 1904 to describe the soil zone surrounding legume roots with intensive bacterial activity. More recently, this term has been broadened to describe the soil zone immediately adjacent to plant roots and influenced by root activities (Darrah 1993). With the influence of root growth and activities (such as water and nutrient uptake, respiration and rhizodeposition), a range of biological, biochemical, chemical and physical properties in the surrounding soil changes, resulting in a very different environment in the rhizosphere from that in bulk soil (Bais et al. 2006; Hinsinger et al. 2005). The so called “rhizosphere effect” is the stimulation of soil microbial populations in the rhizosphere soil owing to the release of root exudates by plants. This effect has been recognized for a long time (Lynch 1987). Rhizosphere is the site of interface between soil, plant roots and soil microorganisms which can all influence the environment and processes (Figure 1.1) (Lynch 1990).

Figure 1.1 The rhizosphere trinity. Taken from Lynch (1990).

Although clear in the definition, the physical extent of the rhizosphere soil is not easily defined because of the complex root-soil interface. In some cases, there is no distinct boundary between the rhizosphere and the bulk soil due to the extension of hyphae of root colonized fungi into the soil (Gobat et al. 2004). But in most cases, rhizosphere soil is

considered to extend only a few millimeters from the root surface (Bertin et al. 2003; Gregory 2006). Although very narrow, the rhizosphere can be further divided into three zones: the endorhizosphere (the various cell layers of the root tissue including the endodermis and cortical layers colonized or potentially colonized by microorganisms); the rhizoplane (the root surface with the epidermis and mucilaginous polysaccharide layers colonized by microorganisms); and the ectorhizosphere (the area surrounding the root which is inhabited by microorganisms) (Lynch 1987; Morgan et al. 2005). As most terrestrial plant roots are colonized by mycorrhizal fungi (mycorrhizosphere zone), the region of soil inhabited and influenced by mycorrhizal roots and mycelia is also commonly present in the rhizosphere (Linderman 1988; Molina et al. 1992).

1.1.2 Root Exudates

Living roots exude a wide range of compounds into the rhizosphere soil. These compounds can be classified into two groups based on their subsequent utilization as microbial substrates, namely low molecular weight organic compounds (such as sugars, amino acids, organic anions (OAs), phenolics and various other secondary metabolites) that can be readily assimilated by soil microorganisms, and high molecular weight organic exudates (such as proteins, pigments, mucilage and miscellaneous other substances) that require extracellular enzymic activity to break them down before they can be assimilated (Meharg 1994). In addition, inorganic compounds (e.g. inorganic ions, H^+ , water and electrons) are also released by plant roots into rhizosphere soils (Bertin et al. 2003). Low molecular weight organic compounds account for the majority of the compounds exuded by plant roots (Bais et al. 2006; Bertin et al. 2003). In this thesis, the term organic anions was used instead of “organic acids”, since these compounds are either partially or fully dissociated in most soil conditions (Jones et al. 2003; Ryan et al. 2001).

Since release of root exudates, which are mainly derived from photosynthesis, is a significant carbon (C) cost for plants, root exudates are believed to have important functions in regulation of plant growth (both directly or indirectly), although most of these functions are just beginning to be investigated (Bertin et al. 2003; Walker et al. 2003). Uren (2007) hypothesized that root exudation was involved in the regulation of internal plant metabolic processes, such as respiration and nutrient acquisition. Additionally, root exudates (e.g. phytoalexins) can also be a mechanism of plant defence against soil-borne pathogens and can stimulate or inhibit interactions with other soil organisms (Bais et al. 2004; Bertin et al. 2003; Rengel 2002). Some of the functions of root exudates are summarised in Table 1.1. The readily assimilated C compounds such as low molecular weight sugars and OAs in root exudates provide an energy source for soil microorganisms

Table 1.1 Organic compounds and enzymes released by plants in root exudates and their function in the rhizosphere. Modified from Faure et al. (2009).

Class of compounds	Components	Functions
Carbohydrates	arabinose, glucose, fructose, galactose, maltose, raffinose, rhamnose, ribose, sucrose, xylose	lubrication, protection of plants against toxin, microbial growth stimulation
Amino acids and amides	all 20 proteinogenic amino acids, aminobutyric acid, homoserine, cystathionine, mugineic acid phytosiderophores	inhibit nematodes and root growth of different plant species, microbial growth stimulation, chemoattractants, osmoprotectants, iron scavengers
Aliphatic acids	formic, acetic, butyric, propionic, maleic, malic, citric, isocitric, oxalic, fumaric, malonic, succinic, tartaric, oxaloacetic, pyruvic, oxaloglutaric, glycolic, shikimic, acetonc, valeric, gluconic, quinic	plant growth regulation, chemoattractants, microbial growth stimulation
Aromatic acids	<i>p</i> -hydroxybenzoic, caffeic, <i>p</i> -coumeric, ferulic, gallic, gentisic, protocatechuic, salicylic, sinapic, syringic	plant growth regulation, chemoattractants
Phenolics	flavanol, flavones, acetosyringone, flavanones, anthocyanins, isoflavonoids	plant growth regulation, allelopathic interactions, plant defence, phytoalexins, chemoattractants, initiate legume-rhizobia, arbuscular mycorrhizal and actinorhizal interactions, microbial growth stimulation, stimulate bacterial xenobiotic degradation
Fatty acids	linoleic, linolenic, oleic, palmitic, stearic acid	plant growth regulation
Vitamins	<i>p</i> -aminobenzoic acid, biotin, choline, <i>n</i> -methionylnicotinic acid, niacin, panthothenate, pyridoxine, riboflavin, thiamine	microbial growth stimulation
Sterols	campesterol, cholesterol, sitosterol, stigmasterol	plant growth regulation
Enzymes and proteins	amylase, invertase, phosphatase, polygalacturonase, protease, hydrolase, lectin	plant defence, Nod factor degradation
Hormones	auxin, ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid, putrescine, jasmonate, salicylic acid	plant growth regulation
Miscellaneous	unidentified acyl homoserine lactone mimics, saponin, scopoletin, reactive oxygen species, nucleotides, calystegine, trigonelline, xanthone, strigolactones	quorum quenching, plant growth regulation, plant defence, microbial attachment, microbial growth stimulation, initiate arbuscular mycorrhizal interactions

and greatly stimulate their growth and activities (Lynch and Whipps 1990). In addition, exudates may act as primers for the degradation of existing soil organic matter (SOM) (Dormaar 1990). Mucilage released by plant roots can help the permeation of roots through soil, improve soil aggregate structure and maintain hydraulic conductivity between roots and soil (Uren 2007). Some compounds such as malate in root exudates are important in the chelating of cations which may be phytotoxic to plant growth (e.g. Al^{3+}) (Delhaize et al. 1993; Rengel 2002; Ryan et al. 2001). Organic exudates, especially OAs, can influence nutrient availability (e.g. iron, phosphorus (P)) in the rhizosphere and assist in nutrient uptake by plants (Dinkelaker et al. 1997; Jones and Darrah 1995; Jones et al. 1996a; Lipton et al. 1987; Rengel et al. 1998a). Allelochemicals in the exudates can benefit host plants in competition with neighboring plants (Bais et al. 2004; Bertin et al. 2003; Uren 2007).

Many factors can affect the quantity and/or composition of root exudates (Grayston et al. 1996; Neumann and Romheld 2007). Some of these factors are summarised in Table 1.2. Root exudates can vary both in quantity and quality between plant species (Curl and Truelove 1986; Rovira 1959; Rovira and Davey 1974). Gransee and Wittenmayer (2000) reported that maize plants released higher amounts of carboxylic acids and less sugars than pea plants. Grayston et al. (1996) compared root exudate composition from several tree species and found considerable variations between tree species, even for closely related species of pine trees. Plant growth stage can also influence root exudates (Hamlen et al. 1972; Juo and Stotzky 1970; Leyval and Berthelin 1993; Singh and Mukerji 2006). For instance, Keith et al. (1986) measured the relative amount of ^{14}C -labelled photosynthate released from wheat roots to the rhizosphere during different developmental stages when wheat plants were pulse-labelled with $^{14}\text{CO}_2$ and grown in the field. At the seedling stage, 8% of ^{14}C was released from roots to the soil, while at the growth stage the percentage went down to 5% and at the flowering stage, only 1% of ^{14}C was released by roots. Gransee and Wittenmayer (2000) also reported that younger (4 leaf stage) maize plants exuded considerably higher amounts of ^{14}C -labelled organic substances per g root dry matter than older ones (6 and 8 leaf stages). The release of exudates is not homogenous along the roots in many cases (Bringhurst et al. 2001; Mathesius et al. 2000). Jaeger et al. (1999) demonstrated that the efflux of tryptophan was associated with branched roots of *Avena barbata*, while sucrose was released around the apical region of primary roots of *A. barbata*.

The amount and composition of root exudates released by plants are also strongly affected by the physicochemical environment (e.g. soil pH, moisture, nutrient availability, soil temperature, soil texture or plant growth media) in the surrounding rhizosphere soils

Table 1.2 Influence of environmental factors of root exudation. Modified from Koo et al. (2005).

Factors	Effect	References
Plant species	Different plants and cultivars have different exudate compositions	Brimecombe et al. 2007; Curl and Truelove 1986; Grayston et al. 1996; Leyval and Berthelin 1993
Plant growth stage	Roots in the early growth stage secrete exudates more frequently	Gransee and Wittenmayer 2000; Hale et al. 1978; Smith 1976
Root locations	Different parts of root release different types of exudate compounds	Bowen 1979; Thornton et al. 2004
Light intensity	High light intensity increases exudation	Hodge et al. 1997; Rovira 1959; Smith 1972
Microorganism	Presence of microorganisms increases exudation; however, microorganisms could also utilize exudates	Hamlen et al. 1972; Krupa and Fries 1971; Leyval and Berthelin 1993; Meharg and Killham 1991; Meharg and Killham 1995; Prikryl and Vancure 1980
Nutrient availability	Nutrient deficiencies (e.g., P, iron) increase exudation	Bowen 1969; Hedley et al. 1994; Lambers et al. 2002; Li et al. 1997; Marschner 1992; Neumann and Römheld 1999
Oxygen status	Composition is different under aerobic and anaerobic conditions	Ayers and Thornton 1968; Grineva 1963; Whipps and Lynch 1986
Soil moisture	Relieving drought stress increases exudation	Reid 1974; Rivoal and Hanson 1994
Soil pH	Acidification changes composition of exudates	McDougall 1970; Meharg and Killham 1990
Temperature	High temperature stimulates exudation	Bekkara et al. 1998; Hale et al. 1978; Meharg and Killham 1989; Rovira 1959
Soil texture/plant culturing media	Mechanical impedance changes root morphology, sandy substrate produces greater amounts of exudates	Barber and Gunn 1974; Boeuf-Tremblay et al. 1995; Kamilova et al. 2006; Mucha et al. 2005; Schonwitz and Ziegler 1982
Stress condition (toxic metals)	Stress changes the composition of exudates	Ahonen-Jonnarth et al. 2000; Donnelly et al. 2004; Jones 1998

(Hartmann et al. 2009). For example, the concentration of OAs, especially oxalate, malate and citrate in root exudates of *Pinus sylvestris* significantly increased in soils containing toxic metals such as aluminium (Al) (Ahonen-Jonnarth et al. 2000). Lipton et al. (1987) showed that alfalfa (*Medicago sativa*) released 80% more citrate under P-stress condition than was exuded by a plant receiving a complete nutrient solution. Fan et al. (1997) reported that quantification of all major compounds (OAs, amino acids, mugineic acid,

phytosiderophores) of barley (*Hordeum vulgare*) under moderate iron deficiency revealed a 7-fold increase in total exudation in comparison with plants grown in complete nutrient solution, with 3-epihydroxymugineic acid (one kind of phytosiderophore) comprising approximately 22% of the exudate. As iron deficiency increased, total quantities of exudate per gram of root remained unchanged, but the relative quantity of phytosiderophore increased to approximately 50% of the total exudate in response to severe iron deficiency.

Soil microorganisms also influence root exudation by degrading exudate compounds and secreting their metabolites into the rhizosphere soil simultaneously (Faure et al. 2009). Several researchers have reported that substances, such as sugars, low molecular weight OAs and peptides are released by bacteria (Rózycki and Strzelczyk 1986) and mycorrhizae (Casarin et al. 2003; Cromack et al. 1979; Griffiths et al. 1994; Sun et al. 1999; van Hees et al. 2006). Some exudates secreted by mycorrhizal hyphae (e.g. phytohormones) generate drastic morphological changes in the host plant roots. Fungal auxins and their derivatives, which are commonly produced by ectomycorrhizal fungi in the root zone of *Pinus sylvestris*, have been shown to modulate root morphogenesis during symbiosis development (Martin et al. 2001; Strzelczyk and Pokojaska-Burdziej 1984). The presence of microorganisms could increase exudation by roots by continuous assimilation of exudate compounds in the rhizosphere (an increase in sink strength) (Barber and Lynch 1977) or production of plant hormones to increase root cell permeability and thus increase root exudation (Bowen 1994). The stimulation of exudation in the presence of microorganisms occurs in a diverse range of plants, including crops and trees (Gardner et al. 1983; Leyval and Berthelin 1993; Schonwitz and Ziegler 1982). Unless under sterile conditions, it is impossible to separate the exudate compounds produced by plant roots from those by microorganisms. Therefore, in most studies, including experiments reported in this thesis, root exudates include compounds derived from both plant roots and rhizosphere microorganisms.

That so many factors can influence root exudates indicates that root exudation plays an important role in response to changes of rhizosphere environmental conditions. Shifts in exudation can modify the biochemical and physical properties of the rhizosphere and thus contribute to the root growth and plant survival (Bais et al. 2004). However, understanding of the exact fate of exuded compounds in the rhizosphere and the nature of their interactions in the soil is very limited (Bais et al. 2004).

1.1.3 Plant-microbe Interactions Mediated by Root Exudates

In the rhizosphere, some microbial activities are critical for plant growth (Table 1.3) (Kennedy and de Luna 2005). For instance, microorganisms play important roles in cycling of C and biogeochemical functions such as nitrogen cycling and solubilisation of P (Cheneby et al. 2004; Mounier et al. 2004; Nehl and Knox 2006; Philippot et al. 2009; Richardson et al. 2009). Some beneficial microorganisms have important plant growth promoting functions (e.g. biocontrol, hormone production, bioremediation) (Dobbelaere et al. 2003; Kuiper et al. 2004; Raaijmakers et al. 2009; Richardson et al. 2009). Mycorrhizae are key beneficial microbes, which allow plants to grow efficiently in sub-optimal environments by obtaining nutrients and water and supplying them to plants (Egerton-Warburton et al. 2005). Microorganisms also contribute to the stability of aggregates in soil via the synthesis of new compounds from root exudates and decomposition of SOM (Degens 1997; Lynch 1995). However, interactions between roots and microorganisms can also be negative, as is the case for plants colonized by pathogenic bacteria or fungi and plant growth-inhibiting microorganisms (Bais et al. 2006; Gregory 2006; Raaijmakers et al. 2009).

Table 1.3 Activities of microorganisms in the rhizosphere. Taken from Kennedy and de Luna (2005).

Root exudates are known as one of the most important factors affecting microbial growth in the rhizosphere. In general, microbial populations are several orders of magnitude higher in the rhizosphere than in the bulk soil (Foster et al. 1983; Koo et al. 2005). The rhizosphere microbial community is normally more diverse and active than that in the bulk soil (Smalla et al. 2001). Some exudate compounds serve as growth substances for soil microorganisms already present near the roots, while others may act as signal molecules or chemoattractants to attract nearby microorganisms present in the bulk soil towards roots (Bais et al. 2006; Grayston et al. 1996). It is believed that some bacteria and mycorrhizal fungi can sense the presence of a compatible host through signal molecules in root exudates (see review by Faure et al. (2009)). Sugars and OAs (e.g. glucose, sucrose, fructose, malate, citrate, quinate, shikimate and oxalate) have been shown to attract beneficial bacteria such as *Azospirillum* spp., *Bradyrhizobium* spp. and *Rhizobium* spp. (Heinrich and Hess 1985; Parke et al. 1985; Reinhold et al. 1985; Tully 1988). Exudates from host plants have frequently been reported to stimulate the colonization of plants by mycorrhizae (Becard and Piche 1989; Buée et al. 2000; Giovannetti et al. 1996). For instance, Fries et al. (1989; 1987) reported that abietic acid exuded from roots of seedling of several tree species induced spore germination of the ectomycorrhizal genus *Suillus*. Martin et al. (2001) reported that quercetin in *Eucalyptus* root exudates induced rapid and striking changes in *Pisolithus tinctorius* hyphal morphology, leading to hyperbranching and successful colonization of *Eucalyptus* roots. In addition, studies have shown that root exudate compounds can modulate the expression of several bacterial genes involved in rhizosphere colonisation and competitiveness (Mark et al. 2005; Matilla et al. 2007; Tamasloukht et al. 2007). Conversely, the presence of root exudates can also inhibit some microorganisms in the rhizosphere. For example, rosmarinic acid in the root exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) has been shown to have an antimicrobial activity against an array of soil-borne microorganisms, including the opportunistic plant pathogen *Pseudomonas aeruginosa* (Bais et al. 2002). Formononetin, an isoflavone released from roots of alfalfa plants stressed with CuCl_2 (Maxwell and Phillips 1990), inhibited the germination of *Glomus etunicatum* and *Glomus macrocarpum* (Tsai and Phillips 1991). A comprehensive review of interactions between plant and soil microorganisms through particular root exudate compounds has recently been published by Hartmann et al. (2009).

Although studies have demonstrated a crucial role of chemical communication in establishing highly specialized relationships between plant roots and specific soil microorganisms, the role for root exudates in regulating overall structures of soil microbial community is poorly understood (Biedrzycki and Bais 2009; Broeckling et al. 2008).

Numerous studies have shown that plants have strong effects on rhizosphere microbial community structures (Baudoin et al. 2001; Costa et al. 2006; Kowalchuk et al. 2006; Smalla et al. 2001). Structures of rhizosphere microbial communities differ between plant species (Grayston and Campbell 1996; Grayston et al. 1998; Haichar et al. 2008; Marschner et al. 2005; Miethling et al. 2000), different root locations (Baudoin et al. 2002; Bringham et al. 2001; Jaeger et al. 1999; Watt et al. 2006a), different growth stages (Egerton-Warburton et al. 2005; Gomes et al. 2001; Marschner et al. 2002; von der Weid et al. 2000) and under different nutrient conditions (Yang and Crowley 2000). Since root exudates are believed to be an important C source for microbial growth in the rhizosphere (Lynch and Whipps 1990), the rhizosphere effect is probably linked to root exudates, which also vary with plant species, root locations, growth stages and nutrient conditions (see Section 1.1.2, Table 1.2). Rengel et al. (1998b) showed that wheat genotypes differed in the extent of bacterial colonization of roots because of soil micronutrient status which could influence the quality and quantity of root exudates released by the wheat genotypes. Yang and Crowley (2000) examined the microbial communities in different locations on roots of barley under iron-limited and iron-sufficient growing conditions. The results showed that approximately 20-40% of the total variation in community structures at all of the root locations could be attributed to the plant iron nutritional status which could have resulted in changes in the composition of the root exudates.

To prove that root exudates are a mechanism by which plants regulate their rhizosphere microbial community, *in vitro* microcosm experiments have been conducted by researchers. In these studies selected compounds were applied directly to soils held under a limited set of environmental conditions (e.g. a selected soil moisture content) for a period of time (Baudoin et al. 2003; Benizri et al. 2002; Benizri et al. 2007; Griffiths et al. 1999; Paterson et al. 2007). All these experiments resulted in development of microbial communities different from those present in control soils, implying the strong influence of root exudates on soil microbial communities. However, it is difficult to assess to what extent these exudates could influence the microbial community in the complex rhizosphere environment in the presence of other potential influencing factors, such as nutrient competition between plant roots and rhizosphere soil microbes. Therefore, the *in vitro* observations must be confirmed under more natural conditions.

Recently, Broeckling et al. (2008) demonstrated that the introduction of novel plant species (*Arabidopsis thaliana* and *Medicago truncatula*) to a soil failed to maintain the soil fungal community previously established under the influence of other plant species, while the communities could be maintained by cultivation of the same species. They further proved that the maintenance of soil fungal communities is mediated largely through plant

root exudates: the fungal community in soil amended with root exudates collected from plants grown in a hydroponic system was similar to those observed for plants grown in soil. Although using exudates from hydroponically-grown plants, which may differ from those released in soils, this study provided strong evidence that plants regulate the structure of fungal communities in soil through root exudation.

1.1.4 Technical Challenges in Rhizosphere Studies

Rhizosphere soil is perhaps one of the most difficult ecosystems in which to conduct research. The heterogeneity of the soil environment (physicochemical properties and nutrient availability), the dynamic activities of plant roots (growth, rhizodeposition), the huge diversity and dynamics of soil microbial communities, and simultaneous and complex interactions in the rhizosphere make understanding of this zone very difficult. In addition, it is difficult to sample root exudates or rhizosphere soil at the narrow soil-root interface without damaging plant roots. Furthermore, rhizosphere processes, such as root exudation and colonization by soil microbes on roots, can respond quickly to a wide range of perturbations and environmental pressures (Paterson et al. 2007). All of the factors above combine to create difficulties in the design of experiments to investigate the impact of root exudates on soil microbial communities *in situ*.

1.1.4.1 Root exudate collection

Root exudates are one of the most poorly quantified compounds of the belowground C cycle, as they only occur in a narrow rhizosphere zone and are rapidly absorbed by soils and/or assimilated by soil microorganisms (Neumann et al. 2009; Paterson 2003; Phillips et al. 2008). Exudates are also constantly in a state of dynamic flux: plants release root exudates and microorganisms consume plant exudates while contributing exudates to soil.

The collection of root exudates presents significant challenges due to difficulties associated with i) accessing the rhizosphere without disturbance or damage to plant roots as a result of the collection system; ii) selecting a suitable collection medium which does not affect root physiology and exudate recovery; and iii) spatial and temporal variations in root and rhizosphere environment (Phillips et al. 2008). Various approaches have been used to collect exudates either directly from nutrient solutions where plants are grown or through accumulation in solid media (generally sand or glass beads) and recovery through different flushing or extraction procedures (Gransee and Wittenmayer 2000; Sandnes et al. 2005; Tang and Young 1982). However, the recovery of exudates by such approaches is generally compromised by various physiological effects on the plant and incomplete leaching or adsorption of exudates by the solid media (Gransee and Wittenmayer 2000;

Neumann and Römheld 2007; Sandnes et al. 2005). These techniques are also not suitable for quantitative calculation of the exudation rates in relation to root properties (Gransee and Wittenmayer 2000). In other studies, whole plants have been removed from solutions or solid media, so that the root systems can be submerged into trap solutions under microbiologically-controlled conditions to allow for release of water-soluble exudates (Mucha et al. 2005; Neumann and Römheld 1999; Roelofs et al. 2001; Wirén et al. 1995). However, the removal of plants from solid media into trap solutions inevitably causes damage to root systems by breakage of roots, rupture of root hairs and epidermal cells or by rapid changes in the environmental conditions (e.g. temperature, pH, oxygen availability) (Neumann and Römheld 2007). While mechanical damage of root systems can be avoided for plants grown in hydroponic systems, root systems grown under such conditions generally show distinct morphological and physiological differences compared to those grown in solid media or soil (Groleau-Renaud et al. 1998; Jones 1998).

As discussed previously, significant spatial and temporal variability in the structure of root systems and in the release of exudates by roots has been reported (Hinsinger et al. 2005; Hoffland et al. 1989; Marschner 1995; Schefe et al. 2008). Without direct access to the rhizosphere in undisturbed systems, these variables cannot be studied appropriately. *In situ* sampling of root exudates from specific regions of roots (also called “localised sampling”) has been achieved by placing filter paper or other collection media (including agarose plugs, chromatography paper, resin bags, resin agar sheets and anion exchange membrane (AEM) strips) directly onto roots after removal of plants from growing media, mainly from hydroponic solutions (Kamh et al. 1999; Kape et al. 1992; Marschner et al. 1987; Neumann et al. 1998; Neumann and Römheld 1999; Schefe et al. 2008; Zhang et al. 2001). Various collection media have also been applied directly onto the root surface of plants grown in rhizoboxes or similar soil-packed containers (Dinkelaker et al. 1997; Kamh et al. 1999; Schefe et al. 2008; Zhang et al. 2001). However, such rhizobox systems are generally limited in size (e.g. 40x15x3.5 cm (Kamh et al. 1999)) and the requirement to grow roots over a planar surface. Microsuction cups have similarly been used either in rhizoboxes or have been applied to root systems under field conditions via access portals (root windows) (Dessureault-Romppe et al. 2006; Dieffenbach et al. 1997; Sandnes et al. 2005; Wang et al. 2004). However, only small volumes of the solution can be collected under most field conditions and exudation rates in relation to root/soil properties are difficult to quantify. Recently, Neumann et al. (2009) reviewed most of the exudate collection techniques with their application ranges and limitations.

1.1.4.2 Root exudate analysis

The lack of comprehensive knowledge of exudate composition is another major barrier to rhizosphere studies (Baziramakenga et al. 1995; Fan et al. 2001; Gransee and Wittenmayer 2000). Recent advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS) or GC-MS coupled with other techniques (e.g. nuclear magnetic resonance) have been used successfully for identification of a wide range of different plant metabolites and root exudates (Fan et al. 1997; Fan et al. 2001; Schauer et al. 2006). However, exudate sampling usually results in small sample volumes, especially when using *in situ* sampling techniques, and/or extremely low concentrations of exudate compounds, and thus the analysis requires sample preparation (e.g. pre-concentration) and sensitive analytical facilities (Neumann et al. 2009). In addition, other compounds (e.g. salts, ions) from growth media, nutrient solutions and extraction solutions may affect the identification or quantification of root exudate compounds (Neumann 2006; Shen et al. 1996; Tang and Young 1982).

1.1.4.3 Corresponding samples for exudate and microbial community analysis

The other major hindrance for studies on the impact of root exudates on rhizosphere microbial communities is the collection and analysis of root exudates and rhizosphere microbial communities in the corresponding samples. Soil samples must be collected from the root-soil interface, so plants grown in hydroponic systems or submerged in trap solutions for exudates collection are not suitable for further rhizosphere microbial community analysis. Micallef et al. (2009) collected root exudates of *Arabidopsis thaliana* grown in hydroponic solutions and analysed rhizosphere bacterial communities associated with roots of *A. thaliana* grown in soils. They did not obtain a statistical correlation between root exudates and bacterial communities. The two different growth media could result in different root morphologies and other rhizosphere processes including exudation (Table 1.2), and the hydroponic system is far too dissimilar to the soil media. Weisskopf et al. (2008) studied the link between root exudates in rhizosphere soils and their bacterial communities by removing wheat and lupin root systems from soil, rubbing rhizosphere soils off the roots and extracting OAs from rhizosphere soils using sterile water. Despite the inevitable risk of damaging fine root and root hairs, this method is very labour intensive and not applicable for large plants such as trees.

Conversely, *in situ* exudate sampling techniques provide an opportunity for subsequent rhizosphere soil collection for microbial community analysis, since the collection process does not change the rhizosphere environment/conditions. This type of sampling was used by Marschner et al. (2002) when they first sampled OAs released from cluster and non-cluster roots of white lupin (*Lupinus albus*) and subsequently sampled roots with attached

rhizosphere soil for microbial community analysis. However, due to the small size of the filter paper (diameter 5 mm) used for exudate collection, the corresponding root segments with adherent rhizosphere soil were too small to separate rhizosphere soil from plant roots and subsequently, deoxyribonucleic acid (DNA) extracted from both soil microorganisms and root cells were used for microbial community analysis. Additionally, in their study, plants were grown in small rhizoboxes which may not be suitable for other plants such as trees to be grown for a long period experiment. Therefore, an improved technique is needed for studying the relationship between exudates and rhizosphere microbial community in the rhizosphere soil.

1.1.5 Rhizosphere Study Systems

Field rhizotrons (also called “root windows”) and minirhizotrons are two of the earliest non-destructive *in situ* techniques for root and rhizosphere studies (Taylor et al. 1990). A field rhizotron is an underground laboratory system containing enclosed columns of soil with transparent plastic windows which allow for recording of root growth and relevant rhizosphere process studies. Minirhizotrons are transparent tubes containing video imaging facilities which can be inserted into undisturbed soil profiles after removal of cylindrical soil cores with corers (McMichael and Zak 2006). Both rhizotron systems were initially designed to study root growth and dynamics and now, with various modifications in types and sizes, have been applied to studies of rhizosphere processes, such as dynamics of the rhizoplane and rhizosphere microorganisms (see reviews by McMichael and Zak 2006; Neumann et al. 2009). These two systems have also been used in forest ecosystems (Hendrick and Pregitzer 1996; López et al. 2001; Wells and Eissenstat 2001) and annual crop plants (McMichael and Zak 2006; Taylor et al. 1990). One of the advantages of rhizotron systems is that they allow for continuous studies of rhizosphere processes in time and space.

Other rhizosphere process study systems include various split-root compartment systems (Chen et al. 2002; Kuchenbuch and Jung 1982) and rhizoboxes (Kamh et al. 1999; Marschner et al. 2002). However, both of these systems suffer from the general disadvantages of pot experiments associated with disturbed soil structure, altered root-zone temperatures and the limited rooting volume. In addition, the high density of roots in the root mat of the split-root compartment system can lead to unrealistically high levels of root exudate accumulation and associated chemical changes in the adjacent rhizosphere compartment and thus to an overestimation of rhizosphere effects (Neumann et al. 2009).

1.2 Research Context and Objectives

1.2.1 Research Context

The overall objective of this project was to investigate the influence of root exudates on the diversity and activity of soil and rhizosphere microbial community using radiata pine as the model system. Radiata pine is the dominant commercial plantation forest tree species in New Zealand, as well as in Australia, Chile and Spain. It occupies more than 90% of the land area under plantation forestry in New Zealand, totalling approximately 1.7 million hectares (Wickham and Watson 1991). In addition, radiata pine produces substantially greater quantities of exudates compared to ryegrass (*Lolium perenne*) (Chen et al. 2002; Scott and Condron 2004), and hence is an ideal system with which to explore effects of root exudates on rhizosphere microbial communities.

In agricultural crops such as maize, sugars are the main components of root exudates, constituting more than 65% of the total C in exudates followed by OAs and amino acids (Krafczyk et al. 1984). In contrast, OAs are the most quantitatively important component of the root exudates in trees and the amount of C in the form of OAs is approximately 2 to 3 times of that of sugars (Grayston and Campbell 1996; Smith 1976). Therefore, this study focused on OAs in root exudates of radiata pine and their influence on rhizosphere microbial communities, although sugars were also included in the microcosm experiment. Due to the limitations in the currently available techniques (Section 1.1.4), a novel *in situ* root exudate sampling technique was first developed in a large-scale rhizotron system which was subsequently used for further rhizosphere studies.

With the development of genetic engineering techniques, a number of different genetic traits, such as insect resistance, herbicide tolerance, disease resistance and stress tolerance, have been incorporated into various crops and trees (Henderson and Walter 2006; James 2003; Thomson 2006; van Frankenhuyzen and Beardmore 2004). The potential environmental and ecological impacts of these genetically modified (GM) plants must be comprehensively studied before broad scale cultivation (Lilley et al. 2006; van Frankenhuyzen and Beardmore 2004). The risk assessment of GM plants has, until recently, been focused on aboveground non-target species (Bruinsma et al. 2003). As plants play important roles in influencing rhizosphere microbial communities and some soil microbes are crucial in functions such as nutrient cycling and plant growth, the potential impact of GM plants on belowground soil microbes should not be neglected (Bruinsma et al. 2003; Lilley et al. 2006). Significant differences between rhizosphere microbial communities associated with GM and unmodified plants were detected in several studies (Castaldini et al. 2005; Dunfield and Germida 2001; O'Callaghan et al. 2008; Tesfaye et al. 2003). These shifts in microbial communities may be caused by unintentional alteration

of root exudates released by GM plants into the rhizosphere, as speculated by several authors (Di Giovanni et al. 1999; Donegan et al. 1999; Milling et al. 2004; Sessitsch et al. 2003; Siciliano et al. 1998). In this project, the potential impact of GM radiata pines on the structure of the soil microbial community and the potential alteration of OAs in root exudates (unintentionally altered) by GM pines were investigated using a novel sampling approach in rhizotrons. Finally, the role of selected radiata pine exudate compounds (sugars and OAs) in shaping soil microbial communities was further examined.

1.2.2 Hypothesis

The most abundant component OAs in the root exudates of radiata pine can significantly influence the diversity and activity of soil microbial community in the rhizosphere.

1.2.3 Objectives

1. To select suitable molecular methods for analysis of active microbial communities in rhizosphere soils (Chapter 2);
2. To develop a novel experimental approach for *in situ* collection of OAs in root exudates of radiata pine grown in a large-scale rhizotron (Chapter 3);
3. To assess the variability in soil microbial communities in the rhizotron system with the aim of providing useful information for the subsequent experiment (Chapter 3);
4. To study the potential impact on rhizosphere microbial communities by GM radiata pine and its potential alteration of OAs in root exudates of GM radiata pine compared to control pines when grown in large-scale rhizotrons under controlled environmental conditions (Chapter 4);
5. To investigate the influence of selected root exudate compounds (OAs and sugars) on the diversity and activity of soil microorganisms (Chapter 5).

1.3 Thesis Format

This thesis comprises six chapters. The first chapter provides the general background information and the context and objectives of this project. Chapter 2 describes the rational selection of the main molecular technique for active microbial community analysis in this study, with the validation of the technique in Appendix 2.5. Chapters 3 - 5 are experimental chapters with research objectives outlined in Section 1.2.2. Each experimental chapter includes introduction, material and methods, results, discussion and conclusion sections. A general discussion of the experiments conducted in this project and suggestions for further work are presented in Chapter 6.

Chapter 2 Molecular Methods for Characterisation and Analysis of Rhizosphere and Non-rhizosphere Microbial Communities

2.1 Introduction

Rhizosphere microbial communities are highly complex and are readily influenced by many factors such as plant species, soil type, environment (seasons etc.) and presence of fauna (Berg and Smalla 2009; Buée et al. 2009; Hawkes et al. 2007). Historically, knowledge of the microbial communities in the rhizosphere and non-rhizosphere was limited to those organisms that could be easily isolated and cultured. However, it is now well accepted that only a small percentage of the entire profile of microorganisms in environmental samples, such as soil, can be cultured in the laboratory (Amann et al. 1995; Head et al. 1998). Culture-dependent methods, therefore, cannot represent the actual *in situ* diversity of microbial communities in most environmental samples (Amann et al. 1995; Dunbar et al. 2000; Wagner et al. 1993; Ward et al. 1990). In contrast, culture-independent techniques are able to profile the microbial community with much higher resolution and are more suitable for the analysis of complex microbial communities (Amann et al. 1995; Entry et al. 2007). Initially, microbial communities were analysed using microbial cell fatty acid profiling (Findlay 1996; White and Findlay 1988) but more recently, nucleic acids (DNA and ribonucleic acid (RNA)) have become the dominant signature molecule for community analysis (Nakatsu 2007; Nocker et al. 2007; O'Callaghan et al. 2006). Now polymerase chain reaction (PCR) amplification is used extensively in microbial community analysis to increase copies of selected target genes for more efficient detection (Nakatsu 2007).

Although a range of molecular techniques are now available for microbial community analysis, only some techniques are practical for ecological studies, in which large number of samples must be analysed in order to differentiate treatment effects from naturally occurring changes in microbial communities caused by environmental conditions, plant growth, etc. (O'Callaghan et al. 2006). In general, methods leading to a detailed view of a microbial community, such as cloning, sequencing and metagenomics, are expensive, time-consuming and labour-intensive (Nakatsu 2007; O'Callaghan et al. 2006). In contrast, the techniques that produce a “snapshot” of a community represented as a genetic fingerprint allow higher throughput and comparative profiling of many samples. Therefore, these techniques are appropriate for ecological research projects (Nakatsu 2007; O'Callaghan et al. 2006). The commonly used genetic fingerprinting techniques are PCR-dependent approaches and include denaturing gradient gel electrophoresis (DGGE),

terminal restriction fragment length polymorphism (T-RFLP), single strand conformational polymorphism and automated ribosomal intergenic spacer analysis.

2.1.1 PCR-DGGE

Although each community fingerprinting technique has its own strengths and weaknesses (see review by Nocker et al. 2007), PCR-DGGE is one of the most frequently used techniques to investigate bacterial and fungal community structures in rhizosphere and soil samples (Kowalchuk and Smith 2004; Marschner et al. 2001; Smit et al. 1999). This technique has been used extensively to monitor differences in microbial community structure associated with GM plants (Heuer et al. 2002; Milling et al. 2004; O'Callaghan et al. 2008), season variations (Gomes et al. 2001; Smalla et al. 2001), plant growth stages (Duineveld et al. 2001; Marschner et al. 2002) and farming practices (Garbeva et al. 2003). DGGE allows the separation of same size but diverse PCR-amplified products in an acrylamide gel composed of a linear gradient denaturant chemicals into a profile composed of bands. The separation of same size PCR products is achieved on the basis of their differing intrinsic stability which depends on the GC content and distribution. As a fragment progresses through the gel and is subjected to increasingly strong denaturing conditions, the double stranded PCR products reach a point where partial strand disassociation occurs. The disassociation results in the physical change of the molecule shape which directly affects its mobility during electrophoresis. Consequently, same size PCR products which differ in sequence are separated on the gel. The profiles from replicate samples can then be compared across treatments to determine the level of similarity in the community structure and to investigate shifts or changes in community composition.

2.1.2 Strengths and Weaknesses of PCR-DGGE

The PCR-DGGE technique has a number of advantages over other techniques. Numerous samples can be analysed on one gel and, with correct use of markers and positioning of treatments across lanes, it is possible to conduct simultaneous comparison between samples. The technique is also affordable for most laboratories. In addition, individual bands of interest can be excised from the gel for subsequent cloning and sequencing (Nakatsu 2007; Nocker et al. 2007; O'Callaghan et al. 2006). However, as with all PCR-based techniques, DGGE profiling relies on the efficiency of nucleic acids extraction from samples and PCR amplification. PCR bias and artifact formation can occur during the amplification process, especially in samples containing multi-templates, as most ecological samples do. PCR bias is caused by differential amplification due to differences in the efficiency of primer binding to templates (Meyerhans et al. 1990; Polz and Cavanaugh 1998), formation of secondary structure of templates (Pallansch et al.

1990), and differences in the kinetics of the PCR reaction (Brunk and Eis 1998). PCR artifacts may arise due to the formation of chimerical or heteroduplex molecules (von Wintzingerode et al. 1997; Wang and Wang 1997). As a consequence, many, if not all, PCR-based techniques will not be totally representative of microbial communities, especially on a quantitative level (Farrelly et al. 1995; Ishii and Fukui 2001). Felske and Akkermans (1998) pointed out that although the most abundant microorganisms are normally represented by the dominant bands on DGGE gels, other important members of the community could be under-represented due to the weaker signals or even absence because of the possible PCR bias and unknown cell lysis efficiencies. Therefore, O'Callaghan et al. (2006) emphasized the importance of selecting suitable nucleic acids extraction methods for each study and optimization of PCR conditions for each analysed gene sequence. In addition to these PCR-based limitations, the DGGE process itself has some specific disadvantages. DGGE patterns derived from environmental samples, such as rhizosphere soil which contain a large number of different bacterial populations, might show as smears on the gel (O'Callaghan et al. 2006). However, this can be avoided by using more specific primers only targeting particular taxonomic or functional groups. Additionally, Kisand and Wikner (2003) stated that the commonly used 16S sequence can contain multiple melting domains which may result in "cloudy bands". It has also been found that a single band in a DGGE gel may be composed of DNA from several species (Sekiguchi et al. 2001; Yang and Crowley 2000) and conversely, several bands are sometimes generated from a single species (Nübel et al. 1996). In addition, comparisons between gels must be carried out with caution because of gel variability (Nakatsu 2007; Nocker et al. 2007). Inclusion of appropriate DGGE markers on each gel is especially important for comparisons between gels (O'Callaghan et al. 2006). Because of the cumbersome determination of signal intensities of all bands which are heavily affected by staining techniques and processes, DGGE is at best only a semi-quantitative analysis when intensities of bands are included in the analysis (Nocker et al. 2007).

2.1.3 Analysis of PCR-DGGE Microbial Community Profiles

The analysis of PCR-DGGE microbial community profiles was initially restricted to visual interpretation of presence and absence of the bands (Felske and Akkermans 1998; Gomes et al. 2001). With the development of specific software packages, the analysis of community profiles has significantly improved through more accurate comparison of both the band position and the relative intensity of different bands within gels. Thus statistical analysis of the data could be achieved. However, because of potential PCR biases and influence of signal intensities by gel staining process, some studies only interpret the data based on presence/absence of the bands rather than relative intensity of the bands

(O'Callaghan et al. 2008). Techniques used in the analysis of DGGE profiles have included computation of the Shannon diversity index (Garbeva et al. 2003), similarity matrices (Kropf et al. 2004), clustering techniques (Duineveld et al. 2001; Morgan et al. 2002; van Verseveld and Røling 2004), and ordination methods such as principal components analysis (Joynt et al. 2006; O'Callaghan et al. 2008), principal coordinate analysis (PCoA) (Pennanen et al. 2004) and non-metric multi-dimensional scaling (MDS) analysis (Feris et al. 2003). Recently, the microbial profiles have also been correlated to the environmental variables using redundancy analysis (Bourne et al. 2008; Tzeneva et al. 2009) or Pearson's correlation based on Shannon diversity index (He et al. 2008).

2.1.4 DNA vs. RNA-based profiling

Until recently, most of the community analyses were based on analysis of microbial ribosomal DNA (rDNA), but the structure of a community does not necessarily reflect its biological activity (Anderson and Parkin 2007; Pennanen et al. 2004). For example, several studies reported that although differences in the metabolic properties of soil microorganisms or soil enzyme activities between treatments were detected, no clear differences between treatments were seen in DNA-based microbial community fingerprints (Duineveld et al. 1998; Engelen et al. 1998; Toyota et al. 1999). This lack of correlation between rDNA-DGGE profiles and other methods used to detect treatment effects on microbial communities could be caused by two factors. Firstly, most microorganisms are thought to be inactive in soil. Olsen and Bakken (1987) reported that 60 to 80% of the cells in soil were extremely small (with volumes smaller than $0.065 \mu\text{m}^3$) under microscopy; these very small cells were believed to be relatively inactive due to limited resources in soil. Secondly, DNA of dead microorganisms can survive in soil by absorption to soil particles which prevents degradation by nucleases. The presence of DNA from dead microorganisms persisting in soil could obscure any changes in the viable microbial community. Lorenz and Wackernagel (1987) reported that up to 59% of DNA could be absorbed onto sand under experimental conditions by physical and chemical interactions between DNA and sand surfaces. The ability of DNase I to degrade DNA was reduced by absorption of DNA to soil (Lorenz and Wackernagel 1987). Demanèche et al. (2001) also demonstrated the plasmid DNA could be absorbed onto the clay mineral and thus effectively protect it against nucleases. Kowalchuk et al. (2003) reported a remarkable similarity between DNA-DGGE fingerprints from sterilized and untreated soils which further indicated that DNA from dead cells or extracellular DNA could persist in soil, be recovered by nucleic acids extraction and PCR, and subsequently be represented on a DGGE gel.

To overcome some of these limitations in DNA-DGGE profiling, and with the improvement in nucleic acids extraction techniques, microbial ribosomal RNA (rRNA) has been used in analysis of microbial communities. RNA is present at a higher level in metabolically active cells than in quiescent cells and thus is directly correlated to cellular activity and growth rate (Manefield et al. 2002; Rosset et al. 1966; Wagner 1994). Several studies have already shown that RNA-based approaches can be more sensitive in detecting shifts in microbial communities (Girvan et al. 2003; Gremion et al. 2003; Mahmood et al. 2005; Nicol et al. 2003; Noll et al. 2005). For instance, Duineveld et al. (2001) found that RNA-derived bands were a subset of the bands detected on DNA-based DGGE gels, indicating that several groups predominant in the rhizosphere were not necessarily the most actively metabolizing groups in the rhizosphere, although differences due to inefficiency of reverse transcription cannot be ruled out. By using rRNA-DGGE technique, Duineveld et al. (2001) detected distinct bacterial communities associated with various growth stages of chrysanthemum (*Dendranthema grandiflora*), which was not detected by rDNA-DGGE and which was consistent with their previous culture-based analysis (Duineveld and Van Veen 1999). In addition, Pennanen et al. (2004) found that bacterial DGGE profiles derived from corresponding DNA and RNA templates were different after addition of a C source, although the predominant bands were common in both profiles. They also showed that some bands only appeared in the rRNA-DGGE profiling but not on DNA-DGGE gels, indicating that some metabolic functions were being carried out by microorganisms which were not dominant in the communities. In some cases, shifts in community structure resulting from a perturbation can be detected earlier using rRNA template instead of rDNA. Hoshino and Matsumoto (2007) found that the rRNA-DGGE detected changes more quickly and showed larger changes in the bacterial community after chloropicrin treatment than did rDNA-DGGE, which showed similar trends to rRNA-DGGE but with a time lag. Differences between the predominant soil fungal communities and those metabolically active at the time of sampling were also detected in fungal communities using 18S or internal transcribed spacer (ITS) as targeted sequences by comparing rDNA-DGGE with rRNA-DGGE in several studies (Anderson and Parkin 2007; Anderson et al. 2008; Bastias et al. 2007).

In contrast to the studies discussed above, other researchers reported no differences between profiles of active microbial communities using rRNA-DGGE in comparison with rDNA-DGGE (Griffiths et al. 2003a; Griffiths et al. 2003b; Izumi et al. 2007; Pennanen et al. 2004; Ros et al. 2009). This may have resulted from the long-term effects of treatments in some of these experiments (Izumi et al. 2007; Ros et al. 2009). With continuous exposure to the same treatments, the active populations may increase in biomass and become the dominant groups in some environments (Nakatsu 2007). Marschner et al.

(2002) also pointed out that rDNA-DGGE profiles only reflect long-term changes in microbial community structure, but cannot detect rapid changes such as could occur in response to short-term changes in root exudates. As the majority of studies indicated that rRNA-DGGE provided the most sensitive microbial community analysis, this method was selected for use in the current project investigating the influence of root exudates on soil microbial communities.

2.1.5 Extraction of RNA

The biggest hindrance of using RNA as a signature molecule for microbial community analysis is the easy degradation of RNA and the widespread distribution of RNase in the environment (Moran et al. 1993; Ogram et al. 1995). As RNases are extremely stable enzymes and are active under most conditions without cofactors (Blumberg 1987), RNA therefore does not persist well in soil, is not stable during extraction process and needs to be stored at -80°C to prevent fast degradation. In contrast, DNA is more stable and persists for long periods in most soils (Trevors 1996). Therefore, the isolation of RNA from environmental samples is technically more difficult than DNA.

Although many RNA extraction protocols have been published and used in practice (Borneman and Triplett 1997; Duarte et al. 1998; Felske et al. 1996; Griffiths et al. 2000b; Hurt et al. 2001; Moran et al. 1993; Ogram et al. 1995; Purdy et al. 1996), the effective extraction of RNA from environmental samples, particularly from soil, remains a challenge. This is in part because inhibitory compounds (e.g. humic acids and clay minerals) are often co-extracted from the complex soil matrix when some published extraction methods are used. Additional purification procedures are then required for successful PCR amplification. These additional steps (such as multiple washing steps, passage through gel matrix Sephadex columns) are both time-consuming and yield lower amounts of RNA (Felske et al. 1996; Griffiths et al. 2000b). Reliable extraction methods have been reported for isolation of RNA from soils (Duarte et al. 1998; Felske et al. 1996; Griffiths et al. 2000b; Moran et al. 1993), but most typically involve multiple steps for purification. Such techniques are impractical for processing large numbers of samples in ecological projects. Like DNA extraction, the efficiency of RNA extraction varies according to soil types (Hoshino and Matsumoto 2007). Therefore, selection and modification of RNA extraction techniques is required for specific soil types.

2.1.6 Primers for Use in PCR-DGGE

The majority of bacterial community studies have used the bacterial 16S ribosomal gene as a molecular phylogenetic marker because of its structural and functional conservation and universal distribution (Pace et al. 1986). Several primer sets targeting general

bacterial 16S rDNA are available and have been used extensively in rhizosphere studies (Heuer et al. 1997; Muyzer et al. 1993). However, in many cases, DGGE profiles of general bacterial 16S rDNA in rhizosphere and non-rhizosphere soils showed complex communities composed of a large number of bands and masked differences between treatments (O'Callaghan et al. 2006; O'Callaghan et al. 2008). To increase the resolution of PCR-DGGE, taxon-specific 16S rDNA sequences can be amplified and subsequently analysed by DGGE. These more specific primers improve detection of less predominant microbial populations. The sensitivity of taxon-specific primers has been clearly shown in several studies examining differences in microbial communities associated with GM and unmodified control plants where profiling of bacterial communities using general bacterial primers did not detect any differences between plant types, but significant differences were found by taxon-specific profiling (Costa et al. 2006; Milling et al. 2004; O'Callaghan et al. 2008).

Two main groups of soil bacteria, α - and β -Proteobacteria, have often been used in studying rhizosphere communities (Gomes et al. 2001; Kennedy and de Luna 2005; O'Callaghan et al. 2008), since the majority of the bacterial communities in the rhizosphere environment fall within these groups. Chow et al. (2002) reported that α -, β -Proteobacteria, as well as Acidobacteria, dominated the rhizosphere of lodgepole pine (*Pinus contorta*) grown under forest conditions. Moreover, a very high proportion of populations belonging to α -, β -Proteobacteria were detected in cloning studies using the rhizosphere soil of GM and control *Pinus radiata* trees grown in the field in New Zealand (J. Lottmann, pers. comm.). As *Pseudomonas* spp. are the predominant group found in root-associated bacteria (Haas and Défago 2005), they have often been profiled in rhizosphere studies (Costa et al. 2007; Costa et al. 2006; O'Callaghan et al. 2008). In addition, Pseudomonads have been reported to be directly influenced and greatly stimulated by plant root exudates, including OAs, sugars, amino acids as well as secondary metabolites (Koo et al. 2005; Smit et al. 2001). For example, certain amino acids (e.g. glutamine, serine, threonine) found in plant root exudates have been shown to attract *Pseudomonas lachrymans* (Chet et al. 1973) and *Pseudomonas aeruginosa* (Nikata et al. 1992). Mark et al. (2005) found that root exudates of sugar beet (*Beta vulgaris*) altered the expression of *Pseudomonas aeruginosa* genes involved in chemotaxis. Thus, profiling of the pseudomonad community is a particularly suitable technique for analysis of potential effects of root exudation on the structure of bacterial communities. Therefore, α -, β -proteobacterial and pseudomonad communities were investigated in the current project to allow for more detailed analysis of the bacterial communities in rhizosphere and non-rhizosphere soils.

Soil and plant-associated fungi are also important components of the rhizosphere microbial community (Kennedy and de Luna 2005). Although some primer sets are available for fungi (18S or ITS region), no single set is suitable for all fungal community analysis and fungal primers must be chosen to suit particular research needs (O'Callaghan et al. 2006). In the current project, general fungal 18S primer set NS1 and FR1-GC designed by White et al. (1990) and Vainio and Hantula (2000), respectively were selected based on the successful amplification of fungal 18S DNA and complementary DNA (cDNA) and separation on DGGE gel carried out in preliminary experimentation (Appendix 2.2, Figure A2.2-3; Appendix 2.5, Figure A2.5-3).

2.2 Methods

2.2.1 Rhizosphere and Non-rhizosphere Soil Preparation

Throughout this project, all the radiata pine root samples collected from soil were shaken gently to remove any loosely attached soil. The soil firmly adhering to the roots was defined as rhizosphere soil. Each root sample with associated rhizosphere soil was weighed in a sterile Petri dish before being transferred into a sterile filter bag (Stomacher® lab system classic 400, A.J. Seward Ltd, London, England) by sterile forceps for extraction of the rhizosphere soil. The sample was suspended in 25 ml of sterile deionised (DI) water and treated in a stomacher blender (Colworth Stomacher 400; A.J. Seward Ltd) for 1 min at high speed. The soil solution was then transferred to a sterile 50 ml Falcon tube. The blending step was repeated with 20 ml of sterile DI water and the soil solution was transferred to the previous Falcon tube. The tube was left on the bench for 15 min to allow sediment to settle out before the solution was transferred to a sterile tube for centrifugation at 10,000 *g* for 30 min at 15°C. The supernatant was carefully discarded and the soil pellet was dissolved in 1.5 ml of sterile 0.85% NaCl and transferred to a sterile 2 ml Eppendorf tube for centrifugation at 13,000 *g* for 30 min at 4°C. The resulting pellets were stored at -80°C for subsequent microbial community RNA extraction. The processing of rhizosphere soil from root samples is outlined in Figure 2.1. Non-rhizosphere soil samples (0.5 g per sample) were collected from areas close to the sampling sites of rhizosphere samples where no roots were visible and immediately stored in sterile 2 ml Eppendorf tubes at -80°C until subsequent RNA extraction. Both the rhizosphere and non-rhizosphere soil samples were processed on the day of sampling.

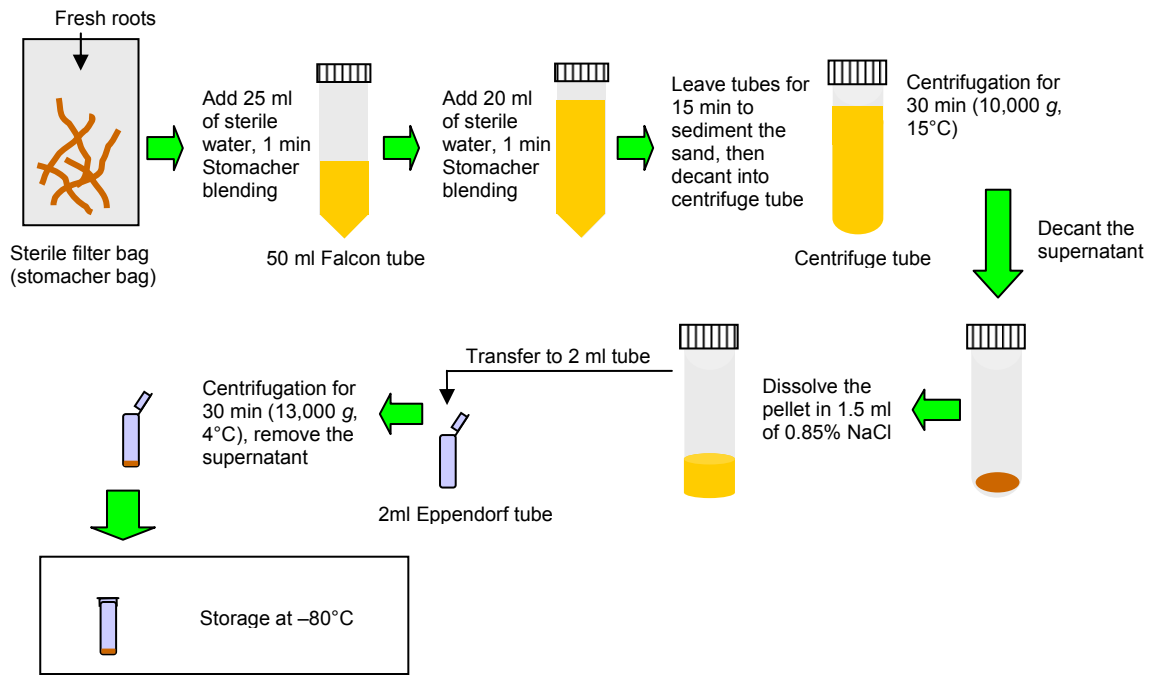


Figure 2.1 The processing procedure of rhizosphere soil preparation from root samples.

2.2.2 Extraction of Microbial Community RNA from Soil

2.2.2.1 Preliminary comparison of extraction methods

Prior to RNA extraction, all solutions and glassware were rendered RNase-free by diethyl pyrocarbonate treatment, and only certified RNase- and DNase-free plasticware was used. The RNA extraction method was developed and tested using small amounts of rhizosphere soil samples collected from *Pinus radiata* grown in a pot (see Appendix 2.1). Two RNA extraction methods were compared (see Appendix 2.2 for details). One of the methods tested was the hexadecyltrimethylammonium bromide (CTAB) extraction method adapted from Griffiths et al. (2000b), in that a vortex mixer was used instead of FastPrep bead beating system, and Lysing Matrix tubes (Q-Biogene, USA) were used instead of Bio-101 Multimix 2 Matrix tubes. The other method was modified from the method described by McKew et al. (2007). Four types of Lysing Matrix tubes were also compared for optimal extraction of both bacterial and fungal RNA using the CTAB extraction method (Appendix 2.2).

Comparison of the RNA extraction methods (Appendix 2.2) showed that the CTAB extraction method yielded better quality RNA as assessed by spectrophotometry using the Nanodrop with ratios of absorbance at 260 nm/230 nm and 260 nm/280 nm. Once optimized, the CTAB extraction method was used throughout the project.

2.2.2.2 Modified CTAB RNA extraction method

Briefly, soil (up to 0.5 g) and 0.5 ml of CTAB extraction buffer were added into a Lysing Matrix B tube and mixed briefly by vortex prior to the addition of 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0) (Sigma-Aldrich Inc., MO, USA). The sample was vortexed for 5 min, incubated on ice for 2 min then vortexed for a further 5 min. The aqueous phase containing nucleic acids was separated by centrifugation at 16,000 *g* for 5 min at 4°C, and transferred to a new 2 ml tube, followed by the addition of an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich Inc.). The contents in the tube were mixed well prior to centrifugation at 16,000 *g* for 5 min at 4°C, after which the aqueous phase was transferred into a new 2 ml tube. The total nucleic acids were then precipitated by adding 2 volumes of 30% (wt/vol) polyethylene glycol (PEG) 6000 -1.6 M NaCl for 2 h at room temperature, followed by centrifugation at 18,000 *g* at 4°C for 10 min. The supernatant was discarded and the pellet was washed with ice cold 70% ethanol and air dried for 5 min at room temperature prior to resuspension in 50 µl RNase free water (Applied Biosystems, CA, USA). All the recipes for reagent preparation are provided in Appendix 2.3.

2.2.3 RNA Samples and Subsequent cDNA Preparation

DNA in the nucleic acid samples was digested using DNase (TURBO DNA-free™ Kit, Applied Biosystems) according to the manufacturer's instructions using samples containing 1 µg of RNA samples which were measured based on the 260 nm absorbance using a Nanodrop. The treated RNA samples were amplified by PCR using different primer sets (341-GC/534R, F203/R1494, F948/R1494, PsF/PsR, NS1/FR1-GC, see Table 2.1) according to the conditions described in Sections 2.2.4 - 2.2.6. Five microlitres of PCR product were run on a 1% agarose gel with ethidium bromide staining to check for the presence of DNA in the RNA samples. Reverse transcriptase-PCR (RT-PCR) conversion of RNA samples into cDNA samples was only carried out when no PCR products were detected on the agarose gel, confirming that no DNA was present in the RNA samples. RT-PCR was carried out using SuperScript™ III reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions. Random primer (Promega Corporation, WI, USA) was used to convert RNA into cDNA for bacterial rRNA. FR1 reverse primer (Table 2.1) was used to convert fungal 18S ribosomal RNA into cDNA. A negative control using DNase/RNase-free distilled water (Invitrogen) instead of RNA sample was included in each RT-PCR run.

Table 2.1 Sequences of the primers used in this project.

Primer ^a	Targeted sequence	Sequence 5'-3'	Reference
341F-GC	Bacterial 16S	cgcccgcgcgcgcggcgggcgggcgggggcacgggggg ccTACGGGAGGCAGCAG	Muyzer et al. 1993
534R	Bacterial 16S	ATTACCGCGGCTGCTGG	Muyzer et al. 1993
F203	α -Proteobacterial 16S	CCGCATACGCCCTACGGGGGAAAGATTTAT	Gomes et al. 2001
F948	β -Proteobacterial 16S	CGCACAAGCGGTGGATGA	Gomes et al. 2001
R1494	α , β -Proteobacterial 16S	CTACGGYTACCTTGTTACGAC	Weisburg et al. 1991
PsF	Pseudomonad 16S	GGTCTGAGAGGATGATCAGT	Widmer et al. 1998
PsR	Pseudomonad 16S	TTAGCTCCACCTCGCGGC	Widmer et al. 1998
F968-GC	α , β -Proteobacterial and Pseudomonad 16S (nested)	cgcccgcgcgcgcggcgggcgggcgggggcacgggggg AACGCGAAGAACCTTAC	Nübel et al. 1996
R1378	α , β -Proteobacterial 16S (nested)	CGGTGTGTACAAGGCCCGGGAACG	Heuer et al. 1997
NS1	Fungal 18S	GTAGTCATATGCTTGTCTC	White et al. 1990
FR1	Fungal 18S	AICCATTCAATCGGTAIT	Vainio and Hantula 2000
FR1-GC	Fungal 18S	cgcccggggcggcgcccggggcgggcgggggcacgggggg AICCATTCAATCGGTAIT	Vainio and Hantula 2000

^a F =Forward primer; R = Reverse primer; GC= GC-clamped primer.

2.2.4 PCR Amplification of Bacterial 16S rRNA Gene Fragment

The V3 region of bacterial 16S rRNA gene sequence was amplified from cDNA samples using the primer set 341F-GC/534R (Muyzer et al. 1993). This resulted in ~200 bp PCR products and could be nicely separated on DGGE gels (Appendix 2.5). Primers used in this study are summarised in Table 2.1. The RT-PCR negative controls were also included in the relevant gene sequence PCR reactions. The bacterial 16S PCR reaction was carried out in 25 µl volumes with 1 µl cDNA template (1:10 diluted after RT-PCR) or RT-PCR negative control, 1 x NH₄ buffer (Bioline, Australia), 0.2 mM dNTPs (Promega Corporation), 0.25 µM each primer (341F-GC, 534R), 3.75 mM MgCl₂, and 1 U Biotaq DNA polymerase (Bioline). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. PCR amplifications were performed in a Bio-Rad iCycler (Bio-Rad, CA, USA) with an initial denaturing step at 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, before a final extension step at 72°C for 7 min. Amplified PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel and ethidium bromide staining.

2.2.5 PCR Amplification of Taxon-specific 16S rRNA Gene Fragments

Alpha- and beta-proteobacterial 16S, and pseudomonad 16S rRNA gene fragments were initially amplified using taxon-specific primers F203/R1494, F948/R1494 and PsF/PsR, respectively (Table 2.1). In the first PCR, taxon-specific 16S rRNA fragments were amplified in 25 µl reaction mixtures containing 1 µl cDNA template (1:10 diluted after RT-PCR) or RT-PCR negative control, 1 x NH₄ buffer (Bioline), 0.2 mM dNTPs (Promega Corporation), 0.2 µM each primer, MgCl₂ (3.75 mM for α- and β-Proteobacteria, 1.5 mM for Pseudomonads), and 1 U Biotaq DNA polymerase (Bioline). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. The thermal cycling programmes were performed with an initial denaturing at 94°C for 5 min, followed by cycles (35 cycles for α-Proteobacteria, 25 cycles for β-Proteobacteria, and 30 cycles for Pseudomonads) at 94°C for 30 s for α-Proteobacteria and Pseudomonads or 1 min for β-Proteobacteria, various annealing temperature for 1 min (56°C for α-Proteobacteria and Pseudomonads and 61°C for β-Proteobacteria) and 72°C for 2 min for α- and β-Proteobacteria or 1 min for Pseudomonads, before a final extension step at 72°C for 10 min. PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel and ethidium bromide staining.

Amplifications of the nested fragments were then carried out using 1 in 100 dilution of the first PCR products incorporating the GC-clamped primer F968-GC for DGGE (Table 2.1). For α- and β- proteobacterial 16S gene fragments, R1378 was used in combination with

the F968-GC primer; and for pseudomonad 16S gene fragments, PsR was used together with F968-GC. The nested PCR reactions were performed in 25 µl volumes with the same recipe as the first taxon-specific PCR reactions except using 1 µl template (1:100 diluted PCR product obtained from the initial taxon-specific PCR) and relevant primer sets. The thermal cycling programmes were performed with an initial denaturing at 94°C for 5 min, followed by cycles (20 cycles for α - and β -Proteobacteria, and 25 cycles for Pseudomonads) at 95°C for 30 s, various annealing temperature for 30 s (56°C for α - and β -Proteobacteria and 62°C for Pseudomonads) and 72°C for 1 min, before a final extension step at 72°C for 10 min. PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel and ethidium bromide staining.

2.2.6 PCR Amplification of Fungal 18S rRNA Gene Fragments

Fungal 18S rRNA genes were amplified from cDNA and selected DNA samples using primers NS1 and FR1-GC (Table 2.1). The PCR reaction was carried out in 25 µl volumes containing 1.5 µl cDNA template (converted by FR1 primer, 1:10 diluted) or RT-PCR negative control, 1 x OptiBuffer (Bioline), 0.2 mM dNTPs (Promega Corporation), 0.25 µM each primer, 4 mM MgCl₂, 1.25 µg bovine serum albumin (BSA; Promega Corporation), 2% dimethyl sulfoxide (DMSO; PCR reagent, Sigma-Aldrich) and 2 U Bio-X-ACT DNA polymerase (Bioline). For all PCR reactions DNase/RNase-free distilled water (Invitrogen) was used. PCR amplifications were performed in a Bio-Rad iCycler with an initial denaturing step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 3 min, before a final extension step at 72°C for 7 min. Amplified PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel and ethidium bromide staining.

2.2.7 DGGE

PCR products were applied on DGGE gels for microbial community analysis using Dcode Universal Mutation Detection System (Bio-Rad) according to the manufacturer's instructions. The DGGE protocol was based on the initial protocol of Muyzer et al. (1993) and was performed using different linear denaturing gradients (100% denaturant contained 7 M urea and 40% (vol/vol) formamide) (recipes of all the solutions for DGGE gels are shown in Appendix 2.4). Aliquots of PCR products (6 to 8 µl) mixed with 5 µl DGGE loading dye (Appendix 2.4) were loaded on DGGE gels. The loading positions for the samples were randomized to avoid any gel position interference.

General bacterial 16S and taxon-specific 16S rRNA PCR products were loaded on 8% polyacrylamide gels (acrylamide/bisacrylamide (37.5:1)) with different denaturing gradients summarised in Table 2.2, topped with 9 ml of stacking gels (0% denaturant).

The gels were performed in 0.5 x Tris-acetate-ethylenediamine tetraacetic acid (TAE) buffer at 60°C at 200 V for 10 min and followed by 80 V for 18 h.

Fungal 18S PCR products were loaded on a 6% polyacrylamide gel with a 0-40% denaturing gradient. To simplify the gel preparation step, no stacking gel was loaded on top of the fungal gradient gel. The gels were run in 0.5 x TAE buffer at 60°C at 200 V for 10 min, followed by 180 V for 17 h (Table 2.2).

Acid silver staining was used to visualise the bands on DGGE gels as described by Sanguinetti et al. (1994). The recipes of reagents used in this staining method are described in Appendix 2.4. Gels were dried for 4 h at 70°C before being scanned using a GS-800 Imaging Densitometer (Bio-Rad).

Table 2.2 Details of the DGGE gradient and running conditions used for separation of the different ribosomal RNA target.

Taxonomic community	Polyacrylamide percentage (%)	DGGE gradient (%)	Running voltage (V)	Running time (h)
Bacterial 16S	8	35-68	80	18
α –Proteobacteria	8	42-65	80	18
β –Proteobacteria	8	45-65	80	18
Pseudomonads	8	40-65	80	18
Fungal 18S	6	0-40	180	17

Bacterial DGGE marker DNA used on gels profiling bacterial communities consisted of bulked bacterial 16S PCR products amplified from DNA samples of pure strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp. (O'Callaghan et al. 2008). All the DNA samples were kindly provided by E. Gerard (AgResearch, New Zealand). The PCR recipe and conditions were described in Section 2.2.4. This DGGE marker was applied on both general bacterial 16S DGGE and taxon-specific 16S DGGE gels. Fungal DGGE marker was made from the composite fungal 18S PCR products of five fungal isolate DNA samples which were kindly provided by Dr. J. Lottmann (AgResearch, New Zealand). These isolates were *Umbelopsis ramanniana* strain FR17, *Trichoderma reesei* strain FR19, *Mortierella chlamydospora* strain FS13, *Phoma cucurbitacearum* strain FS3 and *Clonostachys rosea* strain FS7. The PCR recipe and conditions are described in Section 2.2.6. A volume of 5 µl of either bacterial or fungal DGGE marker mixed with 5 µl DGGE loading dye was loaded on corresponding DGGE gels together with the samples. The DGGE marker was loaded in both the outside lanes and one middle lane on each gel to allow accurate alignment of bands. Incorporation of

DGGE markers on each gel was used to increase the accuracy of the interpretation of band position and the comparison between several gels.

2.2.8 Analysis of DGGE Gels

DGGE gels were scanned with a GS-800 Imaging Densitometer (Bio-Rad) using Quantity One software (version 4.4.1; Bio-Rad). The positions of bands were converted into R_f value using the Diversity Database software (version 2.2.0; Bio-Rad), and analysed based on the presence/absence of bands, using PCoA with software developed by D. Baird (VSN (NZ) Ltd) and as published previously (Clough et al. 2009; O'Callaghan et al. 2008). Lanes were aligned using a Viterbi algorithm for dynamic warping (Glasbey et al. 2005). Peaks were identified by moving a Gaussian model along the series, with the peak position taking all points on the curve, and at each position the amplitude and spread of the peak were estimated. The potential peak positions were then ranked by an index combining amplitude and quality of fit (percentage of variation explained by the model). The top ranked points on this index down to a given cut off value were taken as the peak positions for each curve. The peaks were clustered into bands using a k -means cluster analysis. A similarity matrix between samples was created using the Jaccard similarity measure (Gower 1985) for the presence and absence of bands. The similarity matrix was reduced to 5 dimensions using PCoA, and linear discriminant analysis was used to evaluate the differences between the treatments. The 95% confidence regions around the group means depicted in Figures 3.13 to 3.15 (Chapter 3) were produced by the GenStat DISCRIMINATE procedure (Payne et al. 2007). The significance of the treatment differences were assessed using a Hotelling T^2 -test (Hotelling 1947). Note the confidence regions in the graphs cannot be used to determine whether treatment means are significantly different to each other as these relate only to the variation of a single treatment in two dimensions, whereas the Hotelling T^2 -test uses the joint variation of treatments in the full 5 dimensions of the discriminant space. The confidence regions are presented only to give an indication of the variability of the means. All analysis was done using GenStat™ 10.0 (Payne et al. 2007).

Chapter 3 Rhizotron Sampling Techniques and Spatial Variability in Soil Microbial Communities in a Rhizotron System

3.1 Introduction

Plants release a significant amount of photosynthate into the rhizosphere through root exudates (Grayston et al. 1996; Uren 2007). However, because root exudates occur in a narrow zone of rhizosphere soil around roots and are rapidly consumed by soil microorganisms, these exudates are poorly quantified (Paterson 2003; Wardle 2002). Low molecular weight OAs are important components of plant root exudates, as they contribute to plant growth and are involved in a range of rhizosphere processes (Bertin et al. 2003; Ryan et al. 2001). For example, they can chelate cations in soil, such as Al that may be phytotoxic to plant growth or can dissolve unavailable nutrients (e.g. P, iron) to assist in nutrient uptake by plants (Fan et al. 2001; Jones et al. 1996a; Marschner 1995; Rengel et al. 1998a; Roelofs et al. 2001). In addition, OAs in root exudates are a major C source for microorganisms in the rhizosphere and thus may have a major influence on the structure and function of microbial communities (Jones 1998; Marschner 1995).

The study of root exudates is a significant challenge because of the technical difficulties associated with accessing the rhizosphere without disturbance or damage to plant roots, and significant spatial and temporal variability in the structure of root systems and release of root exudates by roots (Chapter 1, Section 1.1.4.1; Phillips et al. 2008). It is especially challenging to study root exudates of trees in the field where deep roots are difficult to access (Phillips et al. 2008). Therefore, it is not surprising that, of the limited number of studies that have investigated tree root exudates, most have been carried out using seedlings grown in highly controlled environments (Agnihotri and Vaartaja 1967; Bowen 1969; Fries et al. 1985; Krupa and Fries 1971; Leyval and Berthelin 1993; Malajczuk and McComb 1977; Norby et al. 1987; Sandnes et al. 2005; Slankis et al. 1964; Smith 1969; 1970). Only a few studies have been reported where the root exudates were examined in mature trees under field conditions (Malajczuk and Cromack 1982; Phillips et al. 2008; Sandnes et al. 2005; Smith 1970; Smith 1976). Of these, some studies identified the composition of root exudates and quantified specific components in root exudates, with most of these findings based on experiments using seedlings and very simple techniques such as paper chromatography. Although other approaches have been developed to collect root exudate samples, including *in situ* techniques, with their respective advantages and disadvantages (Chapter 1, Section 1.1.4.1; Neumann et al. 2009), there is currently no suitable approach to study root exudates from trees *in situ* without

disturbance of plant growth. Therefore, a new *in situ* collection approach needed to be designed to obtain a better understanding of root exudates processes and their influence on rhizosphere activities.

Due to numerous biotic and abiotic elements, spatial variability in distributions of soil microorganisms is commonly present in the soil environment (Ettema and Wardle 2002). As a subset of the soil environment, the rhizosphere is more complex and influenced by soil as well as plant roots (Buée et al. 2009; Marschner et al. 2004). Spatial and temporal variations have been observed in rhizosphere microbial communities associated with different parts of the roots or plants at different growth stages (Duineveld et al. 1998; Marschner et al. 2001; Yang and Crowley 2000). Therefore, a suitable sampling strategy is needed to obtain meaningful data. In addition, understanding the spatial variability in the rhizosphere community is also important in elucidating rhizosphere processes. Spatial variability in soil microbial communities has been found at different scales in soil, ranging from millimetres to hundreds of metres, in both horizontal and vertical directions (Grundmann and Debouzie 2000; Morris 1999; Parkin 1993; Ritz et al. 2004). As a result, no one sampling strategy is available which is suitable for all situations in which soil or the rhizosphere sampling is required. Therefore, the variability in microbial communities in rhizosphere soils needs to be investigated to allow design of a suitable sampling strategy for each experiment according to the research objectives.

As most of the root mass is present in the topsoil, especially for economically important agricultural crops, many studies (including those of crops and trees) of rhizosphere microbial communities have limited sampling to roots in the top 15 cm of soil (Andreote et al. 2009; Smalla et al. 2001) and thus ignored the rhizosphere microbial communities in the deeper soil layers. Unlike crops with shallow root systems, trees have an average maximum rooting depth of 7.0 ± 1.2 m in most ecosystems (Canadell et al. 1996). Therefore, the rhizosphere microbial community associated with tree root samples located at greater depth may be as important as that in topsoil. In addition, Rosling et al. (2003) reported that ectomycorrhizal species associated with forest root tips at deeper soil layers were different from those in the topsoil and some of the species were only detected with the root tips in deeper soil layers. This finding strongly supports the necessity of including studies of the rhizosphere microbial community in deep soil depths and vertical variability to gain a better understanding of tree rhizosphere processes.

The New Zealand Biotron recently established at Lincoln University is a purpose-built facility for the study of plant-soil interactions. The large-scale rhizotron inside the Biotron was designed with the aim of providing a novel system for studying rhizosphere processes. In this experiment, radiata pine was used as a model plant for development and

evaluation of this novel rhizotron system for investigation of rhizosphere processes. Preliminary rhizotron experiments aimed to i) develop an *in situ* collection technique of OAs and ii) assess variability in soil microbial communities in the rhizotrons. The development of an organic anion (OA) collection system included the establishment of a HPLC method for detection and quantification of selected OAs, together with evaluation of AEM for collection of OAs. Analysis of variability in soil microbial communities in the rhizotron included assessment of the differences between rhizosphere and non-rhizosphere communities, together with variability in rhizosphere communities between and within (at both horizontal and vertical directions) rhizotrons.

3.2 Materials and Methods

Prior to the development of the OA collection technique, a routine HPLC analysis method was set up for profiling of OAs in exudate samples collected from both validation experiments (Section 3.2.2) and the preliminary rhizotron experiment (Section 3.2.4). The selection of OAs for root exudate analysis was based on a GC-MS scan of root exudates of radiata pine seedlings collected in 0.2 mM CaCl₂ trap solution as described in Appendix 3.1. The GC-MS results are also outlined in Appendix 3.1.

3.2.1 Detection and Quantification of OAs by HPLC

A HPLC method was developed to detect and quantify a range of OAs (tartarate, quinate, formate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate) in root exudates of radiata pine. A large peak caused by inorganic ions appeared at the same retention time as the oxalate peak (4.568 min) in HPLC chromatogram (as also reported by Neumann (2006)) (see Appendix 3.6 for detail), so oxalate was excluded from this study. However, its presence in root exudates is acknowledged. The analysis of HPLC was conducted with an Alltech 426 HPLC pump (Alltech, USA), a Waters 717 plus autosampler (Waters Pty Ltd, USA), a prevailTM organic acid column (250 x 4.6 mm, 5 µm particle size) (Grace, USA) with a prevailTM organic acid guard column (7.5 x 4.6 mm, 5 µm particle size) (Grace) used in-line prior to the analytical column, a column oven together with a heat control system and a Waters 490 E programmable multiwave-length UV detector.

Various analysis conditions (including addition of solvent into the eluent, different eluent pH values, column temperature and flow rates), were tested for the separation of OAs and their peak shapes (sharpness), to aid selection of the optimum conditions for OA analysis (detailed in Appendix 3.2). The eluent was degassed 25 mM KH₂PO₄ (pH 2.5) at a flow rate of 0.6 ml min⁻¹. All the samples were thawed immediately before analysis. Sample volumes of 30 µl were injected into the system and separated at 40°C and OAs were

detected according to their retention times and absorbance peaks at 210 nm. Standard mixtures of OAs prepared with various concentrations of analytical grade of tartaric acid, quinic acid, formic acid, malic acid, malonic acid, shikimic acid, lactic acid, acetic acid, maleic acid, citric acid, succinic acid and fumaric acid (Sigma-Aldrich, UK) were used to calibrate the system and generate standard curves to determine the limits of detection and calculate the concentrations of OAs in samples (Table 3.1).

Recovery studies were carried out by adding three different concentrations of the OA mixture (containing final concentrations of 2 mg l⁻¹, 10 mg l⁻¹ or 20 mg l⁻¹ of each anion) to root exudate samples collected from rhizotrons (method see Section 3.2.4) and calculating the recoveries by comparing the samples with the corresponding samples not spiked with OA mixtures. All data were processed using Chromeleon analysis software (version 6.80, Dionex Corporation, USA).

3.2.2 Validation of AEM Collection of OAs

Filter paper and pre-charged AEM have both been used for root exudate collection (Neumann et al. 1998; Neumann and Römheld 1999; Schefe et al. 2008; Zhang et al. 2001) and thus were compared as collection media. The collection mechanism of AEM is the pre-charged HCO₃⁻ on the surface of AEM strips can exchange with other anions present in soil or solution. In a preliminary study, AEM showed a higher efficiency of recovery of OAs from the soil in comparison to Whatman 3MM filter paper (Appendix 3.3). Therefore, AEM was selected for use in further validation experiments carried out in the laboratory as described in the following subsections. Three replicates were used in all the experiments unless otherwise stated. In all cases, samples were stored at -20°C until analysis by HPLC.

3.2.2.1 Recovery of OAs by AEM

Anion exchange membranes (No. 55164 2S, BDH Laboratory Supplies, England) were cut into strips (4 x 6 cm), soaked in DI water for 24 h, followed by loading with four exchanges of 0.5 M NaHCO₃ and stored in 0.1M NaCl at 4°C until use. The ability of AEM strips to collect and retain OAs was tested by loading 250 µl or 500 µl of an OA mixture (12 OAs at a concentration of 500 mg l⁻¹ for each anion) onto the AEM surface evenly with a pipette. Sterile DI water was loaded on the AEM strip as a control. After 1 h, AEM strips were then shaken briefly to remove excess solution before being placed into sterile flasks containing 10 ml of 0.5 M HCl solution and the flasks were shaken at 150 rpm at 4°C for several hours to elute the anions.

3.2.2.2 The effect of elution times on recovery of OAs from AEM

Four different elution times (1, 2, 3 and 4 h) were tested for elution of anions from AEM loaded with 500 µl of an OA mixture (12 OAs at a concentration 500 mg l⁻¹ for each anion). After 1 h, AEM strips were shaken briefly to remove excess solution before being placed into sterile flasks and eluted with 10 ml of 0.5 M HCl solution at 4°C for 1, 2, 3 or 4 h with shaking at 150 rpm.

3.2.2.3 Recovery of low concentration of OAs by AEM

The ability of AEM to collect low concentrations of anions from the solution, as would be expected from root exudates, was tested by soaking strips (6 x 4 cm) in Petri dishes containing 20 ml of an OA mixture (12 OAs at a concentration of 30 mg l⁻¹ for each anion). After incubation for 1 or 3 h, AEM strips were removed from solutions and shaken briefly before being placed into 0.5 M HCl for elution in a shaker at 150 rpm at 4°C for 3 h. As a negative control, AEM strips that had not been soaked in any solution were eluted using 0.5 M HCl.

3.2.2.4 Stability of OAs on AEM in contact with soil

To validate the suitable use of AEM to collect OAs from soil, the retention of OAs captured by AEM strips when in contact with soil (i.e. whether these captured OAs would be stable on AEM during collection period rather than be quickly decomposed by soil microorganisms or absorbed by soil solid phase) was tested. Silt loam soil from Iversen Field, Lincoln University, also used in subsequent rhizotron experiments (Chapters 4 and 5), was used at a moisture content of 16%, which was similar to the soil moisture level maintained in rhizotrons. This was prepared by mixing the soil with DI water. Membrane strips were loaded with 500 µl of an OA mixture (12 OAs at a concentration of 500 mg l⁻¹ for each anion) for 1 h as described previously. The strips were shaken briefly to remove excess solution before being placed onto wetted filter paper (so that the membrane strips were kept moist according to the manufacturer's instructions). Strips were then covered with a thin layer of soil (60 g of evenly spread soil per strip) and placed in the dark at room temperature for 3 h. Both Whatman 3MM and Whatman GB/F were tested for their ability to keep AEM moist during the assay period and possible interference on the stability of anions on AEM strips. Filter paper, cut to the same size as the AEM strips, was washed in methanol followed by sterile DI water before use. For controls, loaded AEM strips or AEM strips backed with filter papers were incubated in the dark in the absence of soil (two replicates each). Following incubation, AEM strips were briefly rinsed with DI water and eluted for 3 h with 0.5 M HCl as previously outlined.

3.2.2.5 Collection of OAs by AEM from soil

In order to collect OAs from the rhizosphere, AEM should be able to collect OAs present in soils, as exudates only occur in a narrow zone at the soil-root interface. Silt loam soil (500 g) was mixed with 77.5 ml of an OA mixture (12 OAs at a concentration of 500 mg l⁻¹ for each anion) or DI water to give a soil moisture content of 16%. Anion exchange membrane strips or AEM strips backed with moistened Whatman GB/F or Whatman 3MM filter papers were placed under thin layers of soil (60 g of evenly spread soil) mixed with either OA solution or DI water as controls. After 3 h, AEM strips were removed from soil and rinsed briefly with DI water to remove soil particles before being eluted with 10 ml of 0.5 M HCl for 3 h as previously described. Controls were also included, which involved placing AEM strips on filter papers only in the absence of soil.

3.2.3 Rhizotron Experimental Set up

The New Zealand Biotron has been specifically designed to operate a controlled environment both above and below the soil surface (Figure 3.1a). Above-ground, there are four Conviron BDW120 plant growth rooms (5 m x 2.4 m) (Conviron, Controlled Environments Ltd, Canada) equipped with metal halide (Model MS400W/HOR, Venture Lighting International Inc., USA) and incandescent bulbs (100W, Phillips, Malaysia), mounted above a clear perspex barrier, and a downward airflow distribution system using sufficient outdoor make-up air to provide ambient CO₂ conditions inside the room (Figure 3.1c). The rooms have controlled light (0 to 1150 μmol m⁻² s⁻¹), temperature (-10 to 40°C), relative humidity (35 to 95%) and CO₂ concentration (ambient to 2000 ppm). The floor of each growth room is fitted with four stainless steel rhizotron cylinders (0.8 m diameter x 0.8 m deep - each containing up to 0.4 m³ of soil), which can be maintained at a range of temperatures (8 to 25°C) and are able to replicate similar vertical temperature profiles to those found under field conditions (Figure 3.1b).

To increase the number of replicates available for use in experiments, each rhizotron container was split vertically to provide two independent growth containers with 0.25 m² in surface area (Figure 3.1d). The independent half rhizotrons are subsequently referred as rhizotron units throughout the thesis. Soil used in this preliminary experiment was supplied by a local garden centre and was sieved through a 5 mm sieve before being packed into each rhizotron unit (0.5 m depth) with a bulk density of 1.2 g cm⁻³ for the top 20 cm of soil and 1.4 g cm⁻³ for soil located between 20-50 cm. The key characteristics of this low fertility soil were analysed by Hill Laboratories Ltd (Hamilton, New Zealand), and are provided in Appendix 3.4. One of eight radiata pine seedlings (approximate 30 cm tall), supplied by Scion Research (Rotorua, New Zealand), was planted in the centre of each of the eight rhizotron units in a growth room after removing the majority of the potting mix

from around the root system. The air temperature in the growth room was maintained at 20/12°C ($\pm 0.5^\circ\text{C}$) during the light (6 am to 8 pm) /dark (8 pm to 6 am) period. Soil temperature was maintained at 14°C ($\pm 2^\circ\text{C}$) at a depth of 30 cm, but was subject to fluctuations in temperature at the soil surface (similar to field conditions) depending on air temperature and lighting. The photosynthetically active radiation at the top of the canopy was maintained at a minimum of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the 14 h photoperiod, but was elevated to $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ for four hours during the middle of the photoperiod. Relative humidity in the room was maintained at 68% during the light period. This condition was selected to mimic spring conditions in Canterbury, New Zealand. The plants were watered three times weekly with 1 l per rhizotron unit at each time. Hand weeding was conducted every two weeks.



Figure 3.1 a). Rhizotron system in the New Zealand Biotron facility. Left: cross section view of a growth room positioned above a rhizotron room. Right: front view of a growth room positioned above a rhizotron room. b). Stainless steel rhizotron cylinder in the downstairs rhizotron room. c). *Pinus radiata* in the upstairs growth room after one year growth. d). Packing the soil into the rhizotron which has been divided into two halves.

After seedlings had been growing in the rhizotron units (one seedling per rhizotron unit) for three months, horizontal soil cores (5 cm diameter x 45 cm depth) were removed at three depths (two cores at each depth of 10, 20 and 30 cm) to create access portals for monitoring root growth and periodic collection of root exudates (Figure 3.2a and d). The process of creating the access portal is shown in Appendix 3.5. The physical integrity of the access portals was maintained using removable inflatable tubes between samplings (Figure 3.2b). The inflatable tubes, which were made from Santoprene (Elastomer Products Ltd, New Zealand), effectively sealed the rhizotron access portal and thereby supported normal root growth. A sealed motorised camera system contained within a perspex tube enabled 360° viewing and recording throughout the access portal (Figure 3.2c and e). The access portals facilitated the monitoring of root growth and morphology, and allowed for collection of roots, rhizosphere soil, and root exudates (Figure 3.2d and e).

3.2.4 *In situ* Collection of OAs in the Rhizotron System

Four months after the creation of access portals in the rhizotrons (seven months after planting), observations of roots through the camera system in the access portals revealed that roots had re-colonized the space around the access portals and appeared healthy (Figure 3.2d). To validate that the developed *in situ* technique for collection of OAs, exudates from a wide range of root types (including root tips, mature roots, etc.) were collected from approximately one-year-old pines in rhizotron units through different access portals located at depths of 10, 20 and 30 cm (Figure 3.1c). Before sampling, a camera system was used to locate roots in access portals (Figure 3.2e). Anion exchange membrane strips backed with moistened Whatman 3MM were placed on the inflatable tubes according to the position of roots observed by the camera system (Figure 3.2f) and maintained in close contact with the area of the selected roots by positioning and inflating the tubes inside the access portals in the rhizotrons. Membrane strips with Whatman 3MM were also placed in contact with a region of adjacent “non-rhizosphere soil” where no roots were visible. After 3 h, the AEM strips were removed and rinsed with DI water before being placed into 10 ml of 0.5 M HCl solution for elution for 3 h as described in Section 3.2.2.2. In total, exudates from 24 root areas and 12 non-rhizosphere soil areas were collected. Root and rhizosphere exudate solutions and non-rhizosphere soil solutions were stored at -20°C until analysis by HPLC.



Figure 3.2 a). The soil corer used for creating access portals in the rhizotron. b). The physical integrity of the access portal is maintained using a removable inflatable tube between samplings. c). The sealed motorised camera system for image and position recording, with a close up of a motorised camera inside the perspex tube. d). The access portal after removing the inflatable tube for root monitoring or sampling. e). The camera system recording the root image in the access portal. f). AEM strips backed with Whatman 3MM (indicated by blue arrows) fixed on an inflatable tube for root exudates collection in the access portal.

3.2.5 Analysis of Variability in Soil Microbial Communities

This experiment was designed to investigate variability in rhizosphere microbial communities between and within rhizotron units, with the aim of identifying some spatially predictable variability of microbial communities in the rhizosphere of radiata pine, and consequently designing an appropriate sampling strategy for the subsequent rhizotron experiment (Chapter 4).

Two of the eight rhizotron units in two different rhizotron compartments in one growth room were selected at random for sampling (Figure 3.3). Rhizotron unit A (located at the front side of the growth room) and rhizotron unit B (located in the middle of the growth room) were both sampled using vertical cores at three sites based on direction and distance to the pine tree (L: left, 25 cm from the tree base, M: middle, 16 cm from the tree base and R: right, 28 cm from the tree base) (Figure 3.3). At each sampling site, soil cores containing soil and roots were taken from the top 15 cm, 16-30 cm and 31-45 cm regions using a soil corer (diameter 10 cm) in three steps with the corer pushed to the corresponding depth in each case. The soil corer was cleaned with a 5% bleach solution and wiped dry between each sampling. On the same day as sampling, duplicate roots associated with rhizosphere soil samples (approximate 1.0 g) were collected from each soil core using ethanol (70%) sterilised forceps and gently shaken to remove non-rhizosphere soil for subsequent rhizosphere soil processing as described in Chapter 2, Section 2.2.1. The amount of rhizosphere soil sampled ranged from 0.08 to 0.29 g per g root sample and was stored at -80°C for subsequent extraction of RNA. Non-rhizosphere soil samples collected from areas free of roots from each soil core were bulked together to reduce the number of samples for processing. Samples (0.5 g) of the composite non-rhizosphere soil (2 replicates) were stored immediately in sterile 2 ml Eppendorf tubes at -80°C until subsequent RNA extraction. The molecular analysis of microbial communities using rRNA-DGGE technique is described in Chapter 2, Sections 2.2.2-2.2.7.

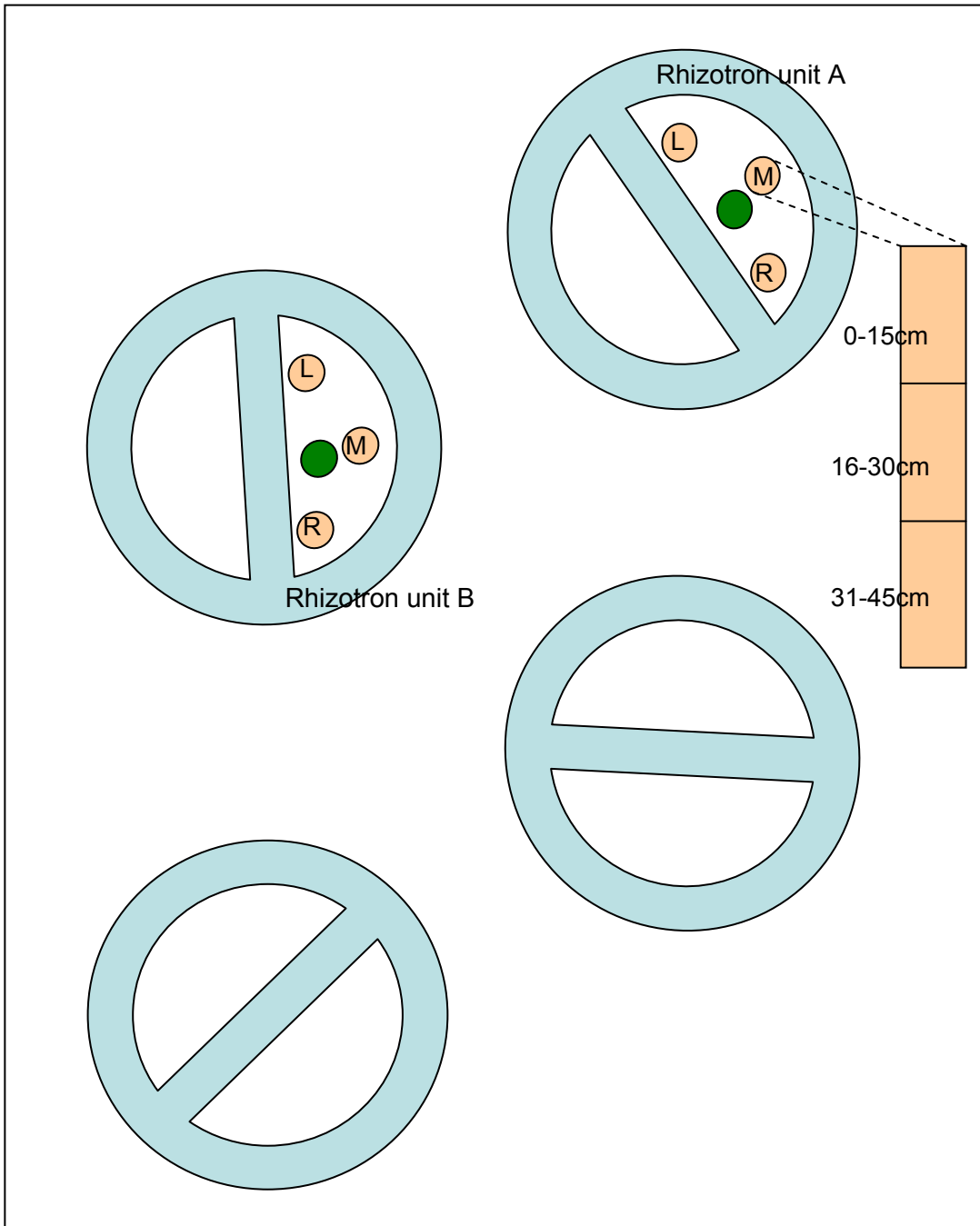


Figure 3.3 Vertical sampling in rhizotron units A and B and their position inside the growth room. Green circle represents radiata pine tree. Yellow circle represents sampling sites. Black line indicates the side of the growth room (5 x 2.4 m). Site L was about 25 cm away from the tree base, site M was about 16 cm away from the tree base, and site R was about 28 cm away from the tree base. Three depths (0-15 cm, 16-30 cm and 31-45 cm) were sampled at each site.

3.2.6 Statistical Analysis

Recovery or amounts of OA collected by AEM strips were compared between the different treatments using one or two way analysis of variance (ANOVA) using Genstat™ 11 (VSN International Ltd, Rothamsted, UK). In an unbalanced experiment design (Section

3.2.2.4), an unbalanced variance analysis was used. For multiple comparisons, treatment means were compared using Fisher's protected least significant differences (LSD) at $P = 0.05$ level. The statistical analysis of DGGE gels is detailed in Chapter 2, Section 2.2.8.

3.3 Results

3.3.1 Detection and Quantification of OAs by HPLC

The optimised HPLC method separated 12 of the selected OAs over a 17 min period with varying degrees of detection sensitivity (Figure 3.4). Amongst them, shikimate showed the largest peak area with 1142 mVxmin; whereas, quinate showed the smallest peak area with only 9 mVxmin, which was only 0.8% of the area of the shikimate peak at the same concentration of 30 mg l⁻¹. The limit of detection of the different anions was derived from calibration curves using six concentrations of OAs in mixed standard solutions with minimum limit of detection being defined by signal to noise ratios being greater than 3 (Table 3.1). The percent recovery of all OAs spiked into root exudate samples was consistently high and ranged between 98% and 106% (Table 3.1). Six different concentrations of each OA in mixed standard solutions for creating calibration curves are presented in Table 3.1.

Table 3.1 Calibration standards for 12 OAs, limit of detection and recovery of anions from spiked root exudate samples using the HPLC analysis method. The recovery from spiked soils are expressed as mean percent recovery ($n=3$) \pm 1 standard error.

Anion	Concentration of standards (mg anion l ⁻¹)	Limit of detection (mg l ⁻¹)	Recovery of anion in spiked samples of root exudate (%)
tartarate	2, 5, 10, 15, 20, 30	0.156	106 \pm 5
quinate	2, 5, 10, 15, 20, 30	0.470	106 \pm 6
formate	2, 5, 10, 15, 20, 30	0.255	99 \pm 4
malate	2, 3, 4, 5, 8, 10	0.300	102 \pm 3
malonate	2, 3, 4, 5, 8, 10	0.232	98 \pm 2
shikimate	0.5, 1, 2, 3, 5, 10	0.004	103 \pm 2
lactate	1, 2, 3, 4, 5, 10	0.415	104 \pm 4
acetate	3, 4, 5, 8, 10, 20	0.426	102 \pm 1
maleate	0.03, 0.04, 0.05, 0.08, 0.1, 0.3	0.006	101 \pm 2
citrate	1, 2, 3, 4, 5, 10	0.200	99 \pm 2
succinate	5, 8, 10, 15, 20, 30	0.750	101 \pm 1
fumarate	0.02, 0.04, 0.06, 0.1, 0.2, 0.5	0.006	103 \pm 3

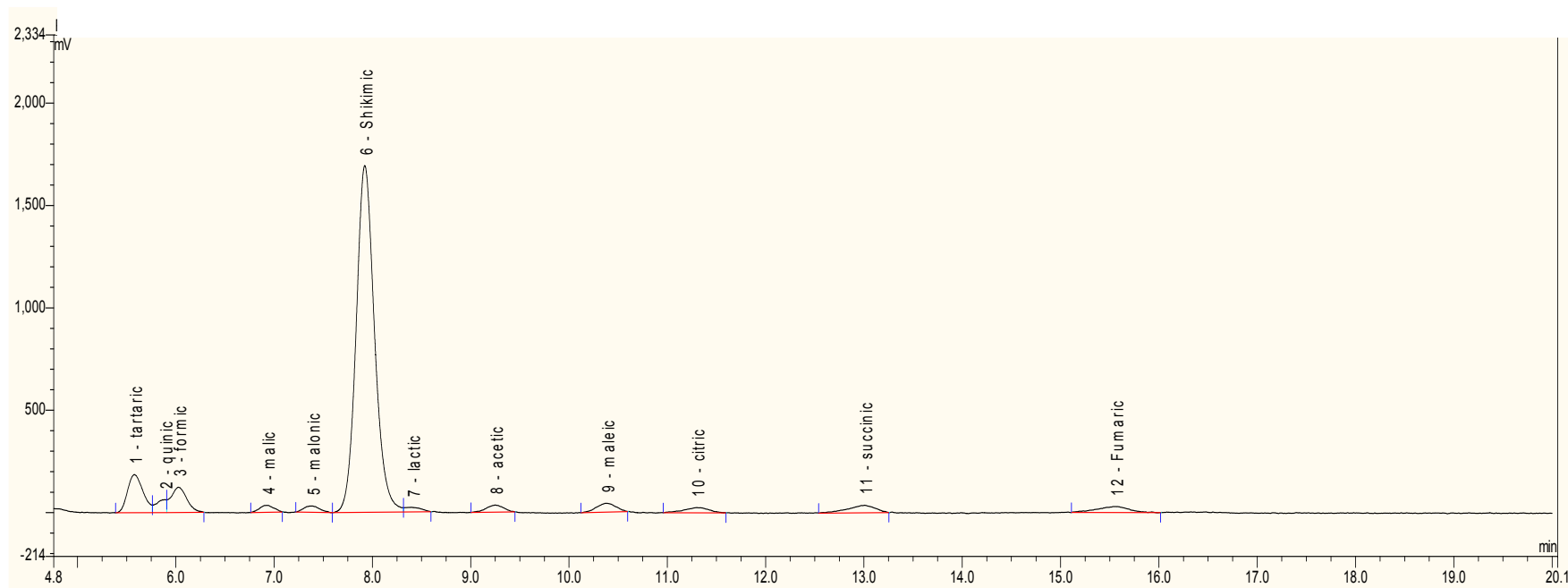


Figure 3.4 Separation of 12 OA standards by isocratic HPLC with 25 mM KH_2PO_4 (pH 2.5) at 0.6 ml min^{-1} at 40°C . The concentration of anions shown in this chromatogram were: 30 mg l^{-1} for tartarate, quinate, formate and succinate, 20 mg l^{-1} for acetate, 10 mg l^{-1} for malate, malonate, shikimate, lactate and citrate, 0.5 mg l^{-1} for fumarate and 0.3 mg l^{-1} for maleate.

3.3.2 Validation of the Use of AEM to Collect OAs

3.3.2.1 Recovery of OAs by AEM

All of the tested anions were captured by AEM strips when loaded with two different amounts of the OA mixture, although levels of recovery varied across anions (Figure 3.5). The recoveries of loaded and eluted OAs ranged from 23% (quininate) to 85% (succinate) when loaded with 250 μl of the OA mixture containing equal amounts of each anion, and from 27% (quininate) to 78% (maleate) when loaded with 500 μl of the OA mixture (Figure 3.5). No OAs were detected in control samples loaded with DI water. The recoveries of lactate, malate, malonate, succinate, as well as the collective average for the total of all anions (58 % and 51%, respectively) were significantly ($P < 0.05$) higher when loaded with 250 μl of the OA mixture as compared to 500 μl , although such a difference was generally considered not to be a major issue in relation to the saturation of the membrane (Figure 3.5).

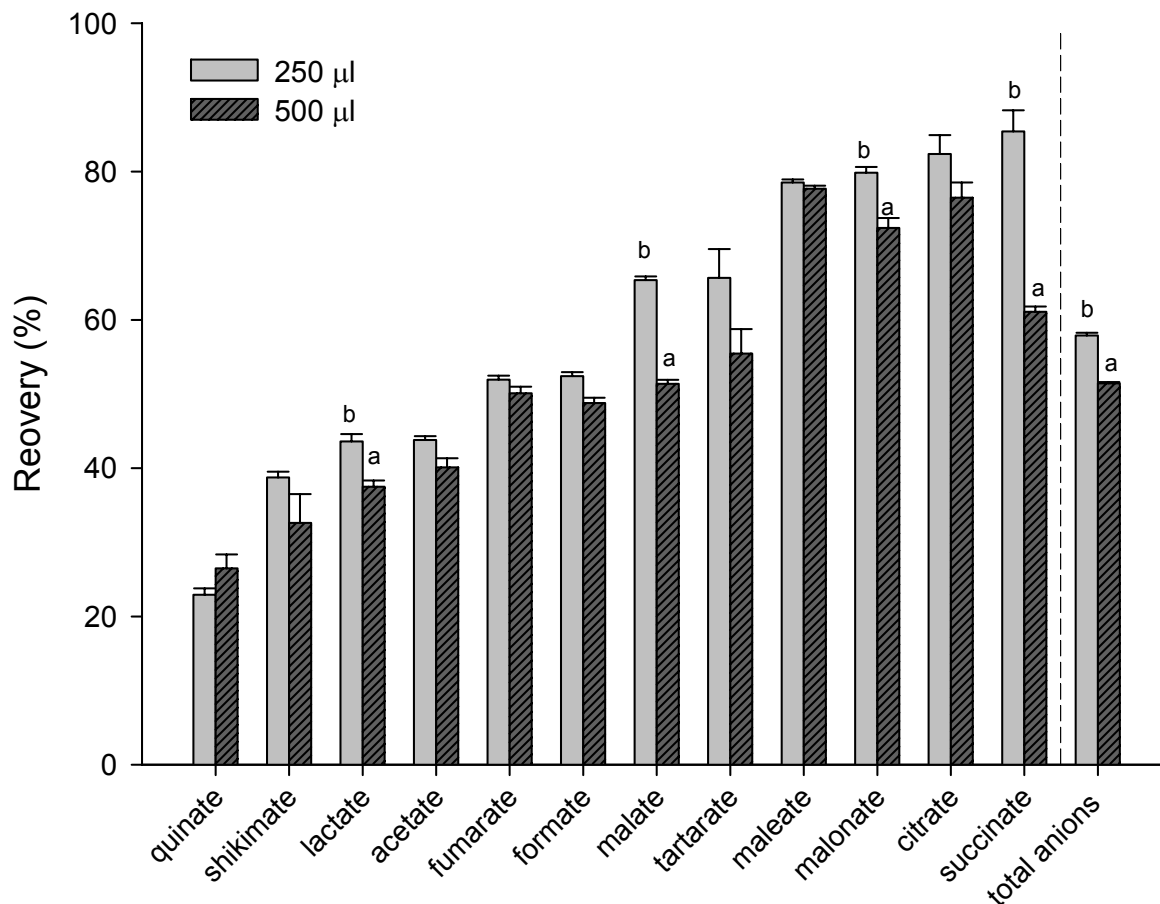


Figure 3.5 Recovery of different OAs from AEM. Shown is the percent recovery (%) of individual anions and the average total amount of anions recovered after loading AEM strips with 2 rates (250 μl and 500 μl) of an OA mixture (500 mg l^{-1} of each anion). Error bars show 1 standard error and for each OA, columns denoted with different letters are significantly different ($P < 0.05$) between the two loadings ($n=3$).

3.3.2.2 The effect of elution time on recovery of OAs from AEM

Elution time had relatively little effect on the recovery of anions from AEM, although in some cases (e.g. fumarate, citrate, malonate, maleate and tartarate) significantly less ($P<0.05$) anion was recovered after only 1 h (Figure 3.6). In these anions which were effected by elution time, elution for 3 h always gave significantly higher ($P<0.05$) recoveries than others (Figure 3.6). However, across all OAs, there was no significant difference ($P<0.05$) in the recovery of total anions over the four time periods and, a 3 h elution time was used in subsequent experiments.

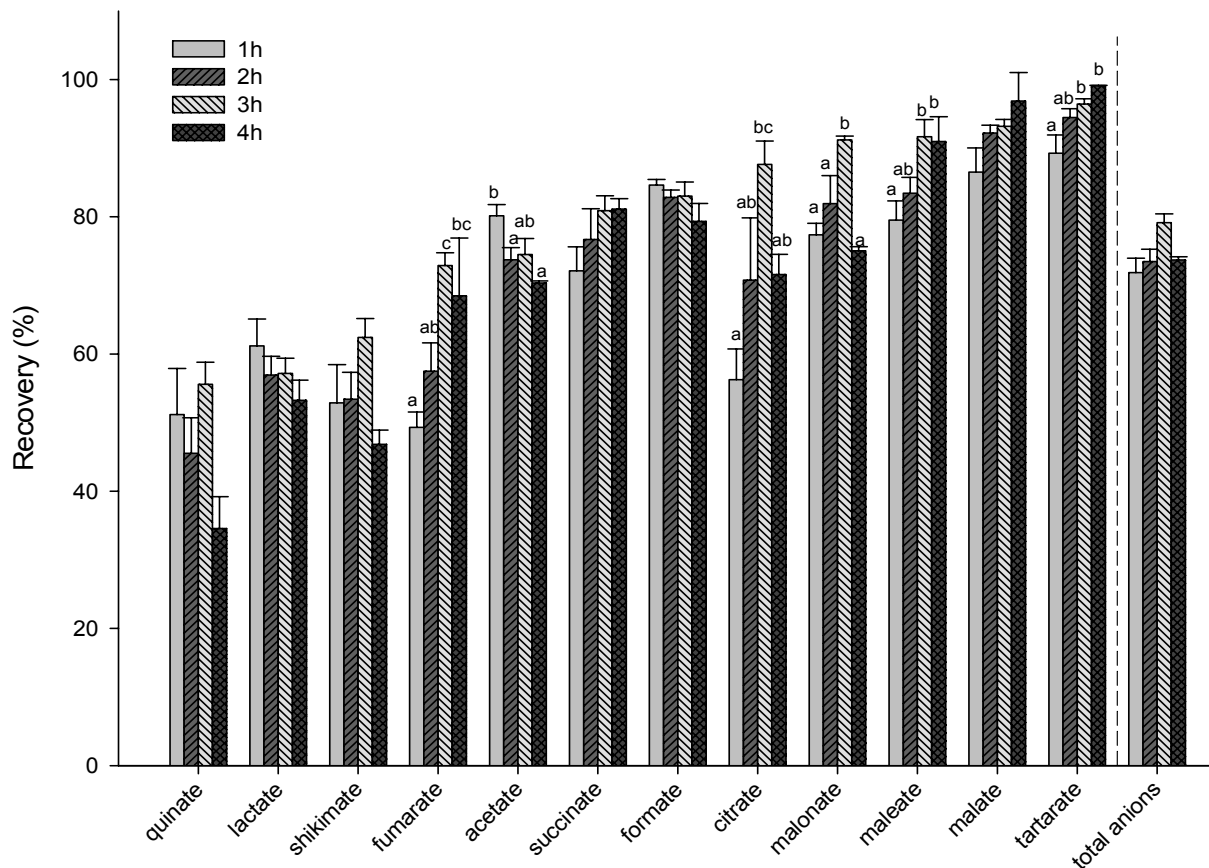


Figure 3.6 Effect of elution time on recovery of OAs from AEM. Shown is the percent recovery (%) of individual OAs and the average total amount of anions recovered after eluting membranes for 1 to 4 h that were loaded with 500 μl of an OA mixture (500 mg l^{-1} of each anion). Error bars show 1 standard error and for each organic anion, columns denoted with different letters are significantly different ($P<0.05$) across elution times ($n=3$).

3.3.2.3 Recovery of low concentrations of OAs by AEM

AEM strips were effective for capturing OAs when present at low concentrations in solution (30 mg l^{-1} for each anion) in Petri dishes over two collection times (1 and 3 h) (Figure 3.7). The amounts of anions collected and recovered from AEM ranged from 0.43 to 12.46 $\mu\text{g cm}^{-2}$ and 0.56 to 14.54 $\mu\text{g cm}^{-2}$ after 1 h and 3 h which represents 2 to 61%

and 2 to 76% of the total amounts of anions present in the solutions, respectively. The highest recovery occurred for maleate, with four other anions (citrate, tartarate, fumarate and malonate) showing more than 20% recovery of the total amounts of anions present in solutions. Averaged across all anions, there was no significant difference in the recoveries of OAs between the 1 h and 3 h collection periods. However, the amounts of lactate and formate were significantly higher ($P<0.05$) when collected by AEM for 1 h in comparison with 3 h (Figure 3.7). In contrast, AEM captured significantly higher ($P<0.05$) amounts of fumarate and maleate from solution for 3 h collection period in comparison with 1 h. A collection period of 3 h was considered to be appropriate for subsequent experiments. No interfering peaks, except for a void peak that appeared in front of all the detected anion peaks, were found in control samples in which AEM strips were placed directly into 0.5 M HCl elution solution.

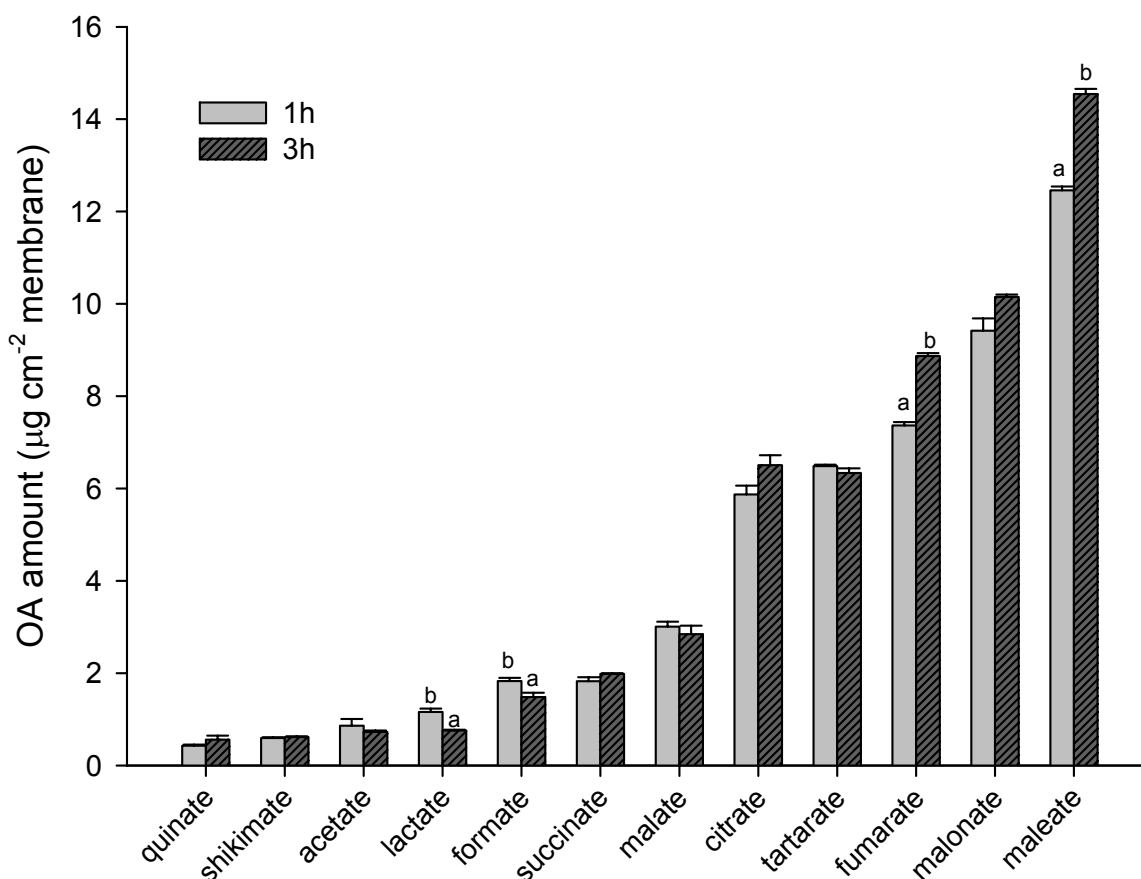


Figure 3.7 Capture of OAs from solution by AEM. Shown are the amounts of OAs collected ($\mu\text{g cm}^{-2}$) on AEM strips (24 cm^2) after 1 or 3 h from 20 ml of an OA mixture containing each anion at a concentration of 30 mg l^{-1} . Error bars show 1 standard error and for each OA, columns denoted with a different letter are significantly different ($P<0.05$) between the 2 collection periods ($n=3$).

3.3.2.4 Stability of OAs on AEM in contact with soil

The stability and subsequent recovery of OAs loaded onto AEM or AEM backed with moist filter papers (Whatman 3MM or GB/F) are shown in Figure 3.8. Irrespective of the collection method used, the recovery of most anions was influenced to a small extent only by the presence of soil and generally remained relatively stable when incubated in soil relative to the no-soil controls. However, for shikimate, lactate and formate, there was a significant reduction in amounts collected by all three methods following contact with soil. The largest effect of incubation of impregnated AEM in soil was a 38% reduction in recovery of shikimate loaded onto AEM backed with Whatman 3MM in comparison with that from the same collection method without soil incubation (Figure 3.8). The recovery of acetate was also significantly decreased ($P < 0.05$) after soil incubation of AEM with either of the filter papers as compared to AEM alone. Interestingly, the recoveries of fumarate and tartarate significantly increased after soil incubation either by AEM alone or AEM backed with filter paper. Across all anions and in all three collection systems, the presence of soil resulted in a net reduction in amounts of anions collected of 10, 13 and 9% for AEM, AEM with Whatman 3MM and GB/F, respectively (Figure 3.8). Across all anions, no significant differences due to the presence or absence of filter paper were evident.

3.3.2.5 Collection of OAs from soil by AEM

Anion exchange membranes were able to capture all the selected anions from the soil samples amended with an OA mixture, although efficiency of recovery of the anions varied (Figure 3.9). As expected, no OAs were detected in control samples, where AEM was incubated with soil amended with DI water or incubated in the absence of soil.

The amounts of anions collected and recovered from AEM over the 3 h incubation period were in the range of 4.38 to 23.6 $\mu\text{g cm}^{-2}$ (Figure 3.9). This represents a recovery of between 1 and 14% of the total amount of OAs added in soil. The anions with highest capture from the amended soils were succinate, formate and maleate, where greater than 10% of the total added amounts of anions were recovered. The use of filter paper backing (either 3MM or GB/F) did not affect the amounts of these OAs captured. The amount of malate, malonate, lactate and quinate recovered were significantly reduced ($P < 0.05$) on AEM with GB/F filter paper as compared to AEM alone or AEM with 3MM paper. With the exception of acetate, amounts of all other anions captured were not different between collection methods (Figure 3.9). Consequently, moistened Whatman 3MM filter paper was used to keep AEM moist during *in situ* collection of OAs from the rhizosphere.

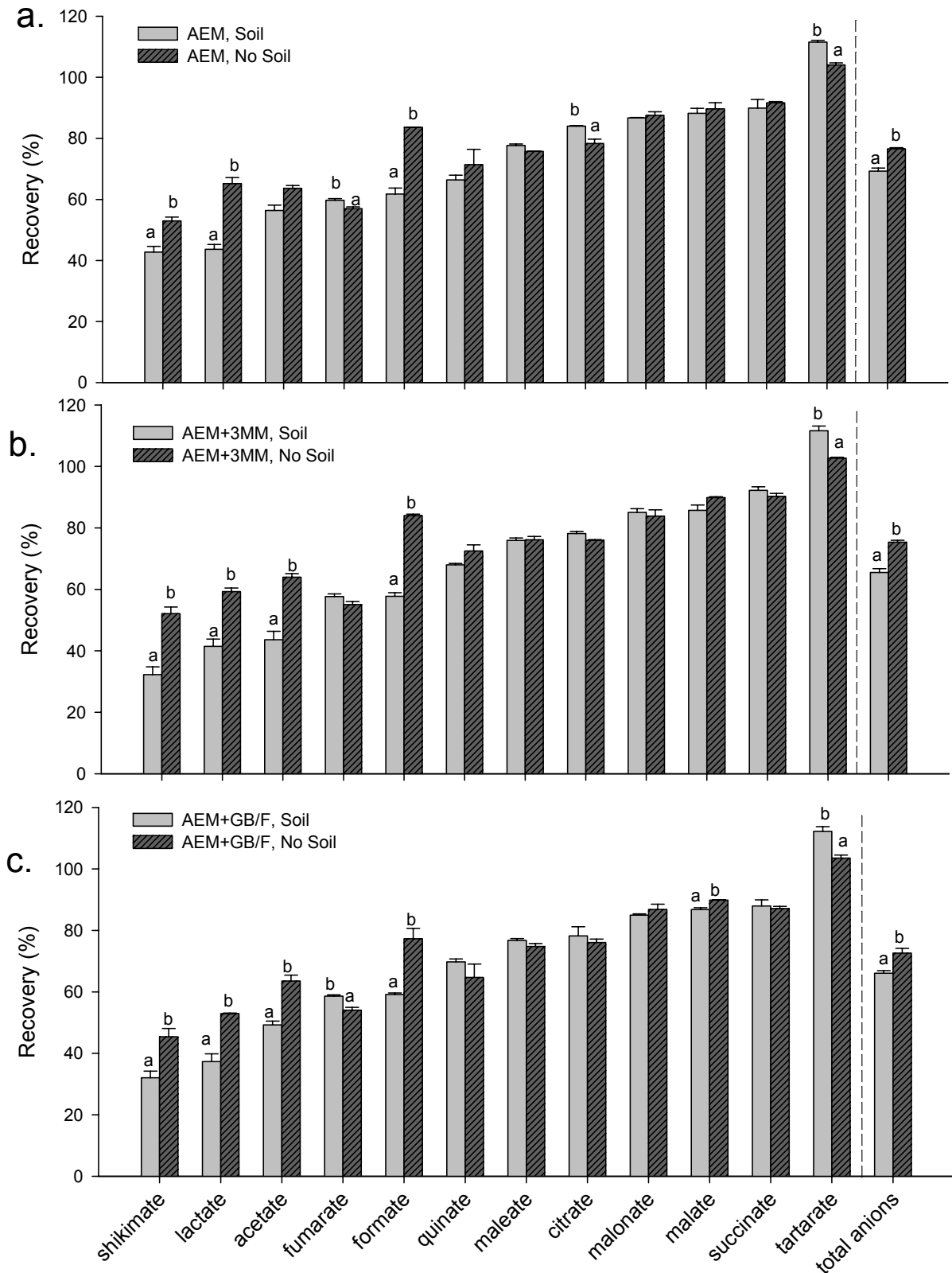


Figure 3.8 The stability and recovery of OAs loaded on AEM when incubated in soil. Shown is the percent recovery (%) of individual anions and the average total amounts of anions recovered after loading membranes with 500 μ l of an OA mixture (500 mg l⁻¹ of each anion). AEM were incubated for 3 h with or without soil either a). alone or backed with moist b). Whatman 3MM or c). GB/F filter papers. Error bars show 1 standard error and for each anion in each panel, columns denoted with different letters are significantly different ($P < 0.05$) between the soil ($n=3$) and no-soil incubations ($n=2$).

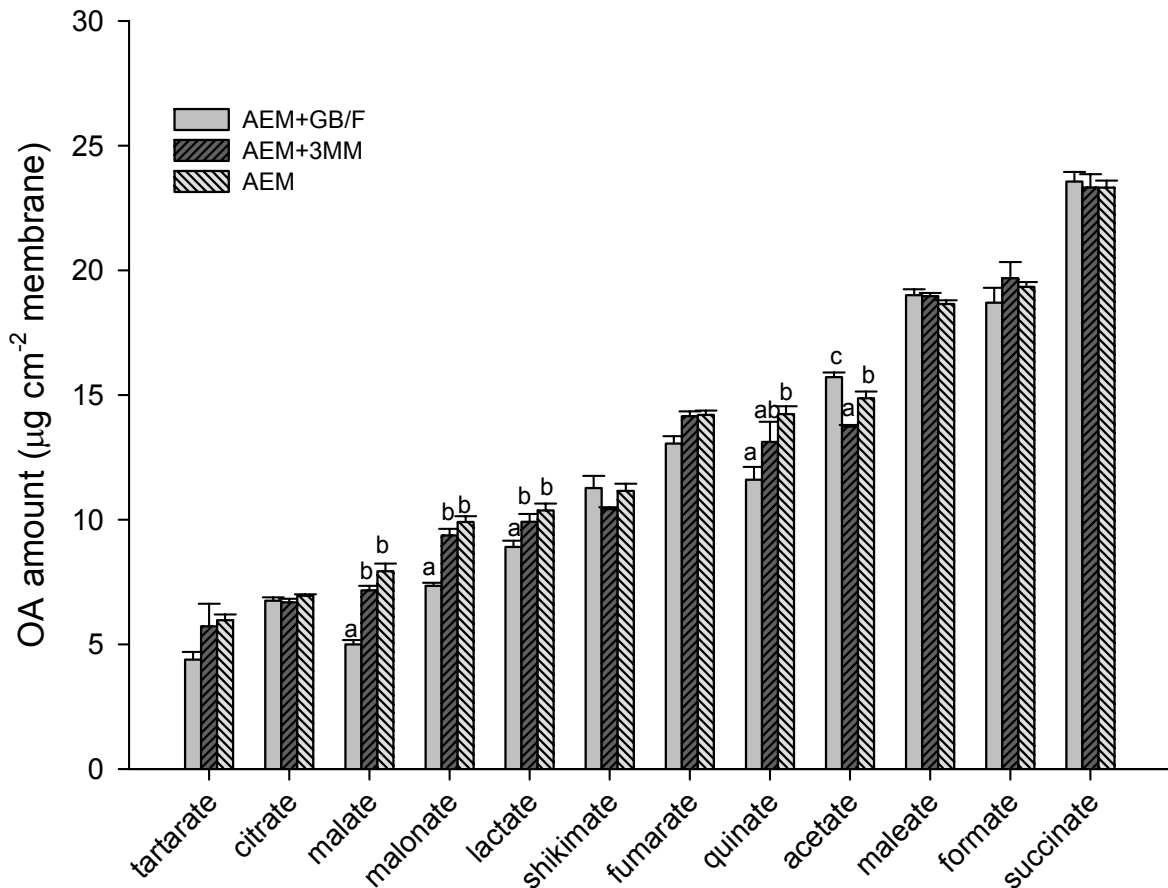


Figure 3.9 Capture of OAs from soil by AEM. Shown is the amount of each anion ($\mu\text{g cm}^{-2}$) collected on AEM strips (24 cm^2) over 3 h of incubation in soil (60 g) containing a mixture of OAs supplied at a concentration of $67.1 \mu\text{g}$ of each anion per g soil. Anion exchange membrane strips were incubated with the soil alone or were backed with either moist Whatman 3MM or GB/F filter papers. Error bars show 1 standard error and for each organic anion, columns denoted with a different letter are significantly different ($P < 0.05$) between collection methods ($n=3$).

3.3.3 *In situ* Root Exudate Sampling in Rhizotrons

3.3.3.1 Morphology of *Pinus radiata* roots in rhizotron units

Four months after creation of access portals, the morphology of radiata pine roots in the access portals in the rhizotron units was observed using the camera system. A number of different root morphologies were observed and a selection of the typical root morphologies as well as ectomycorrhizal colonized roots are shown in Figure 3.10. Ectomycorrhizal colonized roots were frequently observed in the access portals (Figure 3.10a to d). However, as ectomycorrhizae (ECM) was not the focus of this project, no further study was carried out to identify these ECM. In most cases the mantle of ECM could clearly be seen. The mycelia of ECM or other fungi were regularly observed to spread around roots and radiate out into the adjacent soil (Figure 3.10b to d), with a fungal hypha, possibly ECM, seen to connect two roots in one case (Figure 3.10c). Occasionally, fungal

sporulation, possibly by saprophytic fungi was observed on the surface of roots (Figure 3.10e). Apart from roots supporting growth of ECM, young roots with abundant root hairs and root tips, mature roots with root hairs and old roots were also observed using the camera system (Figure 3.10f, g and h). An estimate of scale can be made from Figure 3.10i, which shows a 5 mm thick rhizotron wall imaged by the camera.

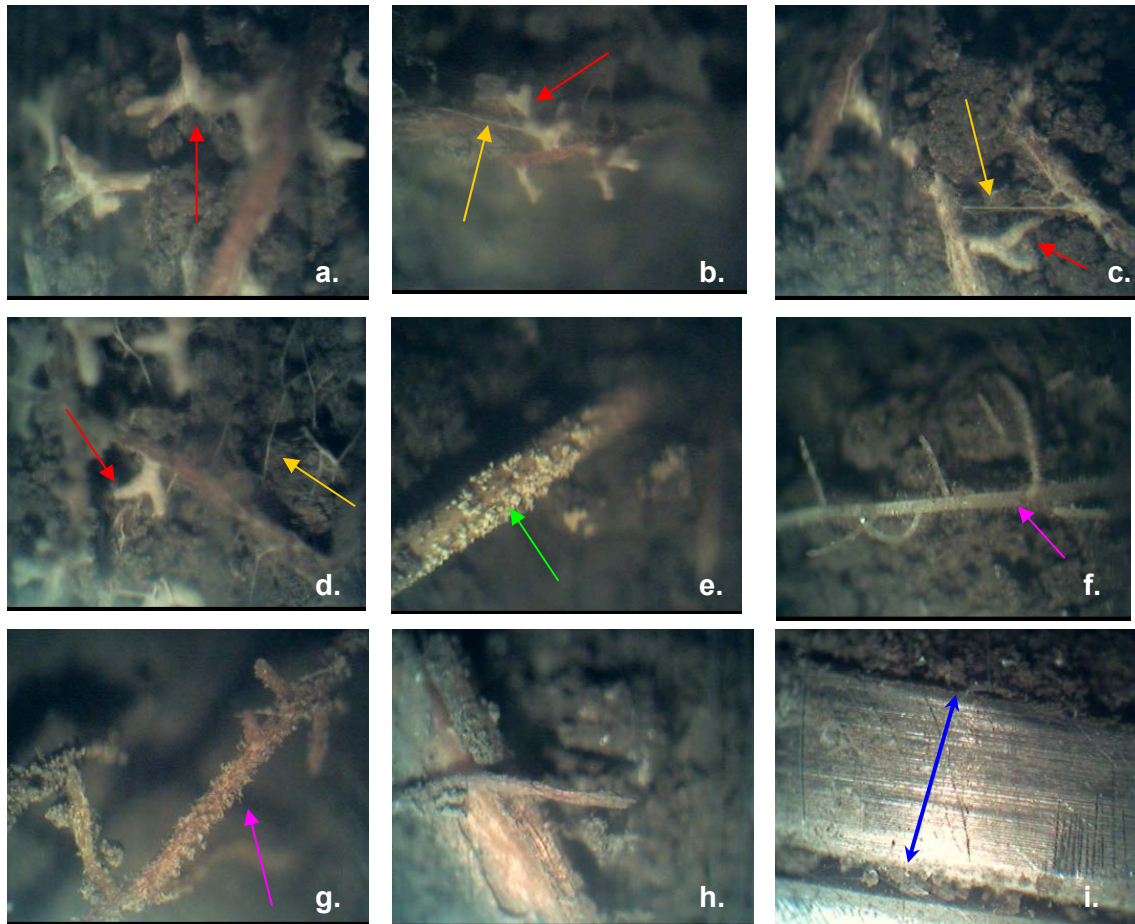


Figure 3.10 Representative images of *Pinus radiata* root morphology observed using the camera system in the access portals in the rhizotron units. a). A root colonized by ECM with the mantle of ECM clearly seen. b). A lateral root colonized by ECM with a different morphology from that in a. Mycelia of ECM spread around the root. c). Fungal hypha, near an ECM colonized root, was observed to connect two roots. d). Roots colonized by ECM with mycelia spreading into the surrounding soil. e). Root partly covered with fungal spores which is indicated by green arrow. f). Young roots with abundant root hairs and presence of several lateral roots. g). Mature root with several lateral roots covered with root hairs. h). An example of a relatively thick and old root in radiata pine root systems. i). Rhizotron wall which is 5 mm thick (as marked by blue line with arrows). The red arrows indicate the ECM in images; yellow arrows indicate the mycelia of ECM or fungi; and pink arrows indicate the root hairs on the roots.

3.3.3.2 *In situ* sampling of exudates in rhizotron units

Twenty-four root exudate samples were collected from root/rhizosphere of eight 1-year-old *Pinus radiata* trees by AEM strips in conjunction with Whatman 3MM. From these, nine

OAs were identified and quantified by HPLC, with formate and acetate being the most frequent detected and most abundant (Table 3.2, Figure 3.11a). Shikimate was also detected in a large number of the samples but was present in only trace amounts. Succinate was detected in only one out of 24 samples, but was present at a high concentration ($2.74 \mu\text{g cm}^{-2}$) in this sample after the 3 h collection period (Table 3.2). A number of other compounds (Figure 3.11a; peaks 6, 7 and 8) with retention times that differed to the organic anion standards were also frequently detected by HPLC; the identity of these remains unknown. The large void peak that appeared at the beginning of the chromatogram may affect the identification of the early eluted anions, such as tartarate and quinate (Figure 3.11a). Four OAs (formate, lactate, acetate and fumarate) were also detected in several samples collected from regions of non-rhizosphere soil (12 samples in total), albeit at lower concentrations in comparison with exudate samples collected from the rhizosphere, with the exception of lactate (Table 3.2). Lactate was detected in four of the 12 non-rhizosphere soil samples; in three samples amounts ranged from 0.5 to $1.6 \mu\text{g cm}^{-2}$, and one sample contained $4.0 \mu\text{g cm}^{-2}$. There was a great deal of variability between samples in both the composition and concentration of OAs in both the rhizosphere and non-rhizosphere soil exudate samples (Table 3.2).

Table 3.2 Quantities of OAs and their frequency of detection from roots and rhizosphere soils collected *in situ* from *Pinus radiata* grown in rhizotron units and from adjacent non-rhizosphere soils. Values are presented as mean \pm 1 standard error for samples ($n=24$ for rhizosphere soil, $n=12$ for non-rhizosphere soil) where positive detection (i.e. greater than limit of detection; Table 3.1) occurred.

OA	Root-rhizosphere soil		Non-rhizosphere soil	
	Concentration ($\mu\text{g cm}^{-2}$)	Number of samples ($n=24$)	Concentration ($\mu\text{g cm}^{-2}$)	Number of samples ($n=12$)
tartarate	0.112	1	nd	0
quinate	nd [#]	0	nd	0
formate	0.87 ± 0.15	20	0.44 ± 0.06	11
malate	1.17 ± 0.29	5	nd	0
malonate	nd	0	nd	0
shikimate	0.09 ± 0.03	16	nd	0
lactate	1.36 ± 0.30	8	$1.66 \pm 0.82^*$	4
acetate	2.40 ± 0.43	23	0.62 ± 0.15	3
maleate	0.27 ± 0.17	4	nd	0
citrate	nd	0	nd	0
succinate	2.74	1	nd	0
fumarate	0.014 ± 0.003	9	0.005 ± 0.001	4

[#] nd: not detectable, the concentration is under limit of detection.

* one sample contained a very large amount of lactate ($4.00 \mu\text{g cm}^{-2}$ membrane).

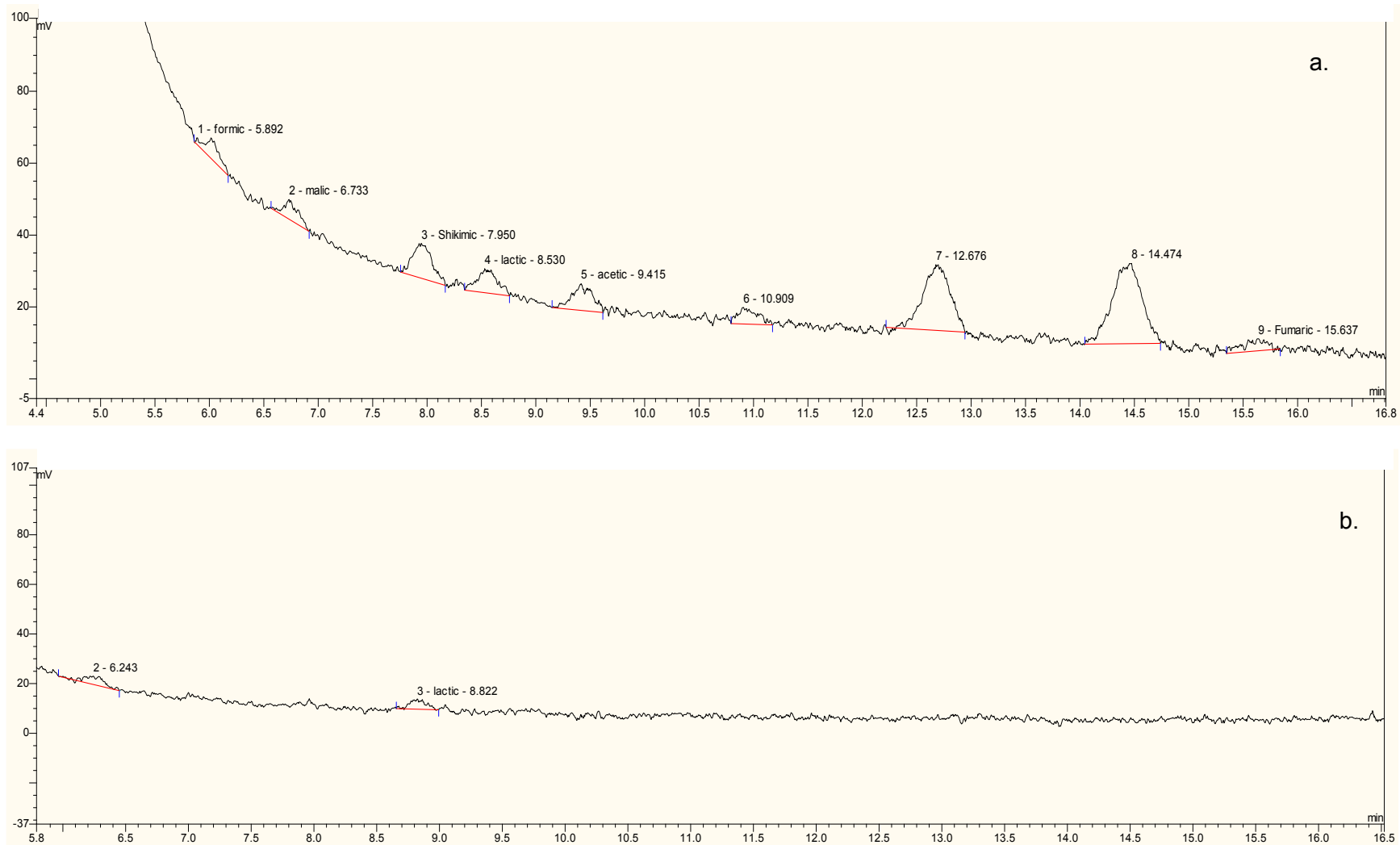


Figure 3.11 a). An example of a root exudate sample which was collected *in situ* by AEM from pine roots and analysed by HPLC. Peaks 1 to 5 and peak 9 were identified by comparing to known standards; peaks 6 to 8 were unidentified. b). An example of an exudate sample which was collected *in situ* by AEM from the non-rhizosphere soil where radiata pine were grown and analysed by HPLC.

3.3.4 Analysis of Variability in Soil Microbial Communities

3.3.4.1 Variability of microbial communities between rhizotron units

Bacterial 16S rRNA PCR products amplified from samples collected from rhizotron units A and B (three sites and three depths per site were sampled from each rhizotron unit) and composite non-rhizosphere soil were loaded onto DGGE gels in a random order (Figure 3.12). Discrimination analysis showed that the rhizosphere bacterial communities in rhizotron unit A were not significantly different from those in rhizotron unit B (Table 3.3). The general bacterial communities in the non-rhizosphere soil were significantly different from those in the rhizosphere soil samples (Figure 3.13, Table 3.3).

The microbial communities in the two rhizotron units were further analysed using α -, β -proteobacterial and pseudomonad taxon-specific rRNA-DGGE with samples collected from rhizotron units A and B in the L site at all three depths (see Figure 3.3). No significant differences were detected between communities in the two rhizotron units, using any of the taxon-specific primers (Table 3.3, Figure 3.13). As seen with the general bacterial primers, the communities of α - and β -Proteobacteria and Pseudomonads in the rhizosphere soil varied significantly in comparison with those in the non-rhizosphere soil in both the rhizotron units.

Table 3.3 Hotelling T^2 probabilities of the level of difference between the communities associated with rhizosphere soils from two rhizotron units A and B and non-rhizosphere soils, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P < 0.05$) are shown in bold.

Taxonomic community	Rhizotron unit/soil	A	B
Bacterial 16S	B	0.0805	-
	Non-rhizosphere soil	0.0000	0.0000
α -Proteobacteria	B	0.1478	-
	Non-rhizosphere soil	0.0016	0.002
β -Proteobacteria	B	0.3781	-
	Non-rhizosphere soil	0.008	0.0076
Pseudomonads	B	0.3211	-
	Non-rhizosphere soil	0.0338	0.0488

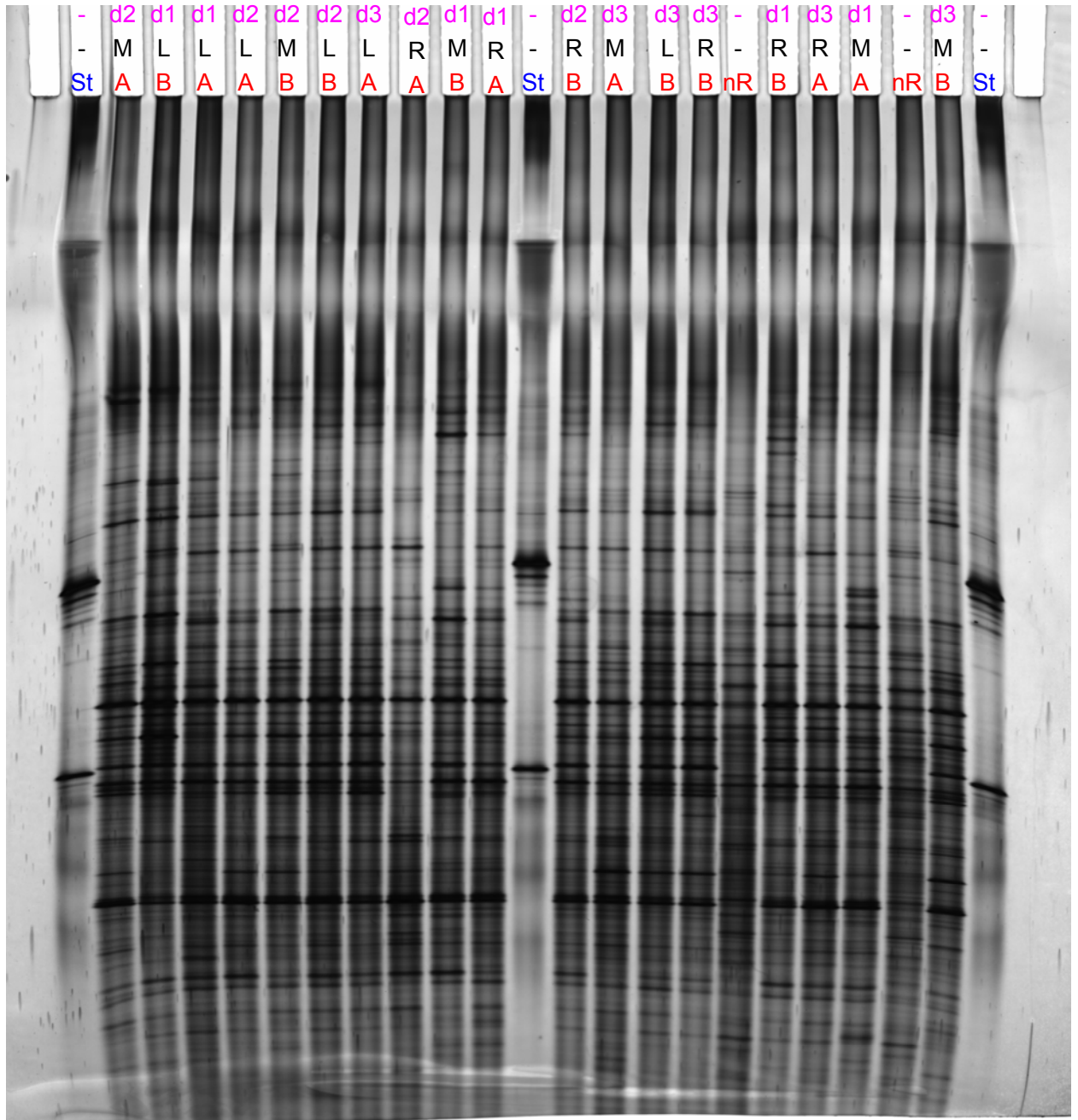


Figure 3.12 An example of rRNA-DGGE gel with samples collected from two rhizotron units A and B, with each rhizotron including samples from the three sites and three depths and composite non-rhizosphere soil. Samples were loaded on DGGE in a random order. Only one replicate from each sampling site were included in this gel. St: bacterial DGGE marker consisted of bulked bacterial 16S PCR products from strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp.. The red letters indicate the samples origin: A: rhizotron unit A; B: rhizotron unit B; nR: non-rhizosphere soil. The black letters indicate the sites where rhizosphere soils were collected from: L: site L; M: site M; R: site R. The pink letters indicate the depth where rhizosphere soils from: d1: 0-15 cm, d2: 16-30 cm, d3: 31-45 cm.

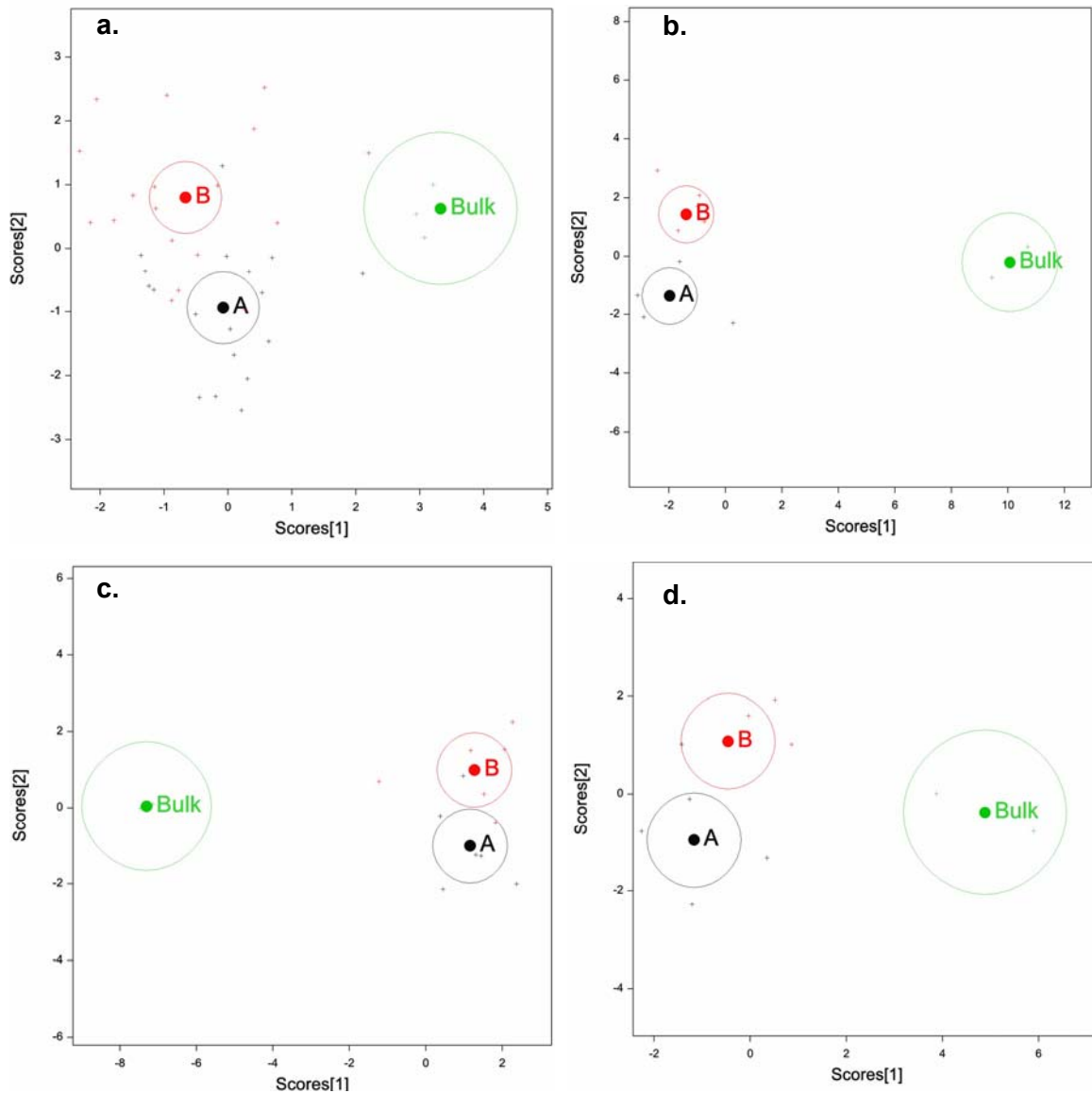


Figure 3.13 Principal coordinates analysis of active bacterial communities from the rhizosphere soil from two rhizotron units A and B and non-rhizosphere soil (labelled as “Bulk” in graphs) amplified with general bacterial and taxon-specific primers. Samples from different depths and different sites but the same rhizotrons were composited for analysis. Open circles represent 95% confidence regions for means of samples ($n=18$ for rhizosphere soil for bacterial community, $n=6$ for rhizosphere soil for taxon-specific communities, $n=2$ for non-rhizosphere soil) which are shown by the solid circles. a). general bacterial 16S; b). α -proteobacterial 16S; c). β -proteobacterial 16S; d). pseudomonad 16S. Significance of differences between treatments are indicated in Table 3.3.

3.3.4.2 Horizontal variability of microbial communities within rhizotron units

The horizontal variability in microbial communities was tested using rhizosphere samples collected from three sites (i.e. L, M, and R) in both rhizotron units A and B from three depths as indicated in Figure 3.3. Bacterial 16S PCR products derived from those samples were randomly loaded across the DGGE gel for microbial community analysis. As there were no difference between samples from rhizotron units A and B (Table 3.3),

samples collected from different depths from different rhizotron units but at the same sites were grouped together for discrimination analysis. As with the previous results, the general bacterial communities in the rhizosphere soil samples from any of the three sampling sites were significantly different from those in the non-rhizosphere soil. However, no significant differences were detected between the rhizosphere soil samples from the three sites L, M and R (Table 3.4, Figure 3.14).

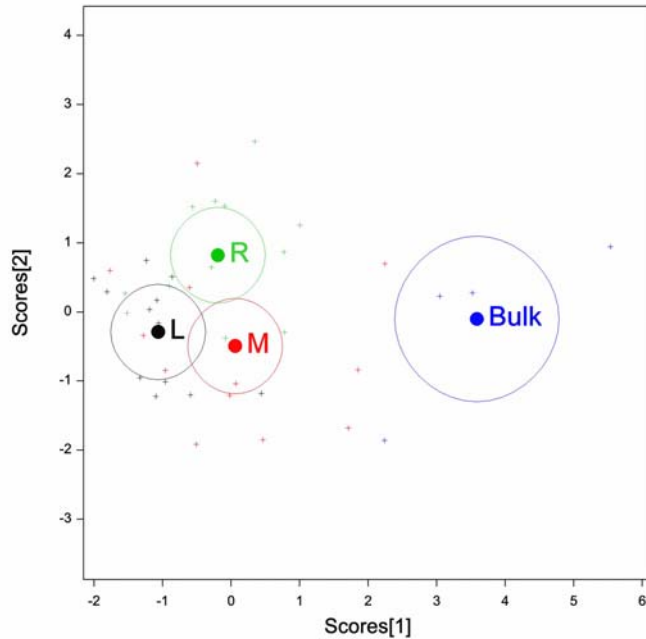


Figure 3.14 Principal coordinates analysis of active bacterial 16S communities from rhizosphere soil associated with roots sampled from three different sites (L, M and R) and non-rhizosphere soil (labelled as “Bulk” in the graph) within the rhizotron. Open circles represent 95% confidence regions for means of samples ($n=12$ for rhizosphere soil, $n=2$ for non-rhizosphere soil) which are shown by the solid circles. Significance of differences between treatments are indicated in Table 3.4.

Table 3.4 Hotelling T^2 probabilities of the level of difference between the general bacterial 16S communities associated with rhizosphere soils from three different sites and non-rhizosphere soils, with each sample under the null hypothesis that all soil samples have the same mean. The probability values with significant difference ($P<0.05$) are shown in bold.

Location with rhizotron unit/soil	L	M	R
M	0.2513	-	
R	0.1460	0.2018	-
Non-rhizosphere soil	0.0000	0.0000	0.0000

3.3.4.3 Vertical variability of microbial communities within rhizotron units

The bacterial 16S rRNA PCR products amplified from the rhizosphere soil samples from three different soil depths from rhizotron units A and B and the non-rhizosphere soil were

compared with samples loaded randomly across the DGGE gels. For robust analysis, rhizosphere soil samples from different sites in different rhizotron units, but same depth were grouped together for discriminant analysis. The bacterial community in the rhizosphere soil in the top 15 cm differed significantly from that at 31-45 cm. However, the bacterial communities in the rhizosphere soil at the middle depth (16-30 cm) were not significantly different to either of the adjacent depths (Table 3.5, Figure 3.15). As previously observed, discriminate analysis showed that the bacterial communities in the non-rhizosphere soil were significantly different from the ones in the rhizosphere soil at each depth.

Table 3.5 Hotelling T² probabilities of the level of difference between various taxonomic communities associated with rhizosphere soils from various depths and non-rhizosphere soils, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P < 0.05$) are shown in bold.

Taxonomic group	Depth/Soil	0-15 cm	16-30 cm	31-45cm
Bacterial 16S	16-30 cm	0.2352	-	
	31-45 cm	0.0401	0.7422	-
	Non-rhizosphere	0.0000	0.0000	0.0000
α -Proteobacteria	16-30 cm	0.0626	-	
	31-45 cm	0.0905	0.3858	-
	Non-rhizosphere	0.0088	0.006	0.0076
β -Proteobacteria	16-30 cm	0.3608	-	
	31-45 cm	0.0047	0.0037	-
	Non-rhizosphere	0.0189	0.0172	0.0151
Pseudomonads	16-30 cm	0.5938	-	
	31-45 cm	0.0316	0.0282	-
	Non-rhizosphere	0.0066	0.0056	0.043

In order to increase the sensitivity of the analysis, primers specific for α -, β -proteobacterial and pseudomonad 16S sequences were used to amplify relevant genes from the non-rhizosphere and rhizosphere soil samples collected at each depth from site L in rhizotron units A and B. Beta-proteobacterial and pseudomonad communities from the rhizosphere soil collected from the top 15 cm and 16-30 cm were not statistically different from each other, while both were significantly different from that at 31-45 cm (Table 3.5, Figure 3.15). However, there was no significant difference in the α -proteobacterial communities in rhizosphere soils collected from the three different depths (Table 3.5). For all the specific bacterial taxa tested, the DGGE community profiles in the non-rhizosphere soil were significantly different to the DGGE profiles in the rhizosphere soil at each depth.

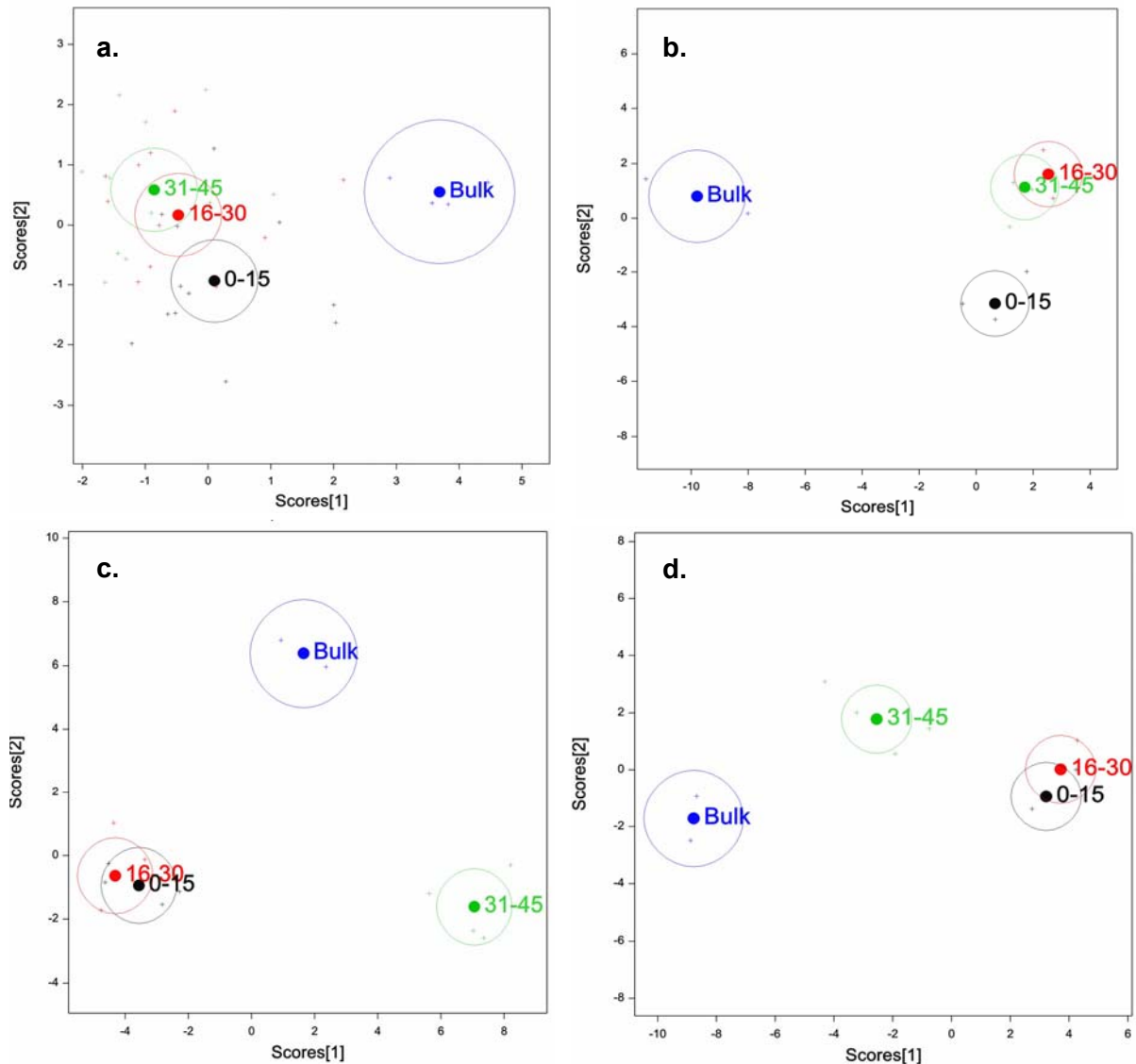


Figure 3.15 Principal coordinate analysis of active bacterial communities from the rhizosphere soil from three different depths (0-15 cm, 16-30 cm and 31-45 cm) and non-rhizosphere soil (labelled as “Bulk” in graphs) amplified with general bacterial and taxon-specific primers. Open circles represent 95% confidence regions for means of samples ($n=12$ for rhizosphere soil for general bacterial community, $n=4$ for rhizosphere soil for taxon-specific communities, $n=2$ for non-rhizosphere soil) which are shown by solid circles. a). general bacterial 16S; b). α -proteobacterial 16S; c). β -proteobacterial 16S; d). pseudomonad 16S. Significance of differences between treatments are indicated in Table 3.5.

3.4 Discussion

3.4.1 Development of *in situ* Method for Collection of OAs

3.4.1.1 Analysis of OAs by HPLC

As a commonly used analytical technique, HPLC has been used for OA analysis in environment samples (Cawthray 2003; Kerem et al. 2004; McCalley 2005; Paleologos and

Kontominas 2005; Simms et al. 2004; van Hees et al. 1999; Wang et al. 2007). Some researchers have reported the separation of more than seven OAs, however, complex sample preparation (such as acidification, passing through cation exchange column and/or ion exchange column) or a second detection at a different column temperature was necessary (Cawthray 2003; van Hees et al. 1999; Wang and Zhou 2006). In the current study, a simple HPLC method, without any sample pre-treatment, was developed which could detect 12 OAs in 17 min. The limits of detection reported here ranged from 0.004 to 0.75 mg l⁻¹ and are among the range reported by others (Cawthray 2003; van Hees et al. 1999; Wang and Zhou 2006; Wang et al. 2007). The large difference in the limits of detection among different OAs found in the current study is also in line with previous studies (Cawthray 2003; van Hees et al. 1999). This is due to differences in molar absorptivity of OAs. The saturated OAs have far greater molar absorptivity than unsaturated ones and thus lower detection limits (Cawthray 2003).

The detection and quantification of early eluted anions, such as tartarate and quinate, in root exudate samples collected from soil using AEM could be affected by the void peak presented at the beginning of the chromatogram. The possible causes of the void peak were examined and are reported in Appendix 3.6. Briefly, the peak was caused by sample injection, Cl⁻ in HCl elution solution and a major contributor was the excess inorganic ions collected from soil. Various modifications, such as changing the elution solution and sample pre-treatment, were carried out to try and reduce the void peak but were not successful (Appendix 3.6). Shen et al. (1996) indicated that the excess of inorganic ions in soil samples can severely disturb the chromatogram and influence the shapes of the early eluting peaks. Neumann (2006) also reported that nitrate and amino acids from soil can affect the early eluted anions, such as oxalate and suggested that pre-treatment of samples with a cation exchanger and acidification of sample may reduce the interference effect at the expense of reducing some OAs. Both of these pre-treatments were tested and resulted in either inefficient recovery of OAs or non-significant reduction of the void peak (Appendix 3.6). Therefore, caution must be taken in interpreting the concentrations of early eluted tartarate and quinate in exudate samples.

3.4.1.2 Suitability of AEM for collection of OAs

The experiments have indicated that AEM is a useful medium for collection of a wide range of low molecular weight OAs from roots and soil. A range of OAs could be captured by AEM from controlled experiments and from rhizosphere and non-rhizosphere soil from pine trees grown in rhizotrons. For most anions, there appeared to be rapid equilibration (<1 h) of organic anion binding and the AEM strips (with surface area of 24 cm²) did not become saturated when 250 µg of each of 12 OAs were loaded onto the membrane

surface. In addition, as much as $25 \mu\text{g cm}^{-2}$ of succinate was collected by AEM strips from soil amended with an OA mixture, indicating that AEM strips were unlikely to become saturated while collecting OAs in root exudates, where anions would be expected to be present in lower concentrations than this range. The highest concentration of any OA detected on AEM in the current study was $10.7 \mu\text{g cm}^{-2}$ for acetate, collected from a region of root and rhizosphere soil.

With the exception of a few OAs, the retention of anions captured by AEM after 3 h was unaffected by incubation in soil, indicating that anions captured by AEM were stable and do not appear to be highly susceptible to degradation by soil microorganisms in the experimental soil. Of the OAs used, shikimate was the most unstable. Oburger et al. (2009) showed that the half life for shikimate in four soil types ranged from 0.6 to 8.6 h with an average at 3.6 h. In addition, as an intermediate of the biosynthesis of aromatic amino acids (the shikimate pathway), shikimate can be transformed to shikimate-3-phosphate and then to chorismate by various enzymes that are common to soil bacteria and fungi (Herrmann and Weaver 1999). Malate and malonate were also shown to have relatively short half-lives in soil (1.9 and 3.4 h, respectively), with decomposition rates varying in different soil types (Oburger et al. 2009). Other studies have shown that OAs such as citrate and malate also degrade rapidly when added directly to soils with half-lives ranging from 1 to 5 h (Jones et al. 1996b; Jones et al. 2003). In contrast, in the current experiment (Figure 3.8), these anions were stable for at least 3 h when immobilized on AEM strips, perhaps because these OAs were not available to microorganisms after being captured by AEM. However, the possibility of low decomposition rates of these anions in the experimental soil cannot be ruled out. As reported by van Hees et al. (2003), strong absorption of OAs to soil solids were found to prevent or delay microbial degradation that otherwise may be very rapid (van Hees et al. 2002). This type of “protection” has also been reported by Jones and Edwards (1998) and Boudot (1992). Similarly, the high stability of most OAs after 3 h in contact with the experimental soil may indicate that the capture of OAs by AEM may protect or delay them from being degraded by soil microorganisms.

Interestingly, the amounts of tartarate increased after 3 h incubation with soil. This may be an artifact associated with tartarate eluting close to the void peak during HPLC analysis (see Section 3.4.1.1 and Appendix 3.6), it may also represent a collection of organic anions from soil. Similarly, the increasing recovery of fumarate may be due to the further collection from soil, as it is present in soil which was confirmed by the presence in the non-rhizosphere soil of radiata pine (Table 3.2).

There was, however, a large difference in the apparent recovery of different OAs following their capture on AEM (e.g. Figure 3.5). Differences in the efficiency of retention or recovery of different OAs from AEM may occur due to the differing strengths of interaction between individual anions and HCO_3^- which was pre-charged on the AEM surface. Since the anions were applied as a mixture in the experiments, interactions between anions may result in inhibited binding efficiency and, subsequently, recovery. In addition, the ability of AEM to capture OAs differed in solution and soil, especially for citrate, tartarate and malate (Figure 3.7, Figure 3.9). This difference may be due to changes in the availability of different anions and their relative solubility from solution to soil. In soil, OAs can be easily absorbed to the soil solid phase, complexed with cationic ligands or metals in soil, transformed by soil enzyme or oxidants in soil, and degraded by microorganisms (Jones et al. 2003; Shen et al. 1996; van Hees et al. 2003). Shen et al. (1996) demonstrated that malic and citric together with some other acids were rapidly immobilized within 20 min of addition to acid forest topsoil. Gardner et al. (1983) proposed that citrate, released from white lupin roots, reacts in soil to form ferric hydroxy phosphate chelates. Citrate and malate also rapidly form complexes with Al in soil solution (Delhaize et al. 1993; Jones 1998), whereas tartarate can chelate with Zn, Cu and other trace metals in soil (Evangelou et al. 2008; Ke et al. 2006). Malate and citrate have also been reported to release Mn from synthetic MnO_2 through a combination of oxidation and complexation (Jauregui and Reisenauer 1982) and consequently decrease the availability of OAs.

Incomplete recovery of OAs by AEM was observed in some experiments (e.g. Figure 3.5). This was not in response to duration of collection period (Figure 3.7) or elution time (Figure 3.6), nor to any apparent instability of the OAs when in contact with filter paper or soil (Figure 3.8, Figure 3.9). Consequently, the incomplete recovery may underestimate amounts of OAs present in both solution and soil (Figure 3.7, Figure 3.9), and therefore most likely in root and rhizosphere and non-rhizosphere soil regions in rhizotron units. Because of variability in the retention of different OAs on AEM and differential recoveries of various OAs under different experimental conditions (e.g. in solution as compared to soil) and with different amounts of available anions, a general correction factor to account for underestimation could not be applied across all anions. However, where required, the level of underestimation for any particular anion concentration may be obtained from the control experiments conducted under similar collection conditions (e.g. Figure 3.5, Figure 3.8).

3.4.1.3 *In situ* collection of OAs from root-rhizosphere and non-rhizosphere soils

The large-scale rhizotron system used in this experiment provides unique opportunities for the study of root exudates and other rhizosphere process *in situ*. The system provides

reasonable space for root growth, convenient easy access to roots and the rhizosphere via access portals for observations and non-destructive sampling, and the possibility for repeated measurements on the same root regions. The inflatable tube used in the access portals in the rhizotron reduced the air gap at the soil-tube interface, a common problem with rhizotron and minirhizotron systems which can lead to unnatural root distribution (McMichael and Zak 2006; Taylor et al. 1990). The black-colored inflatable tube, rather than a clear perspex tube, used in the access portals and the separation of the rhizotron room from the growth room reduced the exposure of roots to light, which has been reported to affect root density in a minirhizotron system (Levan et al. 1987). A major advantage of this rhizotron system compared to the more commonly used rhizobox systems was the stage to which radiata pine trees could be grown (e.g. to 1 year) and the minimization of the interface effect (such as light and soil moisture) on root physiology by sampling through the horizontal access portals (45 cm deep). The study of young trees grown in the rhizotron system can provide the necessary data to link laboratory studies conducted using seedlings grown under controlled conditions to the growth of trees in the field.

During the 6 month observation period, root morphologies of radiata pine in the rhizotron access portals were similar to those that have been observed in field-grown trees (C. Water, pers. comm.; McKenzie and Peterson 1995a; McKenzie and Peterson 1995b; Scales and Peterson 1991). In particular, it was clear that pine roots were effectively colonized by ECM (Figure 3.10). Although only based on morphological observations, ECM that colonized roots in this study were similar in structure to those observed in the field by other researchers (Brundrett et al. 1990; Walbert 2008). All these observations suggest that under the experimental conditions in the rhizotron system, the radiata pine root growth and morphology was similar to those under field conditions.

The rhizotron system together with the AEM strips makes *in situ* sampling feasible and easy to conduct under more realistic plant growing conditions. In the root and rhizosphere samples collected from radiata pine in the rhizotron, acetate and formate were most frequently detected and at the highest concentration. Similarly, Smith (1969) reported that 18-day-old *Pinus radiata* release significant amounts of acetic and oxalic acids when grown in glass beads with a nutrient solution under sterile conditions. Formate has similarly been detected as a dominant OA in tree root exudates in other studies (Sandnes et al. 2005; Shen et al. 1996; Strobel et al. 1999; Strobel et al. 2001). Anions such as malate, lactate, maleate, succinate and fumarate have also been reported in root exudates of trees (Ahonen-Jonnarth et al. 2000; Heim et al. 2001; Sandnes et al. 2005;

Smith 1969; Smith 1976) and were also detected here albeit at lower frequency and at lower concentrations than acetate and formate.

Shikimate was detected in low concentrations in 16 out of 24 root and rhizosphere samples. Sandnes et al. (2005) also reported that low amounts of shikimate occurred in root exudates for both spruce and birch trees when grown in sterile microcosms, but not when sampled from trees grown in either a rhizobox system or roots collected from field-root windows. Ahonen-Jonnarth et al. (2000) detected shikimic acid in both non-mycorrhizal *Pinus sylvestris* and mycorrhizal-infected trees (two out of the five tested mycorrhizal strains) when grown in glass beads under sterile conditions. Given that there is a ~40% reduction in the recovery of shikimate after contact with soil for 3 h, these results further suggest that shikimate may be rapidly degraded in soil. In the current study, AEM strips were placed directly in contact with roots and OAs could be captured rapidly by AEM after being released from roots which may protect them from degradation. Similarly, Zhang et al. (2001) found shikimic acid in the exudates collected in a rhizobox by placing filter paper discs directly onto the root surface. Furthermore, incomplete recovery of shikimate from AEM strips (~30 to 60%) may explain the low amount of shikimate detected in the current study. The shikimate concentration reported here may, therefore, be underestimated by between 2 to 5-fold according to the validation experiments (Figure 3.5, Figure 3.8).

Low concentrations of formate, lactate, acetate and fumarate were also detected in the exudate samples of non-rhizosphere soil. Similar results were reported by Schefe et al. (2008), Koo et al. (2006) and Grierson (1992) for formate, acetate, maleate, fumarate, tartarate and succinate. The presence of OAs in non-rhizosphere soil may originate directly from the degradation of plant material or SOM or from microbial metabolism (Rózycki and Strzelczyk 1986; Stevenson 1967; Vance et al. 1996). As the non-rhizosphere soil samples were collected from areas adjacent to root and rhizosphere samples, it may be possible that OAs had diffused from areas with roots, especially for formate and acetate that were present at relatively high concentrations in the rhizosphere. Alternatively, the high concentration of these anions in the rhizosphere may also be partly derived from microbial metabolism rather than wholly directly exudated from roots.

Both the composition and concentration of OAs in root exudate samples and non-rhizosphere soil samples were highly variable which is consistent with other studies (Phillips et al. 2008; Sandnes et al. 2005; Schefe et al. 2008). Some of the variability might be caused by variability in the roots sampled. In some samples, only root tips were covered by AEM strips, whilst in other cases a range of root types, including mature roots, were present. In addition, the extent to which different roots were colonized by ECM was

also highly variable. Differences in the types and amounts of root exudates released from different parts of root systems are well known (Bringhurst et al. 2001; Hinsinger et al. 2005; Jaeger et al. 1999; Scheffe et al. 2008; Thornton et al. 2004). This variability could be reduced by recording all the sample images and grouping them into different root types/classes for exudate analysis. In addition, the release of root exudates may vary considerably in response to the localized soil environment (Jones et al. 2003). Therefore, the high variability observed in exudation of OAs in a highly spatial heterogeneous soil is perhaps not surprising. Further work to quantify the actual area of the AEM strips in contact with roots (e.g. using image analysis software) may reduce such variability. This type of software was developed and applied in the subsequent experiment described in Chapter 4.

In conclusion, the *in situ* sampling of low molecular weight OAs from the rhizosphere of pine trees and non-rhizosphere soil in the rhizotron system could be achieved using AEM through the access portals with minimal damage to roots. This approach can also be used to study temporal variability by repeated collection of root exudates at the same sites at different times. The recorded images of sampled roots and surrounding soil can be used for studies on the spatial variability of root exudates. In addition, the *in situ* sampling of roots with associated rhizosphere soil and non-rhizosphere soil samples could be achieved using the access portals, camera and sampler as described in Chapter 4.

3.4.2 Variability in Soil Microbial Communities

A rhizosphere effect, in which rhizodeposition from roots enriches for particular microorganisms around the roots from a background community of soil microorganisms, was well demonstrated in the current study. Differences in microbial communities between rhizosphere and non-rhizosphere soil have been commonly observed in numerous studies with different plants using the T/DGGE technique (Costa et al. 2006; de Ridder-Duine et al. 2005; Gomes et al. 2001; Smalla et al. 2001) and other techniques (Marilley and Aragno 1999; Marilley et al. 1998). In contrast, Duineveld et al. (1998) reported similar bacterial communities were present in bulk soil and the rhizosphere of chrysanthemum plants grown in pots in a growth room, as detected by rDNA-DGGE. They explained that the effect of chrysanthemum roots on dominating soil bacterial groups is marginal as opposed to other effects, such as soil type. In subsequent studies, they examined the communities using rRNA-DGGE technique and found the plant root effects did not cause a complete shift in bacterial community but rather subtle changes (Duineveld et al. 2001). Normander and Prosser (2000) also reported that no difference could be detected between DGGE banding profiles of rhizosphere soil and bulk soil for barley grown in pots. However, they observed differences between communities on the rhizoplane (i.e. at the

root surface) and the surrounding bulk soil. Different definitions of root habitats may explain the discrepancies in the observations reported in the literature, although factors such as soil type, plant species and experimental durations may also influence results.

Spatial variability in soil microbial communities can occur both vertically and horizontally (Ettema and Wardle 2002). The variability in the rhizosphere soil microbial communities between rhizotron units and within rhizotron units in both horizontal and vertical directions were studied in the current experiment. There was no significant difference in microbial communities between the samples from two different rhizotron units A and B in different rhizotron compartments within one growth room. This result was confirmed by examining the general bacterial 16S community as well as taxon-specific α -, β -proteobacterial and pseudomonad 16S communities. Although the rhizotron units were located in different positions in the growth room, which may have resulted in slight differences in light density, different fluctuations in temperature, etc., these factors were not as influential as others, such as depth. Importantly, the result validates the utilization of rhizotron units as replicates within the same growth room in the subsequent experiment (Chapter 4).

The horizontal variability was examined using three sites (L, M, and R) within the rhizotron units, and no difference in the rhizosphere bacterial communities was detected by rRNA-DGGE. Considering the small sampling area (0.25 m^2) and the homogeneity of the soil (i.e. sieved and mixed) used in the experiment, the similarity in the rhizosphere bacterial communities from the three sites is not surprising. In the rhizosphere environment, apart from soil, roots are another factor that can influence the bacterial community which has been demonstrated by the clear rhizosphere effect in the current study. However, the roots collected from the three different sites at corresponding depths were similar in morphology (size, mycorrhizal colonization rate). Similarly, Costa (2006) reported that near identical microbial DGGE patterns were observed from rhizosphere soil from the same microenvironment (plant species and sampling field). Felske and Akkermans (1998) also found little variability in bacterial 16S communities from undisturbed soil samples taken at 1 m intervals along a 4 m transect in grassland soil, although in that study soil samples instead of rhizosphere soils were used for microbial communities analysis. However, Nicol et al. (2003) reported the heterogeneity of the soil archaeal community structure in macroscale (2 m interval in a total of 8 m) as well as microscale (a few millimeters) of grassland pasture plots. In comparison with the current study, their study was conducted in the field (with probably much more heterogenous soil compared to the sieved soil used in the rhizotron system) targeting the soil archaeal communities in non-rhizosphere soil (although grass roots were present in the topsoil where sampled).

As expected, the structure of the rhizosphere bacterial communities varied significantly with depth; the general rhizosphere bacterial community at 31-45 cm differed significantly from that in the top 15 cm. Beta-proteobacterial and pseudomonad communities at 31-45 cm were different from those found at 0-15 cm and 16-30 cm. Similarly, depth-variation in rhizosphere ectomycorrhizal communities was reported by Rosling et al. (2003). They examined ectomycorrhizal root tips in seven horizons up to 52 cm and found that most of the ECM were present in only some of the horizons and normally in adjacent horizons rather than in a discontinuous distribution. In addition, some of the ectomycorrhizal taxa were only found in the deeper soil layers. In addition, Dickie et al. (2002) found differentiation in ectomycorrhizal communities by T-RFLP with the samples collected from different layers of the forest floor within the same O horizon. In both of these studies, the soil compositions varied with depth, while a more uniform and well sieved soil, though packed with different bulk densities, was used in this current study. Fungal communities were not examined in the current study, but differences in fungal communities present at different depths of the rhizosphere environment may also contribute to differences in bacterial communities in rhizosphere soil and/or *vice versa*. Izumi et al. (2008) reported that bacterial communities associated with ectomycorrhizal colonized root tips differed significantly from those associated with roots uncolonized by ECM. They explained that roots colonized by ECM could provide a further unique niche for some bacteria, by accommodating them within the hyphae of the ECM or through the availability of organic compounds derived from fungi.

Many studies have found that soil microbial communities and activities varied at different soil depths (Felske and Akkermans 1998; Fierer et al. 2003; Griffiths et al. 2003a; Jackson et al. 2009; Steenwerth et al. 2008; Taylor et al. 2002). The changing physicochemical environment (such as soil moisture, temperature, oxygen availability, pH, soil aggregate size, nutrient availability, organic matter content, etc.) with depth was normally identified or hypothesized as the main cause. As a subset of the soil microbial community, the rhizosphere microbial community can be easily influenced by the surrounding soil environment, in particular the physicochemical environment (Berg and Smalla 2009; Buée et al. 2009; Marschner et al. 2004). Due to the limited amount of rhizosphere soil obtained from root samples in the current experiment, no soil properties were measured to further investigate the possible drivers for the depth variability. However, it is well known that soil conditions and environmental parameters are not uniform with depths, and the soil surface experiences wider fluctuations in temperature and moisture than soils at greater depths (Brady and Weil 2002; Fierer et al. 2003; Fisher and Binkley 2000; Sheppard and Lloyd 2002). In addition, all of these physicochemical properties have been shown to influence

soil microbial communities in one or more studies (Bååth et al. 1995; Bossio and Scow 1998; Kieft et al. 1993; Lundquist et al. 1999; Nicol et al. 2003; Schimel et al. 1999; Wakelin et al. 2008; Watts 1999; Yuste et al. 2007; Zogg et al. 1997). For example, Griffiths et al. (2003a) observed bacterial communities changed with soil depth and this was correlated with a decrease in moisture content. However, they also found that other chemical and physical factors were likely to co-vary which would also influence bacterial communities. Sheppard and Lloyd (2002) detected increased carbon dioxide and methane with depths which indicated an anaerobic environment, and consequently implied a shift in community composition. In the current experiment, the soil temperature in the rhizotron units would be expected to vary with depth. The surface soil exposed to the light, could reach 20°C during the day time and decline to 12°C at the night, whereas the soil temperature at the depth of 30 cm in the rhizotron was consistent at 14°C. In addition, the top 20 cm of soil and 20-50 cm of soil in the rhizotron units were packed to different bulk densities. However, this was not reflected in the general bacterial or three taxon-specific communities as no difference was observed in the rRNA-DGGE profiles of the rhizosphere soils from 0-15 cm and 16-30 cm, although these did differ from the rRNA-DGGE profiles for rhizosphere soil from 31-45 cm. This suggested that bulk density was not a key factor in the rhizosphere bacterial community variability. In contrast, Hillel (1982) showed that bulk density, like other physical factors could affect the soil microclimate, which could further influence the microbial community in bulk soils. Together with the strong rhizosphere effect observed in the current study, the results may imply that plant roots are the primary influence factor in this case.

Apart from the direct effect on microbial communities, the physicochemical environment (including temperature, moisture and nutritional status), can affect the composition and quantity of root exudates released by plants, which can subsequently affect the rhizosphere microbial community (Bekkara et al. 1998; Dijkstra and Cheng 2007; Hughes et al. 1999; Neumann and Römheld 2007; Rivoal and Hanson 1994; Watt and Evans 1999). For instance, Gorissen et al. (2004) demonstrated that C flow from plant roots to soil was significantly reduced under drought conditions and this reduction could be as much as 60% in some cases, causing further reductions in microbial biomass. On the other hand, the presence of microorganisms and their activities can also influence the composition and quantity of root exudates released by plants (Brimecombe et al. 2007). This may enable the maintenance of the selected microbial community at different depths, as rhizosphere microbes could, to a certain extent, influence the root exudate process to maintain their favoured environment.

In the rhizotron units, different types of roots were predominant at the different depths, although roots from the top 0-15 cm were more similar to that from 16-30 cm compared to the deeper layer of 31-45 cm (Appendix 3.7), which may account for some of the differences in bacterial communities observed with depth. When sampling, root colonized by ECM were commonly observed in the top root zone, while mature roots were frequently collected from the bottom root zone. The bacterial 16S microbial communities associated with the different types/ages of roots, i.e. fine root colonized by ECM, middle sized root and big mature root were compared by rRNA-DGGE gel (Appendix 3.7). The analysis of DGGE gels showed different communities associated with different types/locations of roots, which is in agreement with other studies (Clayton et al. 2005; Gochner et al. 1989; Marschner et al. 2001; Yang and Crowley 2000). For example, Duineveld et al. (1999) reported that the bacterial communities associated with young roots were distinct from those from older roots. They explained that young roots released more organic material than old roots which could result in different specific bacterial populations. Therefore, in a subsequent rhizotron experiment (Chapter 4), treatment comparisons were performed with samples taken from each depth from areas which had similar predominant root types.

Non-rhizosphere soil was bulked from different sampling sites and depths to reduce the number of samples for processing in this experiment. However, by pooling all the non-rhizosphere soil, differences in non-rhizosphere communities between depths may have been lost, which may give some indication of whether the cause of these depth-variations was in response to a plant factor or soil factor. Therefore, in the subsequent experiment (Chapter 4), the non-rhizosphere soil was collected and assessed at each sampling site at each depth.

Fungal communities were not investigated in this study due to the failure of fungal PCR amplification, despite the fact that ectomycorrhizal colonized roots was commonly observed. Compared to bacteria, analysis of fungal communities based on the ribosomal small subunit gene is more problematic using PCR-DGGE (Anderson and Cairney 2004). This may be due to the DNA/RNA extraction efficiency and/or PCR primer specificity. Different primers were tested for ability to amplify fungal 18S (NS1/FR1-GC, NS1/FR1, EF3/EF4 (Smit et al. 1999), AU2/AU4 (Vandenkoornhuyse et al. 2002), FF390/FR1 (Vainio and Hantula 2000)) and ITS sequence (ITS1F/ITS4B (Gardes and Bruns 1993)) using cDNA samples from this experiment. However, no consistent results were obtained in any primer set. In contrast, the fungal 18S could be amplified using cDNA derived from soil samples collected from Iversen Field, Lincoln University, with primer set NS1/ FR1-GC using the method described in Chapter 2, Section 2.2.6. Whilst reasons for the poor

fungal PCR amplification from samples collected from the rhizotron units in the present experiment remains unclear, soil from Iverson Field was used in the subsequent rhizotron experiment (Chapter 4).

3.5 Conclusions

This preliminary rhizotron experiment provided valuable information on the preparation and maintenance of rhizotrons for the subsequent experiment. It also showed that the investigation of rhizosphere processes can be carried out in this novel large-scale rhizotron system. Access to the rhizosphere via access portals was convenient and did not damage plant roots or have any observed impact on root physiology. *Pinus radiata* grown in the rhizotron units showed healthy root morphology similar to that observed in field-grown trees. *In situ* collection of low molecular weight OAs from roots of radiata pine trees grown in the rhizotron units as well as non-rhizosphere soil by AEM backed with moistened Whatman 3MM strips and subsequent analysis of exudate samples by HPLC were achievable. Such an approach provides new opportunities for investigating the spatial and temporal variability in root exudate production and composition and subsequent influence on the diversity and function of rhizosphere soil microorganisms.

The microbial community variability study showed that: i). there was a consistent influence of pine roots on the rhizosphere microbial community, as microbial communities in rhizosphere soils differed significantly to those in non-rhizosphere soils in all the cases; ii). there was no variation in the examined rhizosphere communities (general bacteria, α -, β -Proteobacteria and Pseudomonads) between two rhizotron units; iii). there was significant variation in rhizosphere microbial communities with depths, but not horizontally at different sites. Therefore, different rhizotron units can be used as replicates in the subsequent experiment, and root samples collected from different sites but at the same depth could be considered similar and thus could be bulked together if necessary. Depth-variation in the rhizosphere community is present in pine trees, therefore, sampling at various depths is needed to obtain a better understanding of the effect of root exudates on soil microbial community in the subsequent rhizotron experiment.

Chapter 4 Rhizosphere Properties of Genetically Modified and Wild Type Radiata Pine

4.1 Introduction

In the last two decades, interest in the development of GM trees has increased and a number of genetic traits, including herbicide tolerance, insect and disease resistance, wood quality and quantity improvement and altered reproductive development, have been incorporated into more than 30 forest tree species, including commercially important conifers, poplars and eucalypts (see reviews by Henderson and Walter (2006) and van Frankenhuyzen and Beardmore (2004)). Radiata pine is the dominant commercial plantation forest tree species in New Zealand and, due to its exceptionally fast growth and productivity in the New Zealand climate, is a highly valued resource for construction timber, furniture, heating, and pulp and paper. Genetically modified radiata pine trees investigated in this study were developed at Scion Research, Rotorua, and contained the *leafy* gene and selection marker gene *nptII*. The *leafy* gene, originally derived from *Arabidopsis thaliana*, is a flower-meristem-identity gene which is involved in the control of floral development (Weigel et al. 1992). Introduction of this gene may allow radiata pine to flower earlier, thereby reducing time to seed production and shortening of breeding cycles. The modified trees also expressed the antibiotic resistance gene *nptII*, which encodes neomycin phosphotransferase II (NptII) protein which can catalyse the phosphorylation of aminoglycoside antibiotics, including neomycin and kanamycin, enabling the modified plants to grow in the presence of the antibiotics. As NptII protein can inactivate different aminoglycoside antibiotics and, consequently, may affect the growth of microorganisms in soil. Risks associated with the use of this gene and its encoded protein in commercial GM crops have been comprehensively studied; however, no impacts have as yet been reported (Lamarche and Hamelin 2007; Ramessar et al. 2007). The consistent detection of the NptII protein in plant roots indicated the stable expression of introduced genes throughout the project (Appendix 4.1).

While GM trees do not evoke immediate health concerns, as perceived with some GM agricultural crops, the prospect of commercial release of GM trees raises concerns regarding potential ecological impacts (Valenzuela et al. 2006; Walter 2004). Compared to short-lived agricultural crops, GM trees will be growing on a site for many years, and thus have potential to make a long lasting impact on ecosystem processes (Lamarche and Hamelin 2007). In addition, tree roots are more widely spread compared to annual crops, and so they may potentially influence larger areas of the soil ecosystem. Accordingly, there is a need to investigate the potential impact of GM trees on the biodiversity of non-

target microbial communities (Lilley et al. 2006; van Frankenhuyzen and Beardmore 2004).

Numerous studies have investigated the potential influence of GM plants on the structural and functional diversity of soil microbial communities in the rhizosphere, where microorganisms are directly influenced by plant roots. In some studies, GM plant roots were colonized by similar microbial communities to those on wild type (WT) plant roots (George et al. 2009; Heuer et al. 2002; Heuer and Smalla 1999; Schmalenberger and Tebbe 2002). Conversely, other studies have shown that the presence of GM plants significantly affected microbial communities in the rhizosphere (Castaldini et al. 2005; Dunfield and Germida 2001; O'Callaghan et al. 2008; Tesfaye et al. 2003). Several authors have speculated that changes in microbial communities were caused by unintentional alteration of root exudates released into the rhizosphere (Di Giovanni et al. 1999; Donegan et al. 1999; Milling et al. 2004; Sessitsch et al. 2003; Siciliano et al. 1998). Bruinsma et al. (2003) suggested that one way a GM plant can influence microbial community is by introducing novel growth substrates or higher levels of existing substrates into the soil system. A study examining soybean varieties showed enhanced colonization by *Fusarium* spp., a soil-borne pathogen, on glyphosate-tolerant soybean varieties compared to conventional varieties (Kremer et al. 2000). They also detected a transgene-derived protein in root exudates from a glyphosate-tolerant soybean variety, which may be one mechanism for the alteration in the community diversity. However, further characterization of root exudates and additional studies are needed to confirm this hypothesis (Kremer et al. 2000).

Changes in root exudates of GM plants have not been studied in detail, with the exception of those plants modified to intentionally exude altered compounds such as opine (Oger et al. 2000), nodule enhanced malate dehydrogenase (neMDH) (Tefaye et al. 2003; Tesfaye et al. 2001) and phytase (George et al. 2004). Recently, Yan et al. (2007) measured low molecular weight compounds (OAs, sugars and amino acids) in root exudates of *Bacillus thuringiensis* (Bt)-transgenic cotton and unmodified control grown in hydroponic systems and found that their concentration and/or composition exuded from Bt-cottons differed significantly in comparison to those from controls. In addition, unintentional changes in root morphology and root mass in GM silver birch (*Betula pendula*) modified with the 4CL gene involved in lignin biosynthesis compared to unmodified birch were reported by Seppänen et al. (2007). These changes could significantly affect the composition and quantity of root exudates (Koo et al. 2005).

Low molecular weight OAs, which are a major component of tree root exudates, are a significant C source for microorganisms in the rhizosphere, and thus may have a major

influence on the structure and function of microbial communities (Bertin et al. 2003; Jones 1998; Marschner 1995). The alteration in the composition and/or concentrations of OAs in root exudates could result in a shift in the rhizosphere microbial community. Tesfaye et al. (2001) reported that GM alfalfa over-expressing a neMDH released 7 times more OAs compared with WT alfalfa and induced changes in the rhizosphere bacterial community in comparison with WT alfalfa (Teskaye et al. 2003).

Due to the difficulties in rhizosphere sampling as discussed in Chapter 1, Section 1.1.4, study of exudates and microbial communities in corresponding soil samples presents a major challenge. This is particularly difficult when studying trees which have larger and deeper root system than annual crops. To date, there is no published literature examining root exudates of GM plants grown in soil together with its impact on the soil microbial community, with the exception of transgenic proteins and intentionally altered exudate compounds. The objective of the present study was to characterise the microbial communities associated with the roots of the GM and unmodified *Pinus radiata*, as well as determine any alteration in the composition and/or concentrations of OAs in root exudates of GM pines in comparison with control trees when grown in large-scale rhizotron units.

4.2 Materials and Methods

4.2.1 Soil Preparation and Rhizotron Establishment

The soil used in this study was a Templeton silt loam (Immature Pallic soil; NZ Soil Bureau, 1968) collected from the Lincoln University experimental farm (Iverson Field). The soil had been maintained under various pasture-crop rotations, and was under pasture at the time of sampling. Soil from the A (5-15 cm) and B (15-30 cm) horizons was collected and passed through a 10 mm sieve to remove stones and roots. In order to maintain optimal conditions for plant growth over an extended period, washed river sand was mixed with the A horizon soil (4 soil: 1 sand, vol/vol) and B horizon soil (5 soil : 1 sand, vol/vol) to improve the soil drainage. The resulting A horizon soil comprised 3% clay, 50% silt and 47% sand, while the B horizon soil comprised 1% clay, 28% silt and 71% sand. Selected properties of the A and B horizon soil-sand mixes were measured by Hill Laboratories Ltd, Hamilton, and are presented in Appendix 4.2. Each rhizotron cylinder was split vertically to provide two independent rhizotron units with 0.25 m² in surface area to allow increased numbers of replicates in this experiment. All eight rhizotron units (0.25 m² x 0.5 m depth) were packed simultaneously to ensure the uniformity of the soil used in the rhizotron units. Horizon B soil was packed into the rhizotron units at a depth of 26 to 50 cm with a bulk density of 1.2 g cm⁻³. Horizon A soil was then packed into the rhizotron units with a bulk density of 1.1 g cm⁻³ for the top 26 cm of soil. The rhizotron units were placed in a

rhizotron room (dark) at 14°C in the Biotron and watered regularly for 12 weeks before the trees were planted.

4.2.2 Plants and Growing Conditions

Seedlings of radiata pine were developed from cuttings of one parental GM radiata pine (pEM1-4-11) and non-modified isogenic control trees at Scion Research in October 2006, and transferred to the Biotron at Lincoln University in November 2007. Roots and foliage of seedlings were trimmed to uniform sizes to encourage the growth of healthy roots and to create the above-ground uniformity (leaf area). Four seedlings from each tree line were selected for this rhizotron study. Following pruning, seedlings were re-potted into plastic planter bags (size: 16 x 16 x 30 cm) filled with horizon A soil and grown in a growth room with 16 h of light ($560 \mu\text{mol m}^{-2} \text{s}^{-1}$ measured at canopy height) and 8 h in the dark at a constant room temperature of 18°C and air humidity at 75%. The plants were watered 3 times a week.

After seven weeks growth in the planter bags, four uniformly-sized GM pEM1-4-11 seedlings and four WT control seedlings were transferred into the rhizotron units (January 2008) with a GM and a WT seedling planted in rhizotron units adjacent to each other within one rhizotron cylinder (Figure 4.1a and b). The air temperature in the growth room was maintained at 20/12°C ($\pm 0.5^\circ\text{C}$) during the light (6 am to 8 pm) /dark (8 pm to 6 am) period. The photosynthetically active radiation at the top of the canopy was maintained at a minimum of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ during the 14 h photoperiod, but was elevated to $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ for four hours during the middle of the photoperiod (10 am to 2 pm). Relative humidity in the room was maintained at 68% during the light period. Soil temperature was maintained at 18°C ($\pm 2^\circ\text{C}$) at a depth of 30 cm, but was subject to fluctuations in temperature at the soil surface depending on air temperature and lighting. This condition was selected based on the good growth response of radiata pine trees in the previous experiment (Chapter 3). The plants were watered 3 times per week with 1 l per rhizotron unit at each time. Hand weeding was conducted every two weeks. In order to create a uniform environment for each rhizotron unit, the rhizotrons were rotated by 90° every week and randomized within the growth room every 6 weeks during the experimental period.

4.2.3 Bulk Soil Bag Preparation and Installation

To enable the sampling of “pure” bulk soil which was not directly influenced by roots, bulk soil bags which could exclude roots were designed and installed in the rhizotron units. Accordingly, bulk soil exposed to the same environmental conditions as the main rhizotron soil could be obtained at the end of experiment. The bag was made from 20 μm nylon mesh (Schweizer Seidengaze-fabrik AG, Thal, Switzerland) and heat-sealed at two sides

(bottom and side) and a plant tag attached to the top for easy handling during sampling (Figure 4.1c). On the same day that seedlings were planted in the rhizotron units, two bulk soil bags were placed at two corners of each rhizotron unit (Figure 4.1a). A vertical soil core of horizon A soil (4.6 cm diameter) at each corner of the rhizotron unit was removed and directly placed into the bulk soil bag (14.5 cm circumference x 28 cm depth) without disturbance. The bulk soil bag containing horizon A soil was then placed back into the position where the soil core was removed in the rhizotron unit (Figure 4.1b and c).

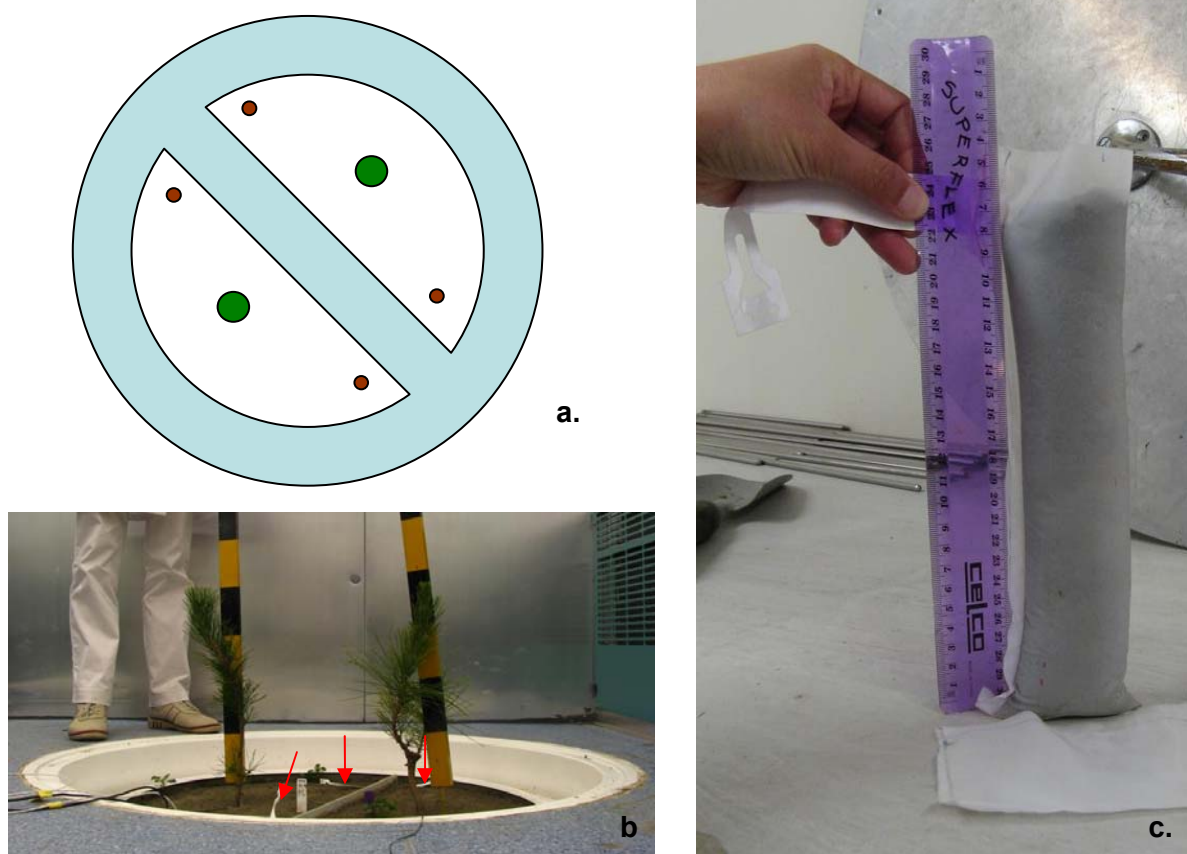


Figure 4.1 a). Sketch of one rhizotron cylinder which was split into two independent rhizotron units. Brown circles represent the bulk soil bags filled with horizon A soil and located at two corners in each rhizotron unit. Green circles represent radiata pine trees planted in the middle of each rhizotron unit. One GM and one WT tree seedlings were planted adjacent to each other in separated rhizotron units. b). Pine trees after one month growth in a rhizotron. The tree on the left was a GM pine and the one on the right was a WT pine. Red arrows indicate tags of the buried bulk soil bags at corners in the rhizotron unit. c). Bulk soil bag filled with horizon A soil core which was sampled from a corner of a rhizotron unit.

4.2.4 Rhizotron Sampling on Three Occasions

Soil and roots were sampled during the experimental period of 10 months growth in the rhizotrons. The first sampling was conducted by taking horizontal cores at two depths (i.e. 10 and 20 cm) after the trees had been growing in the rhizotron units for three months

(April, 2008). The vacant hollow cores in the rhizotron units created by the initial sampling process were maintained by inflatable tubes and used as access portals for subsequent root observations and *in situ* sampling. The second sampling was conducted *in situ* through these access portals six months after their creation (October, 2008). The third sampling was carried out by taking vertical cores at the end of the experiment (November 2008) using the traditional root and soil sampling technique for trees. As vertical variation in bacterial communities in the rhizosphere of radiata pine grown in rhizotron units was previously detected (Chapter 3), samples were also collected from various depths to assess the impact of GM pine trees on soil microbial communities both at the top and within deeper soils. Since soil properties as well as plant roots could influence rhizosphere microbial communities (Berg and Smalla 2009), microbial communities and OAs in root exudates in both rhizosphere and non-rhizosphere soils were analysed at each sampling site.

Due to the destructive sampling techniques used at the initial and final samplings, OAs in the exudates of both root with associated rhizosphere soil (root-rhizosphere soil) samples and non-rhizosphere soils could only be collected *in situ* using AEM-Whatman 3MM strips at the second sampling. However, water-soluble exudate solutions were obtained for samples from all three sampling dates.

4.2.4.1 First sampling after three months growth

Soil and roots of radiata pine were sampled horizontally using a soil corer (5 cm diameter x 45 cm depth) through the side of each rhizotron unit at depths of 10 and 20 cm (two sites at each depth) as described in Chapter 3 (Section 3.2.3; Appendix 3.5). Each soil core was placed in a sealed bag and processed on the same day as sampling. The soil corer was cleaned with a 5% bleach solution and wiped dry between each sampling. The physical integrity of these access portals were maintained using removable inflatable tubes between samplings (Chapter 3, Section 3.2.3). Root samples at the depth of 30 cm were not obtained due to the absence of roots.

All root-rhizosphere soil samples were collected from each soil core using an ethanol (70%) sterilised forceps. Root samples were gently shaken to remove non-rhizosphere soil before collection of root exudates and processing for rhizosphere microbial communities (see Sections 4.2.5 and 4.2.6). The weight of root-rhizosphere soils ranged from 0.155 to 8.414 g. After removing all root samples, the remaining soil in the soil core was considered as non-rhizosphere soil and subsequently processed as described in Sections 4.2.5 and 4.2.6 on the same day as sampling. Based on the preliminary studies described in Chapter 3, samples from the same tree lines at the same depths were similar

and could be considered as replicates. Accordingly, eight replicates were obtained from two sampling sites for each soil category (rhizosphere, non-rhizosphere soil) at each depth (10, 20 cm) from each tree line (GM, WT), providing a total of 64 samples.

4.2.4.2 Second sampling after nine months growth

To ensure pine roots had re-colonized the soil surrounding the access portals and appeared healthy after the disturbance caused by the first destructive sampling, radiata pine roots surrounding access portals were frequently observed using a medical endoscope system (Karl Storz, Germany; Figure 4.2a and b), which replaced the camera system as described in Chapter 3, Section 3.2.3. Because of its small size (1 cm diameter, 45 cm long), the endoscope camera lens could be placed in the access portal during the sampling procedure which enabled all the work to be conducted while being viewed through the camera (Figure 4.2b and d). During the observation, the endoscope camera lens was inserted into the access portal and an image was taken and stored digitally. An endoscope lens-rhizotron adapter was especially designed by S. Larsen (Lincoln University) and R. Cook (Lincoln Ventures Ltd) to hold the camera lens in the middle of the access portal, while still allowing the lens to be rotated 360° and moved freely throughout the access portal (Figure 4.2d).

The second sampling using the *in situ* sampling technique was conducted six months after the first sampling, when reasonable quantities of pine roots were visible surrounding the portals. This sampling used the access portals as sampling sites and adopted the same sampling strategy as for the first sampling. The *in situ* sampling technique using the endoscope system and long arm samplers (Figure 4.2) was developed based on the technique described in Chapter 3, Section 3.2.4.

Prior to the sampling of roots and soils, OAs in root exudates of pine trees were collected using AEM-Whatman 3MM strips through access portals in the rhizotron units. Since a preliminary experiment revealed that radiata pine roots released different amounts of OAs from the same sites at different time periods within a day (Appendix 4.3), the collection of OAs was routinely carried out at the same time period (10 am to 12 pm) from pine roots. Due to the time involved, only two access portals, selected randomly, were sampled per day and sampling was carried out over 16 days. Before sampling, the endoscope camera lens was placed into the access portal for root observation and location after removal of the inflatable tube. Three areas of root samples with different root types (root tip, mature root part, and root colonized by ECM), if available, were selected for root exudate sampling in each access portal using AEM-Whatman 3MM strips as developed in Chapter 3 (Sections 3.3.2 and 3.3.3.2). The selection of three common root types, which were

combined for exudate and rhizosphere community analysis, was to reduce any variability due to the root type (Appendix 3.7).



Figure 4.2 a). The endoscope system. b). The camera lens (45 cm long, 1 cm diameter). c). Long armed scissors and forceps (approximate 45 cm long) used during the second sampling. d). Specially designed endoscope lens-rhizotron adapter that can hold the lens in the access portal and allow the lens be able to rotate 360° and move along the access portal.

Anion exchange membrane strips were cut into small pieces (1 x 2 cm) and charged with NaHCO_3 before use as described in Chapter 3 (Section 3.2.2.1). Whatman 3MM filter paper, cut to the same size as the AEM strip, was washed in methanol followed by sterile DI water before use. An AEM strip backed with Whatman 3MM was fixed onto the soil using a thumbtack with the AEM in direct contact with the root-rhizosphere soil surface. This was achieved using long arm forceps under the light source of the endoscope (Figure 4.3a and b, Figure 4.4a). One non-rhizosphere soil area where no roots were observed through the endoscope camera system was selected for exudate collection by placing an AEM-Whatman 3MM strip on top of the soil using a thumbtack as previously described. After applying four sets of AEM-Whatman 3MM strips in each access portal (three for root-rhizosphere soil samples and one for non-rhizosphere soil), the inflatable tube was re-inserted and inflated to ensure that the AEM strips were maintained in close contact with the root-rhizosphere soil or non-rhizosphere soil samples. After 2 h, the inflatable tube was removed and the camera lens was fixed in the endoscope lens-rhizotron adapter and

positioned inside the access portal. The image of the AEM strip covered root-rhizosphere (Figure 4.4a) or non-rhizosphere soil area was recorded. The AEM strips were then removed using a long arm forceps (Figure 4.3c) and another image of the root-rhizosphere or non-rhizosphere soil area without the AEM strip was recorded immediately with the camera lens located at exactly the same place (Figure 4.4b). The AEM strip removed from the sampling site was briefly rinsed with sterile DI water before being placed in a sterile container with 1.5 ml of 0.5 M HCl and incubated at 4°C for 3 h in a shaker at 150 rpm to elute collected exudate anions. A 500 µl aliquot of the eluted root exudates from each of the three root-rhizosphere soil samples from the same access portals were bulked together in a sterile Eppendorf tube and stored at -20°C until analysis by HPLC as described previously in Chapter 3 (Section 3.2.1).



Figure 4.3 a). Placing the AEM-Whatman 3MM strip on top of roots for OA collection using long armed forceps under the endoscope light source. b). Checking the AEM-Whatman 3MM strip after being placed on top of roots for OA collection using the endoscope camera system. c). Removing AEM-Whatman 3MM from the root/soil samples and sampling root samples with long arm scissors. d). A specially designed soil sampler for non-rhizosphere soil sampling in access portals.

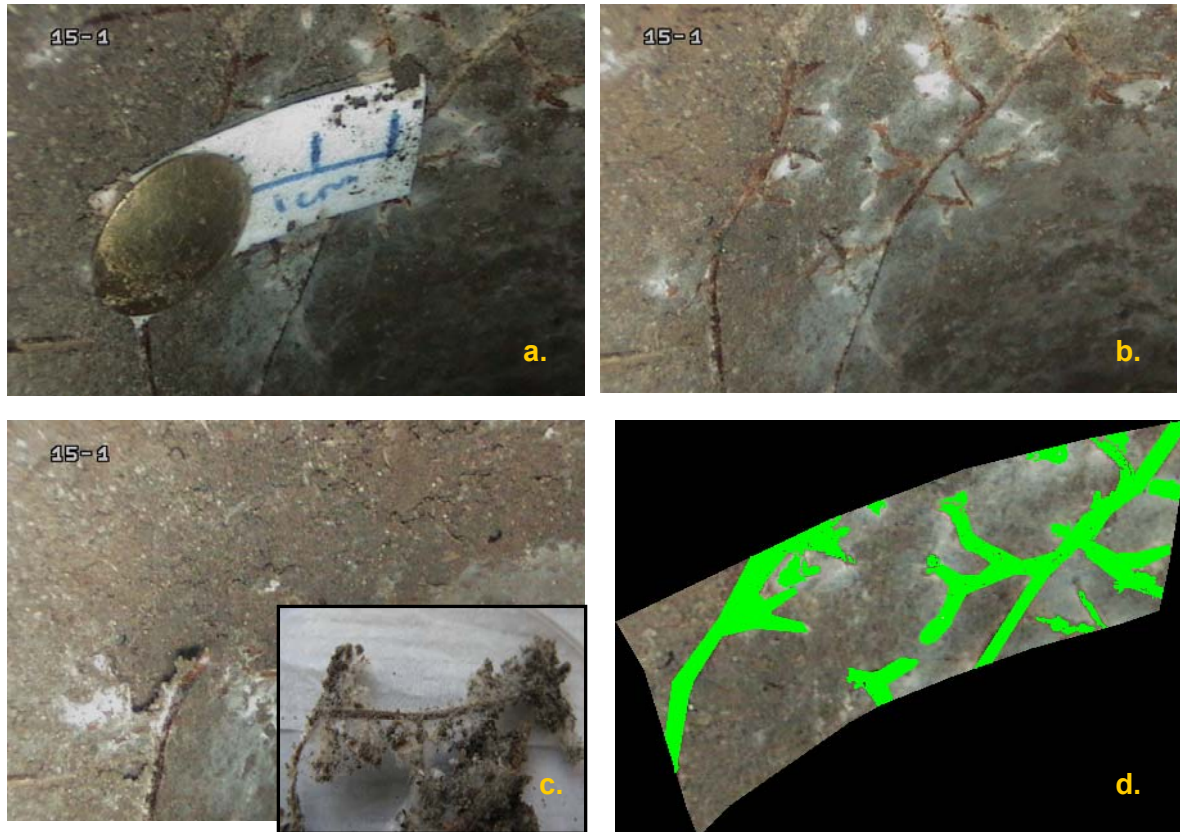


Figure 4.4 a). *In situ* collection of OAs using AEM-Whatman 3MM from roots of radiata pine in the access portal. b). The root area where exudates was sampled from after removal of the AEM-Whatman 3MM strip. c). Roots from where exudates were collected were sampled from the access portal and used for further analyses. d). Calculation of the root area directly in contact with AEM strip using digital imagery designed software. The green area show the roots covered by the AEM strip in figure a. by overlaying images a. and b.

Root samples which were used for exudate collection in the previous step were sampled using long arm scissors (Figure 4.3c, Figure 4.4c) and shaken gently to remove any non-rhizosphere soil before being placed in a sterile 50 ml Falcon tube (one tube per access portal). In order to obtain sufficient rhizosphere soil for microbial community analysis, in most cases, root samples, which were not collected for root exudates in the previous step, were also sampled. The root-rhizosphere soil samples collected from access portals in this sampling ranged from 0.082 to 2.43 g. Non-rhizosphere soil (approximate 2 g) was sampled at the place where exudates were collected using a specially designed sampler and placed in a sterile 50 ml Falcon tube (Figure 4.3d). Root-rhizosphere soil and non-rhizosphere soil samples were processed for collection of water-soluble exudates and RNA extraction (see Sections 4.2.5 and 4.2.6) on the sampling day. The camera lens, soil sampler, long arm forceps and scissors and standard forceps were sterilised with 70% ethanol and wiped dry between each sampling.

In this sampling, eight replicates were obtained from two access portals for each soil category (root-rhizosphere soil, non-rhizosphere soil) at each depth (10, 20 cm) from each tree line (GM, WT), resulting in a total of 64 samples.

Software developed by Prof. Alan McKinnon and Dr. Keith Unsworth at Lincoln University was used to calculate the root area covered by the AEM strip. The programme involved overlapping of two images, with or without the AEM strip, which were recorded by the camera at the same position, identification of roots based on colour, and then calculation of the fraction of root area in the total AEM area (2 cm²) (Figure 4.4d). The position of AEM strip for exudate collection was optimised to minimize errors in root area calculation using the software (e.g. by geometrical distortions) as outlined in Appendix 4.4. As the root surfaces covered by the AEM strips were very variable (ranged from less than 5% to over 65% of the area of the AEM strips) depending on the root growth around access portals, the normalization of exudate concentrations in relation to the sampled root surface areas enabled the variability in exudates caused by the variation in root areas sampled to be reduced.

4.2.4.3 Third sampling after 10 months growth

The third sampling was carried out destructively using a soil corer (diameter 10 cm) inserted vertically from the top to the bottom of the rhizotron (Figure 4.5a). One site which was approximately 20 cm away from the base of the tree in each rhizotron unit was sampled at three depths (D1=0-14 cm, D2=14-28 cm, D3=28-45 cm) in three steps with the corer pushed to the corresponding depth in each case. The soil corer was cleaned with a 5% bleach solution and wiped dry between each sampling. All the root-rhizosphere soil samples (ranging from 0.385 to 5.295 g for this sampling) were collected from each soil core using an ethanol (70%) sterilised forceps and gently shaken to remove non-rhizosphere soil for subsequent collection of water-soluble root exudates and processing for rhizosphere microbial communities (see Sections 4.2.5 and 4.2.6). After removing all the roots, the remaining soil in the core was considered to be non-rhizosphere soil and subsequently processed as described in Sections 4.2.5 and 4.2.6 on the same day as sampling. Four replicates were obtained for each soil category (rhizosphere soil, non-rhizosphere soil) at each depth (D1, D2, D3) from each tree line (GM, WT), providing a total of 48 samples.

Bulk soil bags placed at each corner of the rhizotron units were sampled on the same day by removal from the rhizotron units and dividing the soil representing two depths (D1=0-14 cm, D2=14-28 cm) (Figure 4.5a and b). The bulk soil samples from two bags in each rhizotron unit at the same depth were bulked together to reduce the number of samples

for processing. Accordingly, four replicates of bulk soil samples at each depth (D1, D2) from each tree line (GM, WT) from the bulk soil bags were sampled in this time. In total, there were 16 bulk soil samples.

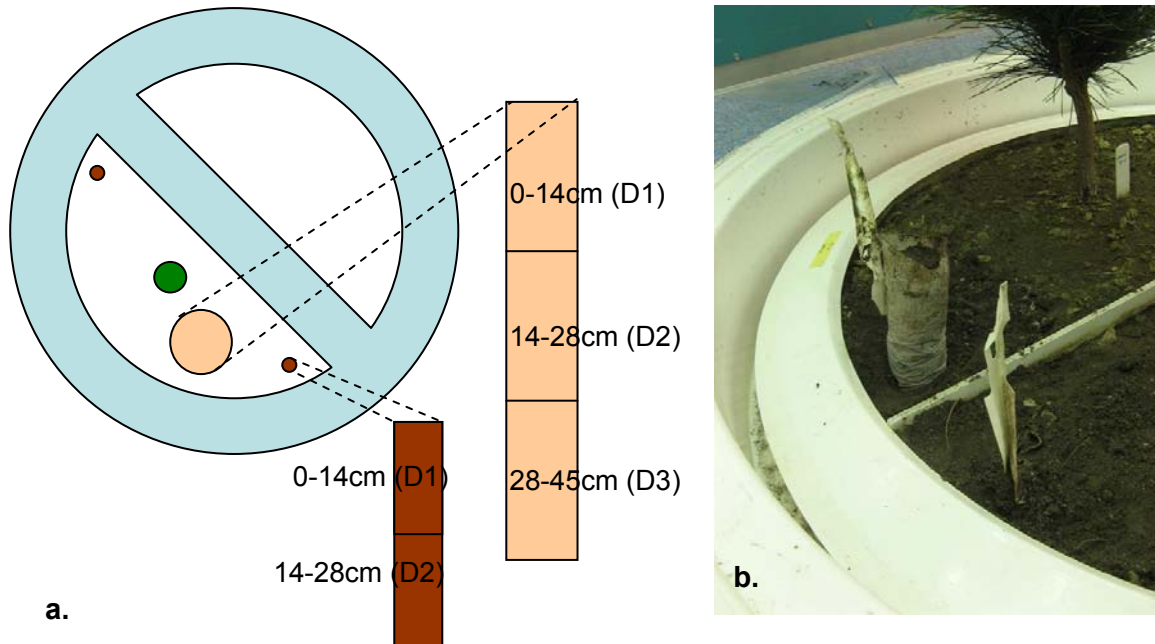


Figure 4.5 a). Sketch of the third sampling strategy. The green circle represents a radiata pine tree. The light brown circle represents a sampling site which was about 20 cm away from the tree base. Three depths (D1=0-14 cm, D2=14-28 cm and D3=28-45 cm) were sampled at each site. The dark brown circles represent the bulk soil bags at two corners in each rhizotron unit. Soil in the bag was split representing two depths (D1=0-14 cm, D2=14-28 cm). b). Removal of the bulk soil bag from each corner of the rhizotron unit.

4.2.5 Extraction and Analysis of Water-soluble Exudate Solutions

Water-soluble exudates were extracted from all the soils (root-rhizosphere, non-rhizosphere and bulk soils) collected from the three samplings. Each root-rhizosphere soil sample was placed in a sterile 50 ml Falcon tube and weighed. One gram of non-rhizosphere or bulk soil sample was placed in a sterile 50 ml Falcon tube using an ethanol (70%) sterilised spatula. Exudate solutions from samples were extracted by addition of sterile DI water at a ratio of 5 ml to 1 g fresh root-rhizosphere, fresh non-rhizosphere or bulk soil in tubes and shaking the tubes for 10 min at 100 rpm, followed by centrifugation at 2,800 *g* for 10 min. This extraction method was modified based on the method described by Schefe et al. (2008) with a different ratio of water to root-rhizosphere, non-rhizosphere or bulk soil samples according to the experiment design (different soil, different amount of soil samples). In addition, the centrifuge-filtration of the supernatant which was conducted by Schefe et al. (2008) was not performed in the current experiment because clear supernatant was obtained after centrifugation. A preliminary test showed

that most OAs in water-soluble exudate samples extracted by this modified method could be detected by HPLC. The supernatant containing water-soluble exudates from each Falcon tube was transferred into a sterile tube and stored at -20°C until analysis by HPLC, as described in Chapter 3 (Section 3.2.1).

4.2.6 Soil Preparation and Microbial Community Analysis

After extraction of the water-soluble root exudate solution, the root-rhizosphere soils in each Falcon tube were resuspended with 25 ml of sterile water and transferred to a sterile filter bag for rhizosphere soil preparation using the method described in Chapter 2 (Section 2.2.1). The rhizosphere soil obtained was stored at -80°C for subsequent RNA extraction. A fresh 0.5 g of non-rhizosphere soil from each sampling site was collected and stored in a sterile 2 ml Eppendorf tube at -80°C on the same day as sampling until subsequent RNA extraction. The bulk soil samples (0.5 g per sampling site) collected from the third sampling were stored in sterile 2 ml Eppendorf tubes at -80°C immediately for further processing and analysis. The molecular analysis of the microbial communities, including general bacterial 16S, taxon-specific α -, β -proteobacterial, pseudomonad 16S, and general fungal 18S, in all the soil samples (rhizosphere soil, non-rhizosphere and bulk soil) using rRNA-DGGE technique is described in Chapter 2, Sections 2.2.2 - 2.2.7.

4.2.7 Statistical Analysis

To reduce the influence of depth-related variability identified in Chapter 3, both OAs in exudates and microbial communities were compared between the soil categories (root-rhizosphere vs. non-rhizosphere vs. bulk soils) and tree lines (GM vs. WT pines) at corresponding depths. Because of the significant difference between rhizosphere and non-rhizosphere soil (and bulk soil at the third sampling) in both DGGE patterns and OA profiles, the tree line treatment analyses were carried out separately on rhizosphere and non-rhizosphere soils.

Some samples contained either no OAs or the levels of OAs were below the limits of detection. For these samples, OA levels were specified as half the limit of detection of the HPLC method (Chapter 3, Table 3.1). To arrive at a comprehensive view of the OAs in each sample, the data was analysed using four different methods (D. Baird, pers. comm.). Firstly, individual OA components were \log_{10} transformed and analysed using ANOVA according to the treatment effects (soil categories, tree lines). Total OAs, calculated as the sum of C of individual OAs, was also \log_{10} transformed followed by ANOVA analysis. In addition, the structure of OA profiles, which considered the components as well as concentrations of OAs present in samples, were analysed using linear discriminant analysis. To reduce the interference caused by infrequently detected OAs in root exudate

samples, only OAs which were frequently (i.e. present in at least 40% of total root-rhizosphere exudate samples in each sampling) detected were selected for OA profile analysis. Furthermore, the diversities of OA profiles were assessed by a Shannon diversity index (H') followed by ANOVA analysis. Shannon diversity index is a commonly used diversity index in ecological analysis (Magurran 1988) and may provide useful information in relation to the soil microbial diversity as analysed in this experiment. The diversity measurement takes into account two factors: richness (i.e. how many OAs are present in each sample) and evenness (also known as equitability, i.e. how equally abundant the OAs are in each sample) (Magurran 1988). Due to the varying limits of detection for the OAs (Chapter 3, Table 3.1), the concentrations of OAs were standardised to a scale of 0 to 1. This was achieved by dividing the concentration of individual OA in each sample by the highest concentration of corresponding OA detected in all the exudate samples at each sampling time. All the OAs were included for the diversity measurement. The Shannon index is calculated using the formula as

$$H' = - \sum_i (n_i / N) \times \log(n_i / N)$$

where n_i are the standardised concentration of each organic anion and $N = \sum_i n_i$.

Due to the infrequent detection of OAs, the total C of OAs and the diversity of OA profiles in water-soluble solutions extracted from non-rhizosphere and bulk soils were not analysed.

Statistical analysis of the structure of microbial communities on DGGE gels is described in Chapter 2, Section 2.2.8. In addition, the diversity of the communities was assessed by a Shannon diversity index based on the presence/absence of the bands on DGGE gels. H' was calculated using the formula above where n_i was 0 (band absence) or 1 (band presence) for any particular band, and N is the total bands (including bands which were not present in this sample but in other samples) detected in each lane by GenStat (D. Baird, pers. comm.). The indices were then analysed using ANOVA between the treatments.

The ANOVA, linear discriminant analyses and calculation of Shannon diversity indices were conducted using Genstat™ 11 (VSN International Ltd, UK). Although ANOVA was carried out across treatments with multi-comparison, only comparison between soil categories or tree lines at each depth was used in this study. For multiple comparisons, treatment means were separated using Fisher's protected LSD at $P=0.05$ level.

4.3 Results

4.3.1 Root Morphology

A number of different radiata pine root morphologies were observed in the access portals of rhizotron units using the endoscope camera system. Selections of the typical root morphologies as well as frequently observed ECM colonized roots are shown in Figure 4.6. In addition, soil organisms such as springtails (*Collembola*) were sometimes seen near roots (Figure 4.6e). The mycelia of ECM or other fungi were observed spread widely around the soil adjacent to roots in several access portals (Figure 4.6f).

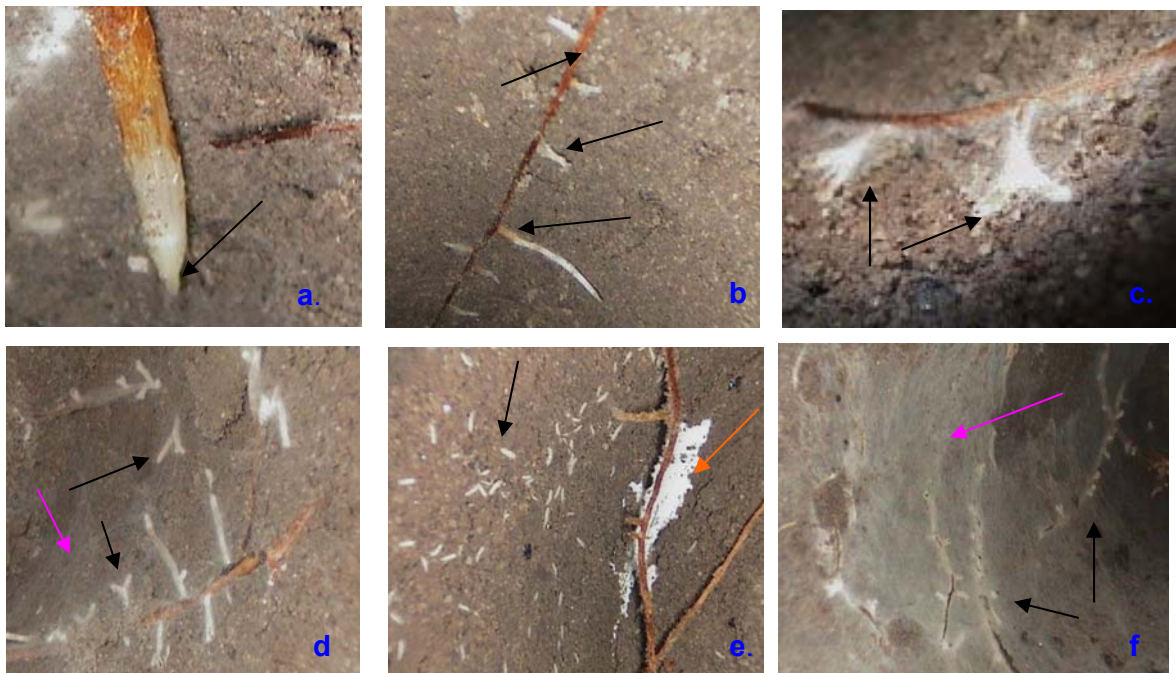


Figure 4.6 Images of radiata pine roots observed using the endoscope camera system in access portals in rhizotrons. a). A root with its root tip (indicated by an arrow). b). A root with several branching lateral root tips (indicated by arrows). c). A root colonized by ECM (indicated by arrows) with the ECM mantle clearly seen. d). Several roots were colonized by ECM (indicated by black arrows) and the mycelia of ECM or saprophytic fungi (indicated by a pink arrow) were seen to proliferate in the soil. e). Numerous springtails (indicated by a black arrow) were seen near two roots with some unidentified white matter (indicated by an orange arrow). f). Large amount of fungal hypha (indicated by the pink arrow) spread around the wall of the access portal with several roots colonized by ECM (indicated by black arrows).

4.3.2 Soil Microbial Community Analyses

4.3.2.1 Microbial communities in different soil samples

Microbial communities were compared in rhizosphere with non-rhizosphere or bulk soils (sampled from bulk soil bags) at corresponding depths (Table 4.1). With the exception of fungal communities at the first and third samplings, discriminant analyses showed that the various taxonomic groups differed significantly between the rhizosphere and the non-

rhizosphere soils as well as the rhizosphere and the bulk soils, indicating the strong rhizosphere effect by radiata pine roots, as was previously observed in Chapter 3.

To assess the potential influence of root activity on non-rhizosphere soil, further analysis was conducted to compare the microbial communities from non-rhizosphere and bulk soils with the samples from the third sampling (Table 4.2). Three out of the five taxonomic groups analysed (general bacteria, β -Proteobacteria and Pseudomonads) showed significant differences between non-rhizosphere and bulk soils at a depth of D1 (Table 4.2). Only the β -proteobacterial community in non-rhizosphere soil differed significantly from that in the bulk soil at the D2 depth. The fungal community did not show any significant difference between non-rhizosphere and bulk soil at either depth (Table 4.2).

Table 4.1 Hotelling T^2 probabilities of the level of difference between microbial communities in rhizosphere (RS) and non-rhizosphere (N-RS) or bulk (BS) soil samples collected at various depths on three sampling periods, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two soil categories are shown in bold.

Sampling time	1 st sampling		2 nd sampling		3 rd sampling				
	RS vs. N-RS		RS vs. N-RS		RS vs. N-RS			RS vs. BS	
Depth	10 cm	20 cm	10 cm	20 cm	D1*	D2	D3	D1	D2
Bacterial 16S	0.0000	0.0000	0.0000	0.0000	0.0007	0.0024	0.0489	0.0013	0.0004
α -Proteobacteria	0.0000	0.0002	0.0058	0.0283	0.0005	0.0035	0.0004	0.0004	0.0008
β -Proteobacteria	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Pseudomonads	0.0000	0.0001	0.0000	0.0000	0.0165	0.0289	0.0001	0.0459	0.0358
Fungal 18S	0.8336	0.7486	0.0003	0.0014	0.5761	0.3858	0.4356	0.1697	0.2115

*D1 = 0-14 cm; D2 = 14-28 cm and D3 = 28-45 cm

Table 4.2 Hotelling T^2 probabilities of the level of difference between microbial communities in non-rhizosphere (N-RS) and bulk (BS) soil samples collected at various depths on the third sampling, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two soil categories are shown in bold.

Treatment	N-RS vs. BS	
	D1*	D2
Bacterial 16S	0.0360	0.9027
α -Proteobacteria	0.1337	0.1697
β -Proteobacteria	0.0027	0.0459
Pseudomonads	0.0211	0.9810
Fungal 18S	0.6709	0.6271

*D1 = 0-14 cm; D2 = 14-28 cm

4.3.2.2 Structure of microbial communities associated with pine tree lines

Significant differences in the structure of the rhizosphere microbial communities were detected between GM and WT tree lines. General rhizosphere bacterial communities

differed significantly between the two tree lines at the first sampling at a depth of 20 cm (Table 4.3a) but when three taxon-specific communities were used, no significant differences between the two tree lines were detected. The α -proteobacterial and pseudomonad 16S communities associated with the GM tree line collected at the depth of 10 cm or D1 from the second and third samplings differed significantly from those in WT pine trees at the corresponding depths (Table 4.3b and c). In addition, β -proteobacterial 16S communities showed significant differences between GM and WT rhizosphere soil samples at a depth of D1 from the third sampling, with no difference detected at other depths or other sampling periods. The significant differences in rhizosphere communities between the two tree lines were also observed in α -proteobacterial 16S and fungal 18S communities at the depth of D2 from the third sampling (Table 4.3c). However, there were no consistent differences in communities between the tree lines over the duration of the experiment.

Table 4.3 Hotelling T^2 probabilities of the level of difference between various microbial communities associated with GM and WT tree lines with soil samples collected at various depths on the a). first sampling period ($n=8$), b). second sampling period ($n=8$), c). third sampling period ($n=4$), with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P<0.05$) between two tree lines are shown in bold.

a. 1 st sampling		Rhizosphere soil		Non-rhizosphere soil	
Taxonomic group	10cm	20cm	10cm	20cm	
Bacterial 16S	0.5588	0.0089	0.1715	0.5552	
α -Proteobacteria	0.062	0.2142	0.4815	0.4823	
β -Proteobacteria	0.7730	0.0525	0.7752	0.0043	
Pseudomonads	0.7284	0.2152	0.2973	0.2659	
Fungal 18S	0.0917	0.0572	0.3057	0.0134	

b. 2 nd sampling		Rhizosphere soil		Non-rhizosphere soil	
Taxonomic group	10cm	20cm	10cm	20cm	
Bacterial 16S	0.5180	0.2054	0.3388	0.0543	
α -Proteobacteria	0.0013	0.1332	0.0067	0.0027	
β -Proteobacteria	0.1586	0.1504	0.3803	0.0277	
Pseudomonads	0.0002	0.6115	0.0676	0.8031	
Fungal 18S	0.1126	0.367	0.4718	0.1445	

c. 3 rd sampling		Rhizosphere soil			Non-rhizosphere soil		
Taxonomic group	D1*	D2	D3	D1	D2	D3	
Bacterial 16S	0.1440	0.4053	0.9350	0.2732	0.8401	0.9175	
α -Proteobacteria	0.0245	0.0427	0.3539	0.2740	0.1938	0.1478	
β -Proteobacteria	0.0285	0.1048	0.4662	0.8173	0.0049	0.3590	
Pseudomonads	0.0014	0.5981	0.8884	0.8463	0.1131	0.9541	
Fungal 18S	0.7422	0.0329	0.1143	0.7132	0.7957	0.1690	

*D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm.

As with the microbial communities in rhizosphere soils, significant differences between non-rhizosphere communities from soil supporting growth of the two tree lines were observed. Beta-proteobacterial communities demonstrated consistently different DGGE profiles between non-rhizosphere samples from GM and WT radiata pine at a depth of 20 cm or corresponding D2 in all three samplings (Table 4.3). Alpha-proteobacterial 16S communities in non-rhizosphere soil samples also differed significantly between the two tree lines at depths of 10 and 20 cm, but these differences were only detected at the second sampling (Table 4.3b).

Table 4.4 Shannon diversity indices of soil microbial communities associated with GM and WT pine trees collected at various depths on the first sampling period ($n=8$), and the P -values of ANOVA analysis across treatments. Values are presented as the mean \pm 1 standard error for samples in each treatment. The probability values with significant difference ($P<0.05$) between treatments are shown in bold. For each row, different letters indicate significant difference ($P<0.05$) between the treatment means.

Soil	Taxonomic group	GM 10cm	WT 10cm	GM 20cm	WT 20cm	P -value
Rhizosphere soil	Bacterial 16S	3.46 \pm 0.07	3.60 \pm 0.03	3.56 \pm 0.05	3.61 \pm 0.03	0.062
	α -Proteobacteria	3.01 \pm 0.05	3.01 \pm 0.03	2.89 \pm 0.03	2.99 \pm 0.06	0.144
	β -Proteobacteria	2.56 \pm 0.08	2.51 \pm 0.12	2.59 \pm 0.07	2.48 \pm 0.11	0.709
	Pseudomonads	3.04 \pm 0.06	3.01 \pm 0.04	3.06 \pm 0.03	3.10 \pm 0.05	0.369
	Fungal 18S	3.07 \pm 0.04 ^b	3.10 \pm 0.04 ^b	3.08 \pm 0.02 ^b	2.94 \pm 0.04 ^a	0.008
Non-rhizosphere soil	Bacterial 16S	3.45 \pm 0.04	3.50 \pm 0.06	3.52 \pm 0.04	3.51 \pm 0.04	0.589
	α -Proteobacteria	3.10 \pm 0.03 ^c	2.96 \pm 0.04 ^a	2.98 \pm 0.04 ^{ab}	3.07 \pm 0.05 ^{bc}	0.028
	β -Proteobacteria	2.51 \pm 0.04 ^a	2.57 \pm 0.07 ^{ab}	2.58 \pm 0.06 ^{ab}	2.71 \pm 0.05 ^b	0.047
	Pseudomonads	3.05 \pm 0.03 ^b	3.06 \pm 0.04 ^b	2.99 \pm 0.04 ^a	3.04 \pm 0.03 ^b	0.011
	Fungal 18S	3.10 \pm 0.05	3.14 \pm 0.04	3.08 \pm 0.05	3.13 \pm 0.06	0.794

4.3.2.3 Diversity of microbial communities associated with pine tree lines

Diversity of the microbial communities was analysed using the Shannon diversity index (Table 4.4, Appendix 4.5). In all three samplings, the diversity indices between taxonomic communities of rhizosphere and non-rhizosphere were not significantly different ($P<0.05$). This however does not indicate that the communities are necessarily similar (Magurran 1988). The diversity indices for the general bacterial 16S communities were highest (around 3.50) compared to the other taxon-specific communities (ranged from 2.37 to 3.10) in both rhizosphere and non-rhizosphere samples (Table 4.4, Appendix 4.5). The lowest H' indices were present in β -proteobacteria across all the treatments. ANOVA analyses showed that apart from the first sampling, the indices did not differ significantly between GM and control trees in all three samplings (Table 4.4, Appendix 4.5). The H' of the rhizosphere fungal 18S community associated with GM pine roots was significantly higher than that associated with the control trees at the depth of 20 cm, indicating a more diverse fungal community colonized in the rhizosphere of the modified pine in comparison with the control pine at that depth (Table 4.4).

In the non-rhizosphere soil samples collected from the first sampling, the H' indices for the α -proteobacterial communities were significantly higher ($P < 0.05$) in GM when compared to the control tree line at a depth of 10 cm, but lower ($P > 0.05$) at a depth of 20 cm (Table 4.4). Diversity indices in pseudomonad communities in non-rhizosphere soil of WT trees at depth of 20 cm from the first sampling were significantly higher ($P < 0.05$) than those of GM trees at corresponding depth, but not in the other depth (Table 4.4).

4.3.3 Organic Anions in Root Exudates Collected *in situ*

4.3.3.1 Identification of organic anions

Individual organic anions

Organic anions in exudates of root-rhizosphere and non-rhizosphere soils from both GM and WT pine trees collected *in situ* in access portals by AEM during the second sampling were calculated as μg per cm^2 of root/soil contacting with AEM for 2 h collection period. Up to 11 OAs, including tartarate, acetate, lactate, formate, quinate, malate, malonate, citrate, shikimate, maleate and fumarate, were detected in exudate samples of root-rhizosphere soils, although some anions were only present in a few samples (Figure 4.7, Table 4.5). Acetate, lactate and formate were the most frequently detected and the most abundant anions in the exudates of root-rhizosphere soil. Maleate, shikimate and fumarate were also frequently detected in the exudates of root-rhizosphere soil samples (> 84%), although were present at lower concentrations. Tartarate was detected in only five out of 32 samples, but was present at high concentrations in some samples (up to $167 \mu\text{g cm}^{-2}$). Nine OAs (tartarate, acetate, lactate, formate, quinate, malate, shikimate, maleate and fumarate) were also detected in the exudate samples from non-rhizosphere soils, albeit at significantly lower concentrations in comparison with those in the root-rhizosphere exudate samples (Figure 4.8). The detection frequencies of some OAs (acetate, lactate, formate, maleate and fumarate) in non-rhizosphere soil were over 50% (Table 4.5). Malate and shikimate were only detected in four non-rhizosphere exudate samples, while they were frequently detected in the rhizosphere exudates (Table 4.5).

As expected, the concentrations of all the individual OAs differed significantly between rhizosphere and non-rhizosphere exudate samples, with all $P < 0.01$ apart from tartarate ($P = 0.013$). Although ANOVA analysis showed that the amounts of malonate collected from WT pines were significantly higher than those from GM trees at both depths, this anion was only detected in two out of 32 samples (Figure 4.7, Table 4.5). Apart from malonate, concentrations of all other anions did not differ significantly in root-rhizosphere exudate samples between the two tree lines. Concentrations of maleate differed significantly

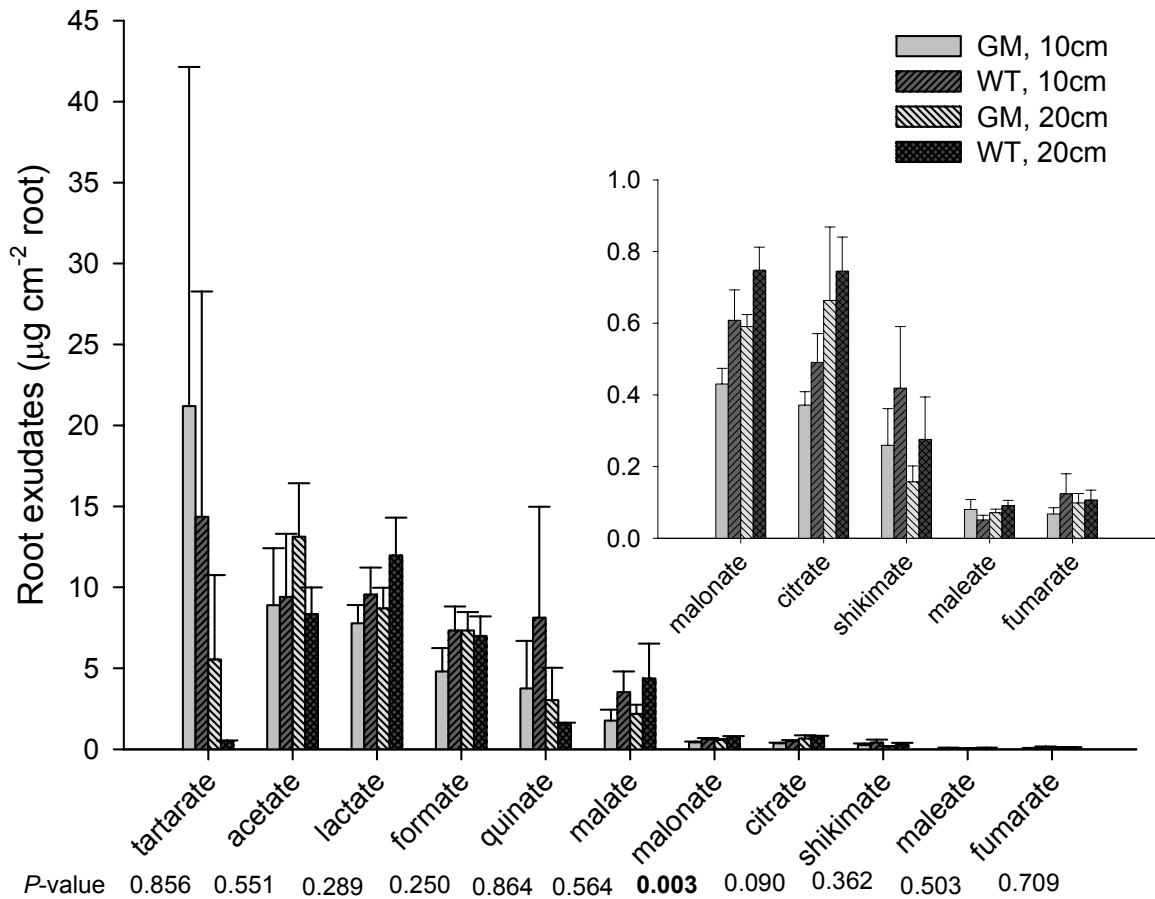


Figure 4.7 The concentrations of OAs collected *in situ* by AEM from root-rhizosphere soils during the second sampling, and respective probability values analysed by ANOVA across the four treatments. Error bars show 1 standard error ($n=8$), and the P -values with significant difference across the treatments ($P<0.05$) are shown in bold. Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph.

Table 4.5 The number of exudate samples collected by AEM where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table 3.1) in four treatments (GM 10 cm, WT 10 cm, GM 20 cm, WT 20 cm) ($n=8$) and in total ($n=32$).

Soil	Root-Rhizosphere soil					Non-rhizosphere soil					
	Treatment	GM 10cm	WT 10cm	GM 20cm	WT 20cm	Total	GM 10cm	WT 10cm	GM 20cm	WT 20cm	Total
tartarate		2	2	1	0	5	0	1	0	1	2
acetate		6	8	8	8	30	8	8	7	8	31
lactate		8	8	8	8	32	7	6	8	7	28
formate		8	8	8	8	32	8	6	8	8	30
quinate		1	2	1	0	4	0	0	0	1	1
malate		3	5	5	3	16	1	1	1	1	4
malonate		0	1	1	0	2	0	0	0	0	0
maleate		6	5	8	8	27	6	2	7	8	23
citrate		0	0	1	1	2	0	0	0	0	0
shikimate		7	7	8	8	30	2	0	0	2	4
fumarate		6	8	8	7	29	2	6	5	8	21

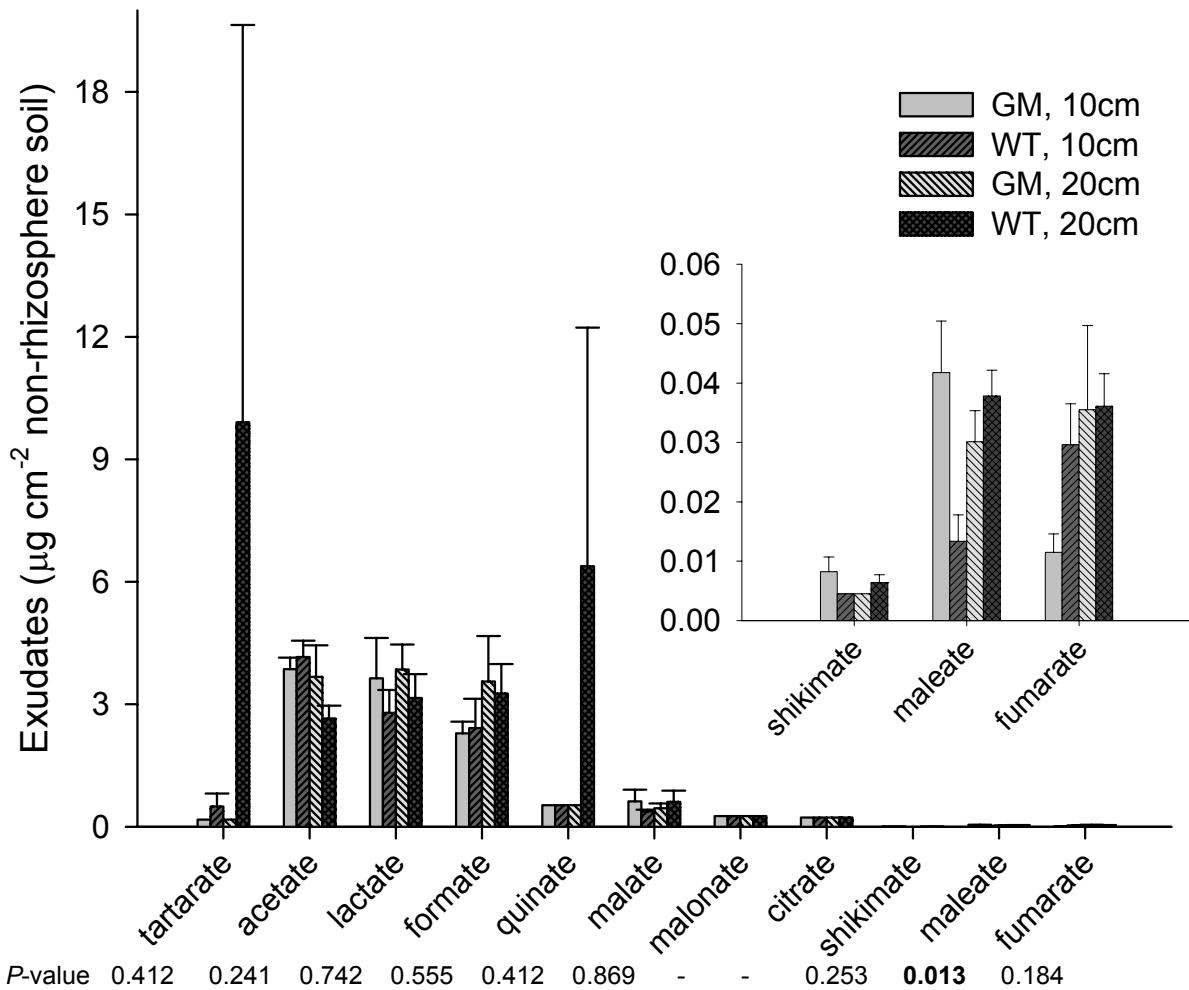


Figure 4.8 The concentrations of OAs collected *in situ* by AEM from non-rhizosphere soils during the second sampling, and respective probability values analysed by ANOVA across the four treatments. Error bars show 1 standard error ($n=8$), and the P -values with significant difference across the treatments ($P<0.05$) are shown in bold. “-” indicates that no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates collected by AEM. Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph.

between exudates from non-rhizosphere soil in which GM and WT trees were grown at the depth of 10 cm, but not at 20 cm depth (Figure 4.8).

Total organic anions

The total C of OAs determined in the exudates of root-rhizosphere soils ranged from 13.1 to 19.6 $\mu\text{g C cm}^{-2}$ of roots (Table 4.6). There was no significant difference ($P<0.05$) between GM and WT trees at either depth. In comparison with those in the rhizosphere exudate samples, the total C of OAs in the exudate samples of non-rhizosphere soil were lower, ranging from 4.2 to 9.6 $\mu\text{g cm}^{-2}$ of roots (Table 4.6). Because of the large variability in amounts of OAs recovered from non-rhizosphere soils, ANOVA analysis was not performed.

Table 4.6 Total C of OAs in exudate samples of GM and WT pine trees collected by AEM ($\mu\text{g C cm}^{-2}$ root) during the second sampling, and the probability values across the treatments analysed by ANOVA. The amounts are presented as the mean \pm 1 standard error for samples from different treatments ($n=8$).

	GM 10 cm	WT 10 cm	GM 20 cm	WT 20 cm	P-value
Rhizosphere	17.5 \pm 8.5	19.6 \pm 7.7	15.2 \pm 4.0	13.1 \pm 1.9	0.905
Non-rhizosphere	4.3 \pm 0.5	4.2 \pm 0.4	4.6 \pm 0.7	9.6 \pm 5.8	n.a.

Note: n.a. = not applicable, ANOVA analysis was not valid for the comparison of total C analysis across the treatments in the non-rhizosphere soil due to the difference in the variance in treatments.

4.3.3.2 Organic anion profiles

The structure of OA profiles, which included both the composition and concentrations of OAs in each exudate sample, were analysed using linear discriminant analysis between tree lines at various depths. The anions acetate, lactate, formate, malate, maleate, shikimate and fumarate, which were detected in at least 50% of the root-rhizosphere exudate samples, were selected for structures of OA profile analysis. The discriminant analysis showed that structures of OA profiles in exudate samples of root-rhizosphere soils differed significantly from those of non-rhizosphere soils ($P=0.000$). Organic anions which showed large differences between the frequency of detection in rhizosphere and non-rhizosphere exudate samples (such as malate and shikimate) may be major drivers for this observation. However, no significant difference was detected between the two tree lines at either depth within either root-rhizosphere or non-rhizosphere exudate samples (Table 4.7).

Table 4.7 Hotelling T^2 probabilities of the level of difference between the structure of OA profiles in the exudate samples associated with GM and WT tree lines collected by AEM at the second sampling ($n=8$), with each sample under the null hypothesis that all the samples have the same mean.

Treatment	GM vs. WT	
	10 cm	20 cm
Rhizosphere	0.8466	0.7198
Non-rhizosphere	0.1350	0.9960

The diversity of OAs profiles in each sample was assessed by Shannon diversity index based on the presence of OAs and their relative amounts are shown in Table 4.8. The diversity indices in the exudate samples of root-rhizosphere soils ranged from 2.10-2.16 and were significantly higher ($P<0.001$) than those in exudate samples of non-rhizosphere soils which ranged from 1.97-2.07. There were no significant differences in diversity indices of OA profiles between GM and WT tree lines at either depth within exudate samples of either rhizosphere or non-rhizosphere soils (Table 4.8).

Table 4.8 Shannon diversity indices of OA profiles in the exudate samples collected by AEM during the second sampling, and the probability values across the treatments analysed by ANOVA. Diversity indices are presented as the mean \pm 1 standard error for samples across the treatments ($n=8$).

Treatment	GM 10cm	WT 10cm	GM 20cm	WT 20cm	P-value
Rhizosphere	2.10 \pm 0.05	2.16 \pm 0.02	2.16 \pm 0.04	2.14 \pm 0.02	0.690
Non-rhizosphere	2.01 \pm 0.03	1.97 \pm 0.03	2.03 \pm 0.02	2.07 \pm 0.02	0.079

4.3.4 Organic Anions in Water-soluble Exudate Solutions

4.3.4.1 Identification of organic anions

Individual organic anion

Between eight and eleven different OAs were detected in water-soluble exudate solutions from rhizosphere soil taken at each of three sampling periods. The compositions and relative amounts of OAs differed between sampling dates. In the water-soluble exudate solutions of root-rhizosphere soils from the first sampling, formate, acetate, quinate, shikimate, malate, lactate, fumarate and maleate were detected, although at different frequencies (Figure 4.9, Table 4.9a). Formate and acetate were recovered in the highest concentrations, with average concentrations of 17 to 39 and 16 to 35 $\mu\text{g g}^{-1}$ of root-rhizosphere soil, respectively across the four treatments (each tree line at depths of 10 cm and 20 cm). Shikimate was detected in 31 out of 32 samples, although at low concentrations that ranged from 7.1 to 18.1 $\mu\text{g g}^{-1}$ of root-rhizosphere soil (Figure 4.9, Table 4.9a). The average concentrations of fumarate and maleate in the four treatments were all below 1 $\mu\text{g g}^{-1}$ of root-rhizosphere soil (Figure 4.9).

In the water-soluble root exudate solutions of root-rhizosphere soils from the second sampling, a total of 11 anions (formate, acetate, quinate, shikimate, malate, succinate, lactate, malonate, citrate, fumarate and tartarate) were detected, and as with the first sampling, concentrations and frequencies varied greatly (Figure 4.10, Table 4.9b). Unlike the first sampling, the OAs present in highest concentrations in water-soluble root-rhizosphere exudate solutions were quinate (ranged from 137 to 290 $\mu\text{g g}^{-1}$ of root-rhizosphere soil) and shikimate (ranged from 70 to 141 $\mu\text{g g}^{-1}$ of root-rhizosphere soil) (Figure 4.10).

In the water-soluble root exudate solutions of root-rhizosphere soils from the third sampling, ten organic anions (quinate, shikimate, malate, succinate, lactate, malonate, citrate, fumarate, tartarate and maleate) were detected (Figure 4.11, Table 4.9c). Succinate, tartarate and maleate were only present in less than 30% of the total root-rhizosphere soil exudate solutions (Table 4.9c). Quinate and lactate were detected in all 24 root-rhizosphere exudate solutions, and were present in the highest concentrations.

Table 4.9 The number of water-soluble root-rhizosphere exudate solutions collected at three sampling periods where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table 3.1) in each treatment ($n=8$ for the first and second samplings, $n=4$ for the third sampling) and in total ($n=32$ for the first and second samplings, $n=24$ for the third sampling).

a. 1 st sampling	GM 10 cm	WT 10 cm	GM 20 cm	WT 20 cm	Total
formate	7	7	7	7	28
acetate	6	6	6	5	23
quinatate	6	6	4	4	20
shikimate	8	8	8	7	31
malate	7	7	4	6	24
succinate	0	0	0	0	0
lactate	3	1	2	0	5
malonate	0	0	0	0	0
citrate	0	0	0	0	0
fumarate	8	8	7	7	30
tartarate	0	0	0	0	0
maleate	2	5	6	4	17

b. 2 nd sampling	GM 10 cm	WT 10 cm	GM 20 cm	WT 20 cm	Total
formate	1	0	2	0	3
acetate	8	8	8	3	27
quinatate	8	7	8	8	31
shikimate	8	8	8	8	32
malate	8	7	8	8	31
succinate	6	4	5	0	15
lactate	7	7	8	8	30
malonate	8	7	8	8	31
citrate	8	8	8	9	31
fumarate	8	8	8	8	32
tartarate	5	1	7	1	14
maleate	0	0	0	0	0

c. 3 rd sampling	GM D1*	WT D1	GM D2	WT D2	GM D3	WT D3	Total
formate	0	0	0	0	0	0	0
acetate	0	0	0	0	0	0	0
quinatate	4	4	4	4	4	4	24
shikimate	4	4	4	4	4	4	24
malate	4	4	3	1	1	2	15
succinate	1	2	0	2	1	1	7
lactate	4	4	4	4	4	4	24
malonate	3	2	1	2	0	3	11
citrate	4	4	4	4	4	4	24
fumarate	4	4	4	4	4	4	24
tartarate	1	0	1	1	1	1	5
maleate	0	0	1	1	0	0	2

*D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm.

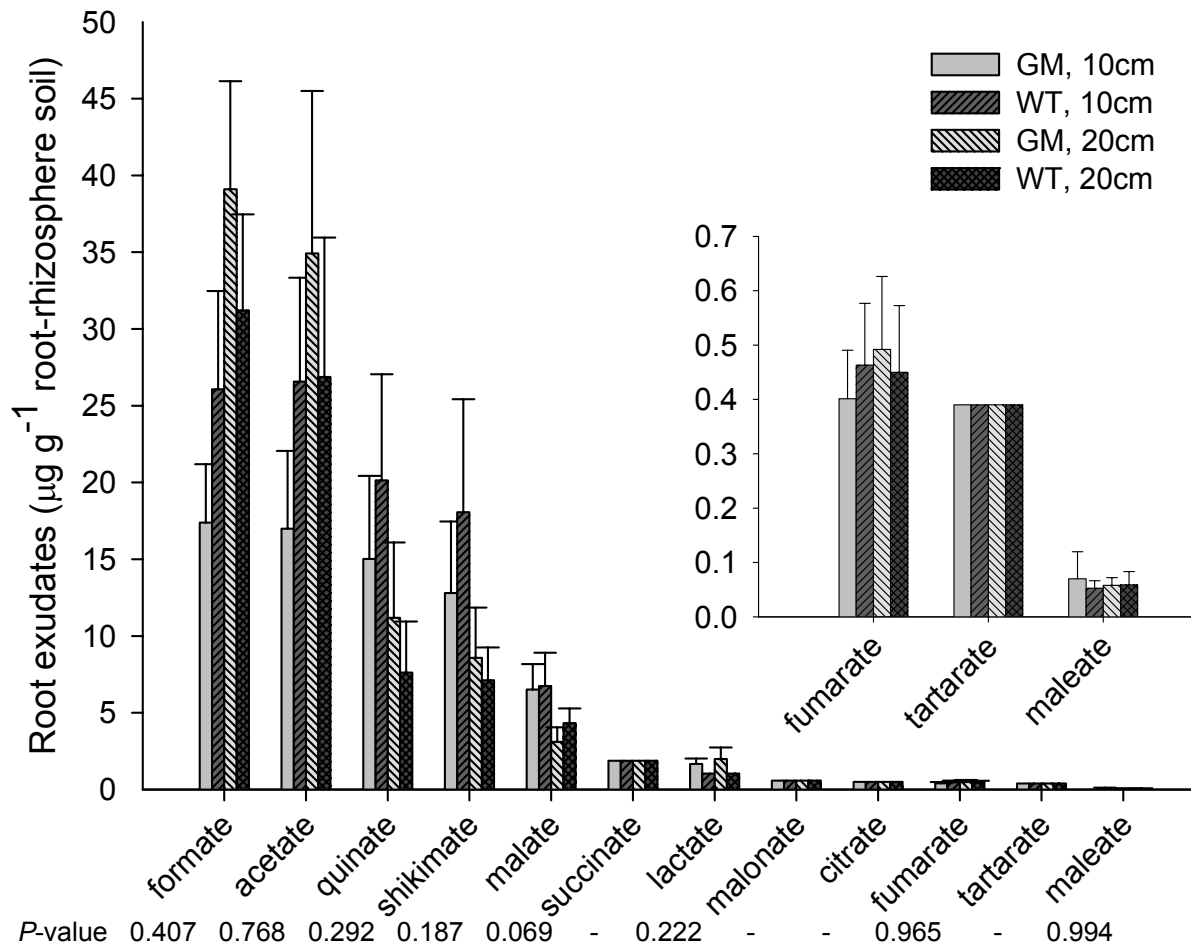


Figure 4.9 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the first sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph. “-” indicates no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.

Shikimate, citrate and fumarate were also detected in all root-rhizosphere soil exudates, but at lower concentrations (Table 4.9c, Figure 4.11).

Shikimate and fumarate were most consistently detected in water-soluble exudate solutions from root-rhizosphere soils over all three samplings (Table 4.9). Other anions varied greatly in frequency. For example, formate was detected in 28 out of 32 samples at the first sampling, compared with only three out of 32 samples and none of the 24 exudates at second and third sampling dates, respectively. Lactate was detected in 5 out of 32 exudate solutions at the first sampling, compared with 30 out of 32 and 24 out of 24 exudates at second and third sampling periods, respectively. Acetate was detected in over 70% of exudate solutions at the first and second sampling periods, but was not detected at all at the third sampling date (Table 4.9).

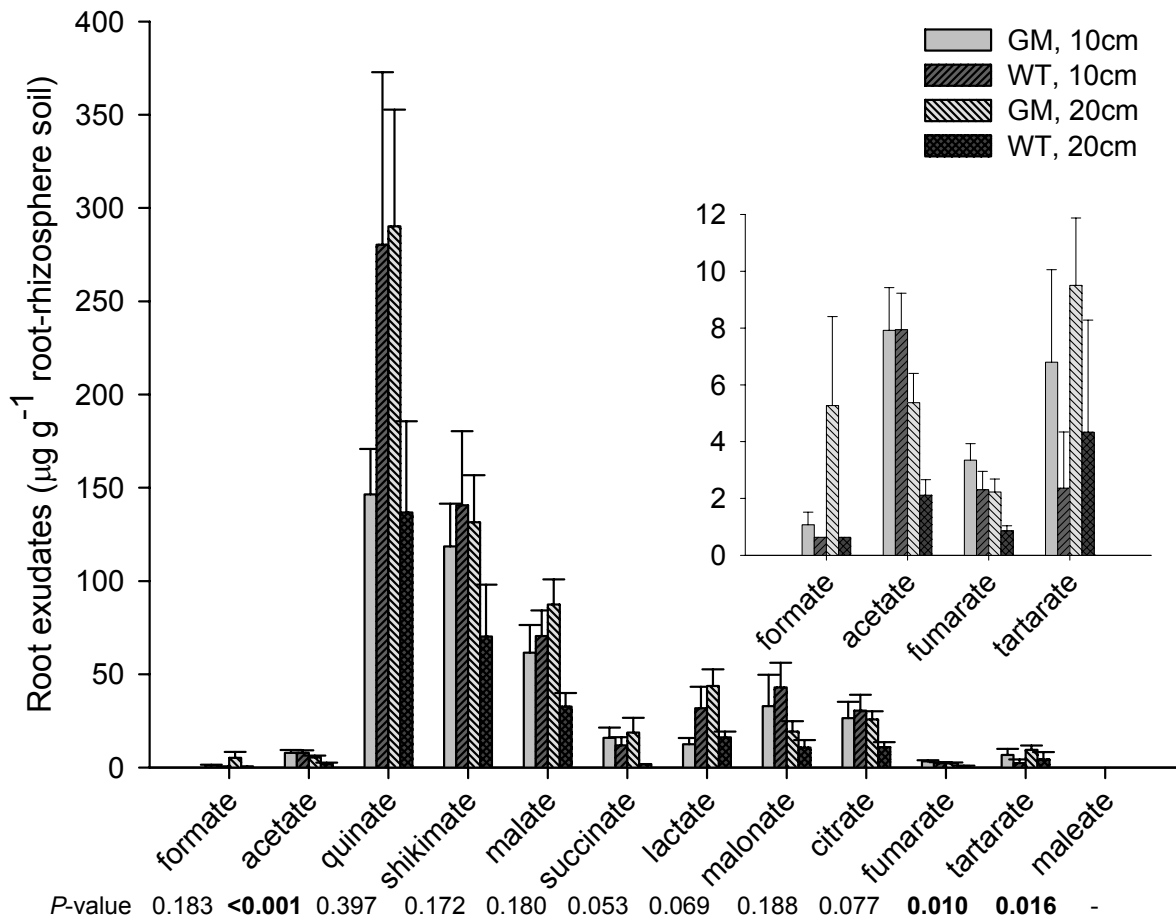


Figure 4.10 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the second sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown within the graph. The P -values with significant difference across treatments ($P<0.05$) are shown as an insert in bold. “-” indicates no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.

None of the individual anions significantly differed in concentrations present in water-soluble exudate solutions collected from GM and WT trees at either depth at the first sampling (Figure 4.9). While the concentrations of acetate collected from the depth of 20 cm of WT pine trees at the second sampling was significantly lower than those collected from GM tree line at the same depth (Figure 4.10). Acetate was detectable in only three out of eight root-rhizosphere exudate samples from the WT trees at the depth of 20 cm, while this was detected in all eight samples from the GM trees at the second sampling (Table 4.9b). Similarly, tartarate was only detected in one of eight root-rhizosphere exudate solutions of WT tree line at the depth of 20 cm, and this resulted in the significant lower average concentration than that collected at the same depth from GM trees (Table 4.9b, Figure 4.10). Fumarate was detected in all the extracts from the root-rhizosphere soil at the second sampling, and ANOVA analysis showed that the amount of this anion in

exudate solutions collected from WT tree line at 20 cm was significantly lower than that collected from the GM tree line at that depth (Table 4.9b, Figure 4.10). At the third sampling, no significant difference in individual anion concentrations in water-soluble root-rhizosphere exudate solutions from GM and WT trees were detected (Figure 4.11).

A few OAs were detected in water-soluble exudate solutions extracted from non-rhizosphere soils at all three samplings, although with lower concentrations compared to those from root-rhizosphere soils. Only two OAs (formate and acetate) were detected in one of 32 exudates samples from non-rhizosphere soils at the first sampling. Acetate, shikimate, malate, succinate and lactate were detected in less than 40% of non-rhizosphere soil extracts at the second sampling (Figure 4.12, Table 4.10). With the exception of fumarate ($0.54 \mu\text{g g}^{-1}$ soil) which was detected in only one out of 24 extracts, no anions were detected in the non-rhizosphere exudate solutions at the third sampling. Similarly, no OAs were detected in water-soluble exudate solutions extracted from bulk soils at the third sampling.

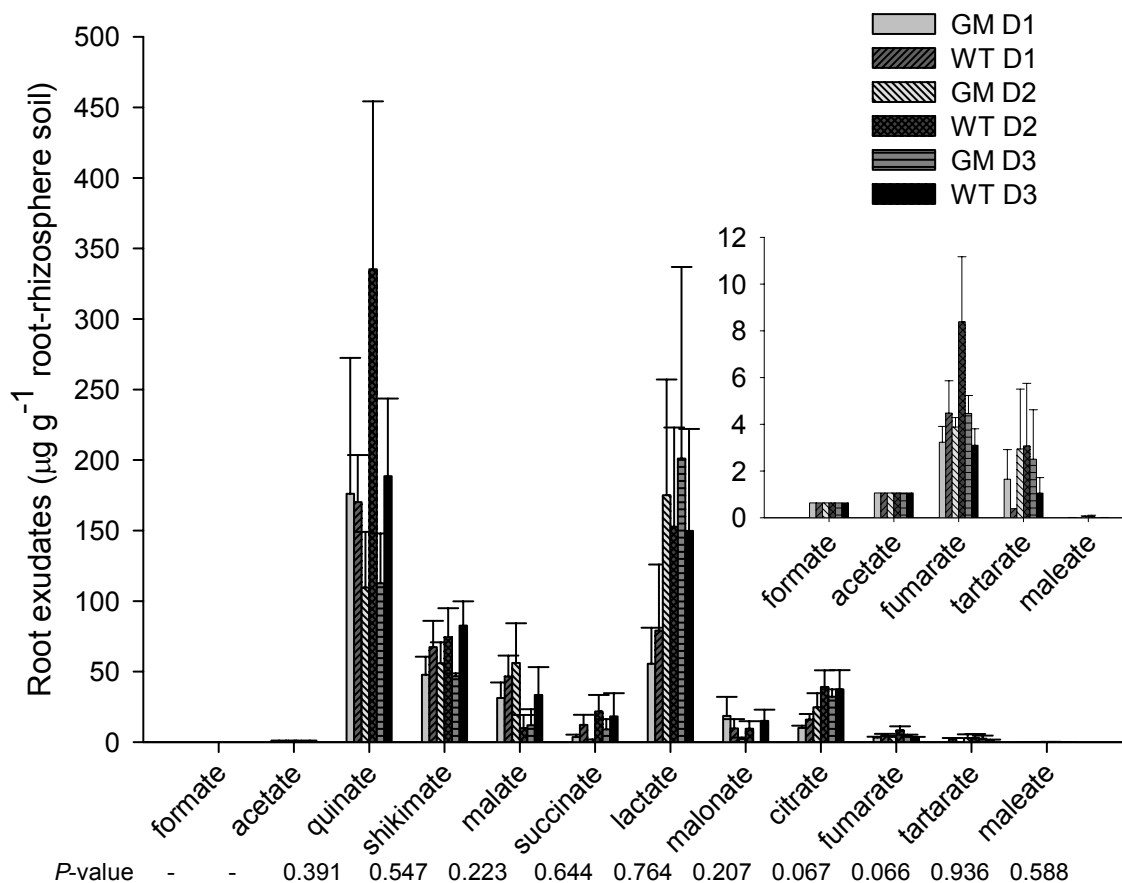


Figure 4.11 The concentrations of OAs detected in water-soluble exudate solutions extracted from rhizosphere-root soils which were collected at the third sampling, and respective probability values analysed by ANOVA across the treatments. Error bars show 1 standard error ($n=4$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the graph. D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm. “-” indicates no P-value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.

Table 4.10 The number of water-soluble exudate solutions from non-rhizosphere soils collected at the second sampling where OAs were detected by HPLC (i.e. greater than limits of detection; Chapter 3, Table3.1) in each treatment ($n=8$) and in total ($n=32$).

2 nd sampling	GM 10 cm	WT 10 cm	GM 20 cm	WT 20 cm	Total
formate	0	0	0	0	0
acetate	3	3	3	3	12
quininate	0	0	0	0	0
shikimate	1	1	1	0	3
malate	3	1	0	2	6
succinate	1	0	3	0	4
lactate	1	0	1	0	2
malonate	0	0	0	0	0
citrate	0	0	0	0	0
fumarate	0	0	0	0	0
tartrate	0	0	0	0	0
maleate	0	0	0	0	0

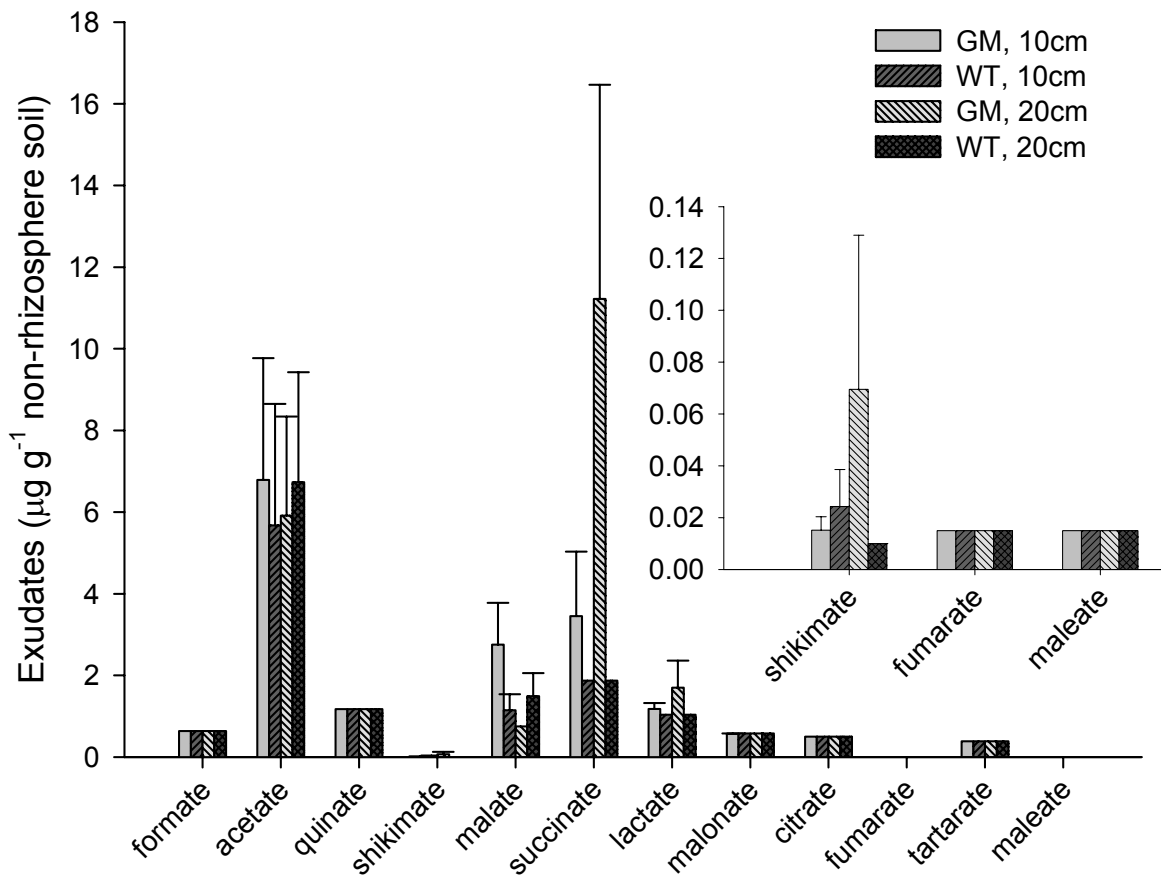


Figure 4.12 The concentrations of OAs detected in water-soluble exudate solutions extracted from non-rhizosphere soil collected at the second sampling. The levels of those OAs under detection limits were specified as half the limits of detection of the HPLC method (Chapter 3, Table 3.1). Error bars show one standard error ($n=8$). Due to the low level of some anions, they were plotted in a small scale graph with the same unit and shown as an insert within the figure.

Significant differences in the amounts of individual anions present in exudate solutions between rhizosphere and non-rhizosphere soils were found in majority of the anions at all three sampling times (Appendix 4.6). However, the concentrations of formate ($P=0.082$), acetate ($P=0.102$) (at the second sampling) and maleate ($P=0.233$) (at the third sampling) did not differ significant in water-soluble exudate solutions between root-rhizosphere and non-rhizosphere soils.

Total organic anions

The total C of OAs in the water-soluble exudate solutions of root-rhizosphere at the first sampling ranged from 27 to 38 $\mu\text{g C g}^{-1}$ of root-soil. This was significantly lower ($P<0.001$) in comparison with those at the second and third samplings which were in the range of 123 to 279 $\mu\text{g C g}^{-1}$ of root-soil (Table 4.11). The highest total C, amongst root-rhizosphere exudate solutions at the second sampling, was detected in samples of GM tree line at 20 cm (271 $\mu\text{g C g}^{-1}$ of root-rhizosphere soil). At the third sampling, the highest total C was present in the exudate solution of WT pine root-rhizosphere soils at the depth of D2 (279 $\mu\text{g C g}^{-1}$ of root-rhizosphere soil) (Table 4.11). There was no significant difference ($P<0.05$) in total C of OAs between GM and WT tree lines at either depth at any sampling period (Table 4.11). The total C of OAs in the water-soluble exudate solutions from non-rhizosphere soil samples were not calculated as few OAs were detected.

4.3.4.2 Organic anion profiles

The structure of the OA profile based on the anions with more than 40% detection frequency in the root-rhizosphere soil extracts were analysed by linear discriminant analysis. All the selected OAs at each sampling period are shown in Table 4.12. The structure of the OA profiles in the exudate samples of root-rhizosphere soil differed significantly from those of non-rhizosphere soil at all three samplings ($P=0.000$) and those of bulk soils at the third sampling ($P=0.000$). Amongst root-rhizosphere samples, there was no significant difference ($P<0.05$) in the structure of OA profiles in water-soluble root exudates between GM and WT tree lines at any depth at any sampling period (Table 4.13).

The diversity of OA profiles in exudate solutions assessed using Shannon diversity index are showed in Table 4.14. There was no difference detected in the diversity indices of root-rhizosphere exudate samples between the two tree lines at the corresponding depths at all three sampling dates (Table 4.14). The Shannon diversity indices of OA profiles in root-rhizosphere exudate solutions at the D1 depth were significantly different from those

at the depths of D2 and D3 at the third sampling in GM trees but not in WT trees (Table 4.14).

The Shannon diversity indices of OAs in the water-soluble exudate solutions from non-rhizosphere soil samples were not calculated because of infrequent detection of OAs.

Table 4.11 Total C of OAs in water-soluble root-rhizosphere exudate solutions ($\mu\text{g C g}^{-1}$ root-soil) collected at all three samplings, and the probability values across the treatments in each sampling period analysed by ANOVA. The amounts are presented as the mean \pm 1 standard error for samples at different treatments ($n=8$ for the first and second samplings, $n=4$ for the third sampling).

Sampling period	GM 10 cm (D1*)	WT 10 cm (D1)	GM 20 cm (D2)	WT 20 cm (D2)	GM D3	WT D3	P-value
1 st	27 \pm 7	38 \pm 10	35 \pm 9	28 \pm 7	NA	NA	0.6670
2 nd	183 \pm 31	265 \pm 69	271 \pm 50	123 \pm 39	NA	NA	0.175
3 rd	147 \pm 49	171 \pm 28	178 \pm 47	279 \pm 45	175 \pm 62	222 \pm 64	0.608

NA = not applicable; *D1 = 0-14 cm; D2 = 14-28 cm; D3 = 28-45 cm

Table 4.12 Organic anions selected for the analysis of structure of OA profiles in water-soluble root exudate solutions at three samplings.

Sampling	Selected OAs
1 st sampling	formate, acetate, quinate, shikimate, malate, fumarate and maleate.
2 nd sampling	acetate, quinate, shikimate, malate, succinate, lactate, malonate, citrate, fumarate and tartarate.
3 rd sampling	quininate, shikimate, malate, lactate, malonate, citrate and fumarate.

Table 4.13 Hotelling T² probabilities of the level of difference between the structure of OA profiles in the water-soluble root-rhizosphere exudate samples from GM and WT pine trees at all three sampling periods ($n=8$ for the first and second sampling, $n=4$ for the third sampling), with each sample under the null hypothesis that all the samples have the same mean.

Sampling	1 st sampling		2 nd sampling		3 rd sampling		
	10 cm	20 cm	10 cm	20 cm	D1*	D2	D3
P-value	0.7965	0.3989	0.0917	0.2273	0.8598	0.6634	0.2199

*D1 = 0-14 cm; D2 = 14-28 cm; D3 = 28-45 cm

Table 4.14 Shannon diversity indices of OA profiles in the water-soluble root-rhizosphere exudate samples at all three samplings, and the probability values across the treatments analysed by ANOVA. Diversity indices are presented as the mean \pm 1 standard error ($n=8$ for the first and second samplings, $n=4$ for the third sampling). The probability values with significant difference ($P<0.05$) across the treatments are shown in bold. Different letters indicate significant difference ($P<0.05$) between the treatment means.

Sampling period	GM 10 cm/ D1*	WT 10 cm/ D1	GM 20 cm/ D2	WT 20 cm/ D2	GM D3	WT D3	P-value
1 st	2.11 \pm 0.07	2.16 \pm 0.07	2.10 \pm 0.09	2.07 \pm 0.08	NA	NA	0.755
2 nd	1.99 \pm 0.10	1.90 \pm 0.17	2.16 \pm 0.04	2.07 \pm 0.04	NA	NA	0.364
3 rd	1.92 \pm 0.05 ^b	1.93 \pm 0.03 ^b	1.77 \pm 0.03 ^a	1.85 \pm 0.02 ^{ab}	1.81 \pm 0.02 ^a	1.86 \pm 0.05 ^{ab}	0.016

NA: not applicable; *D1 = 0-14 cm; D2 = 14-28 cm; D3 = 28-45 cm

4.4 Discussion

Although studies have been carried out to investigate the potential impact of GM crops on non-target soil microbial communities (Heuer et al. 2002; Lottmann et al. 1999; O'Callaghan et al. 2008), there have been few studies on the effects of GM trees on soil microbial communities in the rhizosphere. In this study, changes in root exudates were also investigated as this is generally considered to be a major driver for the microbial community structure. Moreover, this is the first study to examine the potential influence of GM trees on the communities of metabolically active soil microorganisms (by virtue of rRNA-DGGE) together with the potential shifts in OAs in exudates released by GM plants grown in soil.

4.4.1 Experimental Design

It is well documented that soil microbial communities fluctuate naturally in response to seasons, plant growth or changes in physicochemical parameters (Di Cello et al. 1997; Dunfield and Germida 2004; Grayston et al. 2001; Smalla et al. 2001). Therefore, caution is needed when drawing conclusions about root-associated microbial community changes based on results from a single sampling time (Grayston et al. 2001). Smalla et al. (2004) also suggested that baseline data is needed to assess potential changes in the context of natural fluctuations. However, to date most published studies investigating the influence of GM trees on soil microbial communities were based on a single harvest point (Andreote et al. 2009; Kaldorf et al. 2002; Lamarche and Hamelin 2007; LeBlanc et al. 2007). In addition, Dunfield and Germida (2003) questioned the biologically significant influence of GM plants on soil microbial communities if their impact was only detected at one sampling time. Accordingly, they emphasized the importance of conducting samplings at several time points for risk assessment of GM plants. The current study with GM radiata pine has confirmed the importance of repeated sampling in assessing impacts of GM plants on associated microbial communities.

Three separate samplings were taken from soil and roots of GM and WT pine trees grown in large-scale rhizotron units under controlled conditions for 10 months in the current experiment. Initially, several *in situ* samplings were planned after the creation of horizontal access portals by the first destructive sampling. Unfortunately, because of the slow growth of pine root mass, this could not be achieved in the limited time available. Accordingly, the *in situ* sampling was only conducted once at the second sampling. A final destructive sampling (the third sampling) was also carried out on completion of the experiment. The first sampling was conducted when pine trees were initially being settled in rhizotrons and the following two samplings were conducted when trees were more established.

4.4.2 Soil Microbial Communities

4.4.2.1 Microbial communities associated with GM and control trees

From visual observation, tree morphology (shoot height, root mass, mycorrhizal colonization) and growth rates did not differ between GM and WT trees during the experiment. However, some significant differences in rhizosphere microbial communities between GM and control pine trees were detected using rRNA-DGGE technique, although there were no consistent trends over the duration of the experiment. Similar results have also been observed in other studies using different plant species with various transgenic traits. For example, Sessitsch et al. (2003) detected a transient, but significant effect on the diversity and community structure of culturable *Bacillus* spp. associated with GM potato expressing antibacterial lytic peptide cecropin in comparison with an unmodified parental variety. Heuer et al. (2002) detected differences in DGGE profiles in rhizosphere samples of a senescent GM line of T4 lysozyme-modified potato (DL4) in comparison with the unmodified control line but not at other sampling times. The microbial community associated with the rhizosphere of the herbicide-tolerant GM canola (Quest) was significantly different from the control canola; however, the differences were not consistent throughout six samplings carried out during the entire field season (Dunfield and Germida 2003). Some researchers considered these transient differences as natural fluctuations among samples rather than GM plant effects (Blackwood and Buyer 2004; Dunfield and Germida 2004; Heuer et al. 2002).

Transient effects of GM plants on soil microbial communities has been attributed by some researchers to the unintentional alteration of plant characteristics due to the interruption of particular genes (Donegan et al. 1995; Sessitsch et al. 2003). However, more recently, these effects were attributed to a genotype and/or environment interaction (e.g. seasons, plant growth stages, soil moisture) leading to different exudates being released in the soil (Dunfield and Germida 2004; Griffiths et al. 2000a). Dunfield and Germida (2003) reported that the fatty acid profiles of the rhizosphere communities and communities from unplanted fallow soil at one field site in Saskatchewan, Canada, but not at another site, changed significantly across six samplings over the field season. They suggested that environmental factors, such as soil moisture content, may play a role in changing the fatty acid profile of the soil microbial communities in this location rather than GM plants. In the current study, a controlled environment (temperature, light density and air humidity) was maintained consistently throughout the experiment. However, changes in soil physicochemical parameters, such as soil moisture and pH (Appendix 4.7), as well as tree growth could be important in triggering or amplifying the difference between GM and control tree lines through exudate input in rhizosphere soil.

Several studies found that the effect on rhizosphere microbial communities by environmental factors, such as season, plant growth stage and field site, was much greater than the minor difference caused by the presence of GM plant in comparison with WT (Gomes et al. 2001; Gyamfi et al. 2002; Heuer et al. 2002). Although in the current experiment samples collected at different times were not compared directly on the same DGGE gels, comparison between gels with the alignment of internal markers indicated that the differences in rhizosphere communities between the two tree lines were minor in comparison to the difference between the first and the following two samplings (data not shown). J. Lottmann (pers. comm.) studied the same GM radiata pines grown in the field over two years in New Zealand and found the seasonal shifts in bacterial and fungal communities were more significant than the minor differences detected between GM and WT tree lines.

With the exception of fungal communities, the rhizosphere effect was apparent in all the communities at the three sampling times, as the rhizosphere communities differed significantly in comparison with those in non-rhizosphere or bulk soils. The clear rhizosphere effect in bacterial communities confirms the influence of pine roots. However, in some cases, soil also plays an important role in determining the rhizosphere community structure (da Silva et al. 2003; Duineveld et al. 2001; Marschner et al. 2004). Few studies investigating potential impacts of GM plants have included the non-rhizosphere soils. In the current experiment, communities of non-rhizosphere soils collected from adjacent sites of rhizosphere soils were also investigated at all three samplings. Similar to rhizosphere microbial communities, there were some significant but inconsistent differences observed in the DGGE profiles between GM and WT tree lines, with the exception of β -proteobacterial community at 20 cm or corresponding D2 depth which showed consistent difference between the two tree lines at the three samplings. These differences in microbial communities of non-rhizosphere soils may be caused by the heterogeneous physicochemical factors in soil environment. As discussed in Chapter 3, Section 3.4.2, numerous studies have shown that the soil microbial communities could be influenced by soil environmental parameters, such as pH, moisture, aggregate size, gas conditions, nutrient availability, organic matter content, etc. Although every effort (using well-prepared soil, rotating the rhizotrons and randomizing the rhizotrons within the growth room) was made to create a uniform environment in the rhizotrons, differences in soil conditions (such as soil aggregation, moisture and pH) could have developed during the 10 months duration of this experiment with the growth of plant roots (Hinsinger et al. 2005; Watt et al. 2006b). Activities of soil organisms (Glare et al. 2004) such as springtails which were frequently observed in the rhizotrons may also have differed across replicates and tree lines. Furthermore, the soil pH and moisture in non-rhizosphere soils between the first

and third samplings did vary (Appendix 4.7). The soil moisture showed variability within replicates at the third sampling and this variability was larger when compared to those at the first sampling. This implies that with time, the physicochemical parameters in the soil microenvironment may have become more heterogeneous with the activities of roots and soil organisms, even though there was a uniform soil environment to start with. Accordingly, this may explain some chance variation that was observed in non-rhizosphere such as with the β -proteobacterial communities at 20 cm depth or corresponding D2 samples.

In most cases, the detection of shifts in microbial communities by GM pine trees in rhizosphere soils and in the adjacent non-rhizosphere soils were not consistent. This inconsistency ruled out the possibility of indirect influence of rhizosphere effect or the extension of rhizosphere effect to the non-rhizosphere soil by GM pine. This further indicates that the differences in non-rhizosphere soil may be due to the spatial variability in soil environment. Microbial communities present in samples described as “non-rhizosphere” may have also been influenced by roots that were not visible at time of sampling. Due to the sampling technique used, especially for the second sampling, non-rhizosphere soil was collected from the surface wall of access portals which may have been influenced by roots that were not visible. On occasion, previously concealed roots appeared after collection of non-rhizosphere soil samples from the surface of portals. Moreover, the non-rhizosphere soil may have been influenced by pre-existing roots sampled previously. Thus the non-rhizosphere may contain the pre-existing rhizosphere community. The differences detected in some of the communities between bulk soil and non-rhizosphere soil at corresponding depths, especially at the D1 depth, indicate the possibility of the presence and influence of roots on non-rhizosphere soil. However, the possibility that these differences were caused by different physicochemical parameters between non-rhizosphere and bulk soil cannot be excluded. The bulk soil bags were located in the corners to ensure that they were not in the way of access portals. However, soil pH and moisture did not show any significant difference between non-rhizosphere and bulk soils. In addition, soil organisms (i.e. springtails) could be another source of influence on the non-rhizosphere soil but not bulk soil as they could not enter the bags.

Interestingly, the general bacterial 16S communities, but not the three selected taxon-specific communities, showed significant difference between GM and control tree lines in rhizosphere soils taken at the first sampling. This may indicate that the affected bacterial groups did not belong to the taxon-specific groups tested here. In the field study conducted by J. Lottmann (pers. comm.) using the same GM and control trees, it was found that α - and β -Proteobacteria had the greatest representation followed by Bacilli in a clone library study using the rhizosphere soil from one summer sampling period. However,

they did not detect any specific bacterial groups or operational taxonomic units associated with either GM or control tree lines. Apart from α -, β -Proteobacteria and Pseudomonads, other groups, such as Acidobacteria and Actinobacteria, are commonly present in the rhizosphere soil of pine trees. Chow et al. (2002) reported that α -, β -Proteobacteria (24 and 19%, respectively) and Acidobacteria (19%) groups dominated the rhizosphere of lodgepole pine grown under forest conditions in Canada. Fillion et al. (2004) also identified Proteobacteria, Acidobacteria and Actinobacteria as main bacterial groups in the rhizosphere of *Picea mariana* seedlings.

The absence of significant differences between fungal communities present in rhizosphere and non-rhizosphere soils may have resulted from extension of fungal mycelia/hyphae from the rhizosphere to surrounding non-rhizosphere soil. The dominant active fungal community in this system is likely to be ECM, which due to their extraradical mycelium, will not be restricted to the rhizosphere. Widespread fungal mycelia and even the formation of mycelia nets were observed in several access portals (Figure 4.6f). In addition, large variability in DGGE band patterns between replicates within both rhizosphere and non-rhizosphere/bulk was observed (data not shown). This variability among replicates may have resulted from the more heterogeneous distribution of fungi than bacteria and low recovery of fungal rRNA during RNA extraction or following PCR steps. Costa et al. (2006) also observed the high variability in DGGE profiles among replicates of fungal fingerprints in rhizosphere and bulk soils of two crops (strawberry (*Fragaria ananassa*) and oilseed rape (*Brassica napus*)) in the first season and consequently could not identify the ribotypes with increased abundance in the rhizosphere community in some fields. However, the influence of crops on rhizosphere fungal communities was much clearer in the second season in their study. Several other studies also reported a high variability among replicates of fungal fingerprints (Girvan et al. 2004; Klamer et al. 2002; Oros-Sichler et al. 2006).

Apart from the general fungal 18S community, Basidiomycete communities in samples from the third sampling were also analysed (Appendix 4.8). Basidiomycetes is a typical phylum of the ECM which could be influenced by the GM pine trees, although ectomycorrhizal colonization of both GM and WT pine trees was observed during the experiment. The analysis of DGGE communities of ITS region of Basidiomycetes which was amplified by primer set ITS1F (Gardes and Bruns 1993) and ITS4B-GC (Landeweert et al. 2005) from DNA samples showed similar profiles across all the samples. This finding is in agreement with other studies. Kaldorf et al. (2002) also did not detect any differences in the mycorrhizal diversity between GM aspen (*Populus tremula* x *P. tremuloides*) lines and control parental line in a field trial assessed using PCR-RFLP. However, they observed poor establishment of one of the four common ectomycorrhizal morphotypes on

roots from one transgenic aspen line compared to the control line. Pasonen et al. (2005) reported that all the GM silver birch lines with sugar beet chitinase IV expression were able to form normal ectomycorrhizal associations. Seppänen et al. (2007) reported that GM silver birch (genetically modified in lignin biosynthesis) formed normal ectomycorrhizal associations with the fungus *Paxillus involutus*, although the GM silver birch showed significant differences in root biomass and morphology in comparison to the control line.

The inconsistent shifts in communities associated with GM and WT tree lines may indicate that the tested GM pine trees did not have any biologically significant impact on soil microbial communities. Recently, it has been proposed that the assessment of the influence of GM plants should focus on functional microorganisms in soil to further assess the potential influence on soil processes (Dunfield and Germida 2004; Heuer et al. 2002; Smalla et al. 2004). Functional bacterial groups involved in processes, such as nitrogen-fixation, organic and inorganic P-solubilisation and potassium-solubilisation, in the rhizosphere soil of GM plants in comparison to the control have been investigated (Hu et al. 2008; Lamarche and Hamelin 2007). Costa et al. (2007) developed a PCR-DGGE system to target the *gacA* gene (global antibiotic and cyanide control gene) fragments in *Pseudomonas* for the simultaneous analyses of *Pseudomonas* community structure and function in soil. Soils collected at the first sampling in the current study were analysed for the presence and diversity of the *gacA* gene which was amplified using primers and conditions as described by Costa et al. (2007) (Appendix 4.9). However, most of the samples only showed one dominant band with two faint bands, and minor variation between samples. Therefore, due to this low diversity of the *gacA* gene in samples in this study, no further analysis was carried out using this functional gene.

4.4.2.2 Variations in DGGE profiles between replicates

Variations in DGGE banding patterns were observed among replicates in the rhizosphere in all tested communities, although the bacterial communities showed relatively smaller variability than fungal communities. The DGGE profiles in non-rhizosphere or bulk soils were more similar among replicates compared to rhizosphere soils, with the exception of fungal communities. Although a reasonable number of replicates were collected ($n=8$ for the first and second samplings and $n=4$ for the third sampling), the variation among replicates may reduce the power to detect the potential statistical difference in the communities between GM and WT tree lines. Variations between replicates may be caused by natural heterogeneity of the soil and rhizosphere soil environment (Ettema and Wardle 2002; Hinsinger et al. 2005). In addition, different root locations could be inhabited by different microbial communities (Duineveld et al. 2001; Marschner et al. 2001; Watt et al. 2006a). Therefore, the variability in rhizosphere communities could also be due to

differences in root types sampled. In the second sampling the choice of root samples was limited by the presence of the root types within access portals. Efforts were made to sample three common types of roots, i.e. root tips, mature root part and root colonized by ECM, in each access portal. However, in some cases, only one type of root was available.

Furthermore, the RNA extracted from the limited amounts of rhizosphere soil obtained from several root-rhizosphere soil samples may not well represent the *in situ* microbial communities in the rhizosphere soils. On average, 0.35 g of rhizosphere soil (ranged from 0.06 to 0.5 g) was used for microbial community analysis. Although the impact of soil sample size on microbial communities was not examined in this project, rhizosphere samples with amounts around 0.1 to 0.5 g showed good reproducibility among the replicates, with most of the replicates showing near identical band patterns on rRNA-DGGE gels using rhizosphere soils collected from radiata pine grown in a pot (Appendix 2.1, Appendix 2.5, Figure A2.5-1 and Figure A2.5-2). However, when using extremely small amounts of rhizosphere soils (e.g. 0.03 g), differences in band profiles in rRNA-DGGE were observed in comparison with a 0.16 g replicate of the same rhizosphere soil (Appendix 2.5, Figure A2.5-2). Nicol et al. (2003) investigated the reproducibility of archaeal DGGE profiles using different soil sample size (10, 1 and 0.1 g soil, respectively). They found that the variation among triplicates of 1 g and 0.1 g soil samples was greater than the variation among the 10 g samples.

RNA instead of DNA used in this study may also account for some of the variability among the replicates. As discussed in Chapter 2, Section 2.1.4, RNA is directly related to the metabolic activity of microorganisms, and therefore, responds rapidly to the environment whereas DNA is relatively stable. Any alteration in microscale soil environment or microbial activity could be revealed in the RNA molecule and consequently, may introduce more variability among replicates.

4.4.3 Organic Anions in Exudates

Apart from the significant difference between OAs in rhizosphere and non-rhizosphere soil in exudates both collected by AEM *in situ* or extracted by sterile DI water, four different statistical analyses showed that only a few OAs in the exudates of rhizosphere soils differed significantly between GM and WT tree lines at some sampling periods. Those differences were mainly due to the infrequent detection of OAs in exudate samples (such as malonate in exudates collected by AEM *in situ*). However, some potential differences in OAs released by GM and WT pine trees may have been masked by the variability among replicates in all the exudate samples collected by either method. Although the exudate concentrations collected by AEM *in situ* were normalized by the actual root surface used

for collection, the variability across replicates were still too great to detect any tree line effect. Similarly, Phillips et al. (2008) were unable to detect significant effects of sampling months or seasons on exudation by loblolly pine (*Pinus taeda*) in the field because of variability among replicates, although efforts were made to reduce some of the variation in exudation by normalizing exudate rates to fine root surface area which correlated relatively well with the exudation of C. Interestingly, the significant difference detected in the diversity of OA profiles between depths of D1 and D2/D3 at the third sampling in water-soluble exudate solutions of GM tree lines, but not WT line. This may further indicate some significant differences in OAs between GM and WT lines were masked by large variability. Yan et al. (2007) reported that the total organic acids (targeted to measure oxalic, citric and acetic acids) released by Cry1A modified Bt-cotton (*Gossypium hirsutum*) were about 35% more than that of WT cotton in full nutrient solutions, whereas in the absence of P and K, the differences were reduced to 5 to 10%. They also found different components of OAs were detected between GM and WT cottons in the nutrient solution without nitrogen. Direct comparisons between the study by Yan et al. and the current study is not appropriate because of different experiment designs (transgenic traits, plants, culturing environments, collection techniques) and analyses (different targeted OAs, data analysis).

The composition and relative concentrations of OAs in water-soluble exudate solutions varied across the three sampling periods. The concentrations of individual organic anions and the total C of detected OAs in exudate solutions at the first sampling was lower than those at the second and third sampling periods. This difference may be due to the fact that sampling was conducted when pine trees were at two different growth stages (initial establishment stage vs. relatively mature stage) with different amounts of leaf area and root development. With foliage development, photosynthesed carbon would be expected to increase and result in greater transfer of C into the rhizosphere through exudation. Smith (1972) studied the influence of defoliation on exudates using sugar maple (*acer saccharum*) and found that defoliation, as a reducer of photosynthetic capacity, can affect the amounts of compounds in the root exudates. It is also well documented that plant growth stages can significantly affect the release of exudates (Grayston et al. 1996; Hale et al. 1978; Leyval and Berthelin 1993). Sandnes et al. (2005) found that some OAs, such as malic and butyric acids as well as dissolved organic C, increased with spruce and birch seedlings grown in microcosms (sampled at 8, 9, 10 and 11 weeks) and rhizoboxes (sampled at 5, 9, 14 and 18 weeks) with time. Gransee and Wittenmayer (2000) also showed that with the development of maize plants the relative amount of carboxylic acids increased, whereas sugars decreased. Smith (1976) found that young maples exude

greater amounts and more diverse carbohydrates compared to mature trees, while mature trees exude greater and more diverse amino acids.

Variability in the quantity and detection frequency of OAs in exudate samples is consistent with the previous study (Chapter 3, Table 3.2) and numerous studies in the literature (Grierson 1992; Lipton et al. 1987; Phillips et al. 2008; Sandnes et al. 2005; Scheffe et al. 2008; Smith 1976). Marschner et al. (2002) reported that OAs exudation from cluster roots of white lupin collected *in situ* in rhizoboxes varied, ranging from 0 to 189 mg l⁻¹. In addition, Dessureault-Romppe et al. (2006) also found wide variability in the amount of OAs (citrate, oxalate, malate and acetate) released by cluster roots and normal roots of white lupin in a microsuction cup/rhizobox system. For instance, they detected a very wide concentration range for citrate (3.8 to 2056 µM) during the active periods of cluster roots.

As mentioned in Chapter 1, Section 1.1.2, various environmental factors, including soil pH, moisture, temperature, nutrient availability, plant growth stage and the presence of microorganisms, have been documented that can affect the quantity and/or composition of root exudates. In the current experiment, soil pH values in the non-rhizosphere soil samples from different depths were different and changed between sampling times (Appendix 4.7). Meharg and Killham (1990) reported that the amount of ¹⁴C (expressed as a percentage of the total ¹⁴C fixed by plant) released from perennial ryegrass increased from 12.3 to 30.6% with increasing soil pH values from 4.3 to 6.0. In the current study the soil moisture in the non-rhizosphere was relatively consistent (18.4 to 20.4%) across all the samples at the first sampling and moisture level reduced by half with a wider range (5.6 to 12.7%) at the third sampling (Appendix 4.7). Reid (1974) has shown that the exudation of sugars, amino acids and organic acids from ponderosa pine (*Pinus ponderosa*) seedlings in hydroponic systems changed under different levels of water stress (decreasing water potential of root bathing media) from 0 to -11.9 bar and levels of particular compounds released may increase or decrease depending on the level of stress. Microbial communities around roots could also influence root exudates (Cromack et al. 1979; Griffiths et al. 1994; Meharg and Killham 1991; Meharg and Killham 1995; Prikryl and Vancure 1980) and thus could be another additional source of the variation. For example, Leyval and Berthelin (1993) showed that mycorrhizal colonized beech (*Fagus sylvatica*) roots exuded different OAs from non-mycorrhizal colonized beeches. However, Krupa and Fries (1971) found that Scots pine (*Pinus sylvestris*) roots exuded the same types of volatile compounds in the presence or absence of mycorrhizal fungi, although the mycorrhizal roots exuded a greater amount. As different microbial species vary in their impact on OAs exuded by plants (Ahonen-Jonnarth et al. 2000; van Schöll et al. 2006), the colonization by different microbial communities with each root sample, as

shown on DGGE gels, may also cause the alteration in OAs released by plants. Based on research results, Jones et al. (2003) hypothesised that even slight changes in the chemistry of the soil or physiology of the plant may induce rapid shifts in the composition and quantity of exudates.

As different types of root exudates are released at different root zones (Bringhurst et al. 2001; Jaeger et al. 1999; Mathesius et al. 2000)), the sampled roots, especially at the second sampling, could also partly account for the observed variability. As discussed previously, the sampled roots were restricted to what was available in access portals for exudate collection, although efforts were made to collect three common types of roots in each access portal. However, this type of variability could be reduced to minimum if sufficient root mass was present in access portals with a longer growth period. With the advantage of the endoscope used in this *in situ* sampling approach, exudates could be collected from exactly the desired root zones.

Variability of OAs, both composition and amounts, among replicates was found with both annual crops (Dessureault-Rompere et al. 2006; 2007; Lipton et al. 1987; Schefe et al. 2008; Smith 1976) and trees (Phillips et al. 2008; Sandnes et al. 2005), indicating that this feature is not plant species related. In addition, variability among replicates was observed in exudate samples collected by both methods used in the current study as well as other sampling techniques (Lipton et al. 1987; Phillips et al. 2008; Sandnes et al. 2005), indicating that the variability is not related specifically to sampling techniques. However, in general, *in situ* sampling tends to show greater variability in comparison with “whole root system” sampling. This further indicates the majority of the variability is caused by the microscale heterogeneity of soil environment and/or plant roots. The removal of trees from soil, despite the difficulties, would inevitably damage root systems and rupture fine roots and root hairs. Therefore, *in situ* sampling is a preferred option for precise tree root exudate sampling. The wide natural variability in exudate production presents a significant challenge in this area of research (Phillips et al. 2008), but further experimentation will assist in determining how many samples and how frequently the exudates need to be collected and when they should be collected.

As with the previous experiment (Chapter 3, Section 3.3.3.2) and other studies (Grierson 1992; Koo et al. 2006; Schefe et al. 2008), OAs were also detected in some of the non-rhizosphere samples, although at lower concentrations and frequency compared to the rhizosphere samples. Some of the possible sources for these anions were discussed in Chapter 3, Section 3.4.1.3. In the current experiment, the activity of soil organisms, especially springtails were often observed in soil in the rhizotron (Figure 4.6e), could also account for some OAs detected in the non-rhizosphere soils. In addition, in some cases,

the widespread fungal mycelia (Figure 4.6f) may also contribute OAs in non-rhizosphere samples.

The relative abundances and/or detection frequencies of OAs detected in root exudate samples collected *in situ* by AEM strips were different from those extracted by DI water at the second sampling. Although the water-soluble exudate solution was not collected from intact roots and thus has inherent limits, this method (referred as rhizosphere soil solution in some literature) has been widely used with various extraction solutions (Baziramakenga et al. 1995; Fox and Comerford 1990; Grierson 1992; Schefe et al. 2008; Shen et al. 1996; Strobel et al. 2001; Weisskopf et al. 2008). Apart from the risk of releasing some OAs inside root cells from excised sections into water-soluble exudate solution, the collection mechanisms could also contribute to some of the differences seen between the collection techniques. Collection of OAs on AEM strips relies on the strength of interaction between the various anions and HCO_3^- , which were pre-charged onto the AEM surface. As demonstrated in Chapter 3, Section 3.3.2, OAs showed different efficiencies of recovery by AEM from soil and solutions. Water-soluble root exudate solutions could only extract the soluble OAs from root and soils. Insoluble anions, absorbed onto soil solid phase, complexed with cationic ligands or metals in soil (see discussion in Chapter 3, Section 3.4.1.2), could not be extracted by water used as extraction solution in this study. In addition, Cl^- from the elution solution in AEM collected exudates contributed to the void peak at the beginning of the HPLC chromatogram which could affect the earlier eluted anions, as compared to water-soluble exudate solutions (Appendix 3.6). The void peaks in water-soluble exudate solutions were smaller when compared to those in exudate samples collected by AEM (data not shown). Apart from these, the sampling process itself may also account for some of the variability. The OAs collected by AEM (sampled from three sites of root-rhizosphere soils in each access portals) were bulked for analysis. In most cases, water-soluble water exudates contained not only these root-rhizosphere soil samples but also others collected at the same access portals in order to obtain adequate rhizosphere soil for microbial community analysis. Moreover, the root-rhizosphere samples were collected by AEM *in situ* first and then sampled roots were extracted by sterile DI water. Therefore, some accumulated OAs may have all or partly been collected by AEM before water extraction. Despite these differences, these two collection methods could compliment each other and strengthen confidence in the results of the study that there was no clear difference between GM and WT pine. As suggested by Phillips et al. (2008), complementary experimental approaches should be used for better understanding the exudation process in the rhizosphere because of inherent limitations in all available exudate collection and analysis methods.

The relative efficiency of OA collection by AEM or extracted by DI water methods can be made by comparing the OAs detected between the non-rhizosphere exudate samples collected by the two methods at the second sampling. Nine OAs were detected in the exudate samples collected by AEM strips (Table 4.5), while only five were detected in the water-soluble exudate samples of non-rhizosphere soils (Table 4.10). Apart from malate and succinate which were only detected in less than 20% of the water-soluble exudate samples, the other three OAs were also detected at lower frequencies in DI water extracts in comparison with those collected *in situ* using AEM strips. The average total C of OAs in the water-soluble non-rhizosphere exudate samples was $3.87 \mu\text{g C g}^{-1}$ soil. While the average total C of OAs in the non-rhizosphere exudate samples collected by AEM strips was $5.06 \mu\text{g C cm}^{-2}$ of AEM. It would be expected that only OAs in soil in close proximity to the AEM strips could be collected by the membrane. Thus, assuming that the AEM strips could collect OAs from a soil region within a depth of 1 mm, the average total C of OAs in exudate samples collected by the AEM (2 cm^2 per strip) would be $\sim 23 \mu\text{g C g}^{-1}$ soil. This represents a 6-fold increase of that in exudate samples collected by DI water. Collectively, these results suggest that using AEM is a relative more efficient technique for collection of OAs from soils compared to extraction by DI water.

4.4.4 Linking the Exudates with Microbial Communities

Although some significant differences in rhizosphere microbial communities were detected between GM and WT tree lines across the various sampling times and depths, there was no overall trend that suggested the communities were affected by the genetic modification. Similarly, there was no clear difference in OAs (individual, total C or as a profile) in exudate samples (both collected *in situ* and water extracted) between the two tree lines. The transient difference in communities may be due to the natural variability in exudation of OAs. Correlation of microbial communities with OA profiles was not performed because of the variability among replicates in this study. However, a subsequent experiment conducted under more controlled condition investigated the impact of selected OAs and sugars from radiata pine exudates on soil microbial community (Chapter 5).

While several studies have been carried out to investigate the direct influence of root exudates on rhizosphere microbial communities *in vivo*, most studies were inconclusive due to difficulties inherent in the experiment design (Chapter 1, Section 1.1.4). Recently, Micallef et al. (2009) investigated the root exudate profiles and rhizobacterial communities associated with eight various accessions of *Arabidopsis thaliana* and reported that despite accession-specific differences in both rhizobacterial communities and root exudates, no statistical correlation was evident between these two components. The authors attributed

the lack of correlation to inherent limitations of the techniques employed and/or lack of a temporal component in the sampling method used. In that study, the root exudate samples were collected from hydroponic-grown plants while the rhizosphere communities were examined from soil-grown plants. Plants have distinct root morphologies and can release different exudates during growth in solution in comparison with soil (Barber and Gunn 1974; Groleau-Renaud et al. 1998; Schonwitz and Ziegler 1982), and this may explain the lack of correlation. Nonetheless, several plant-derived factors, such as root system architecture, contribute to bacterial selection in the rhizosphere (Micallef et al. 2009). Weisskopf et al. (2008) reported that the variability among the rhizosphere communities (both RNA- and DNA-based) of wheat and lupin could not be correlated to the OA contents extracted by water from excised roots, although the authors reported that OA contents of exudates did explain 15% of the variability in active endophytic microbial communities (Weisskopf et al. 2008). In contrast, Marschner et al. (2002) reported that bacterial community structures of the cluster roots of white lupin were correlated to the concentration of several organic acids (cis-aconitic, citric and malic acids) exuded by plants, while the bacterial community structure of the non-cluster roots was only influenced by malic acid as shown by canonical corresponding analysis. They also reported that the eukaryotic community of cluster roots was correlated with citric acid, while the community of non-cluster roots was not affected by any OAs. However, they did not report how much variability in the communities was explained by the variability of OAs. Instead, a DGGE gel of eukaryotic communities loaded with PCR products derived from the root-rhizosphere soil samples containing different levels of citrate were shown in the paper. Several bands that increased or decreased in intensity with increasing citrate exudation were claimed by the authors. However, these results could be disputed with no clear trends observed. In addition, caution is needed to interpret DGGE data using intensity, as this is only a semi-quantitative technique (Chapter 2, Section 2.1.2).

The findings of studies discussed above, together with the current study, highlight the difficulties involved in studying root exudates and its influence on microbial communities. Biedrzycki and Bais (2009) pointed out that the colonization of microorganisms on roots and development is complex and not well understood due to the dynamic nature of the plant root surface and microbial diversity. However, the current study provides a novel technique to collect root exudates *in situ* and study the corresponding rhizosphere soils of plant grown in soil in a large-scale container.

4.4.5 Microbial Communities Associated with GM and WT Pine Trees

In this study, all the four GM radiata pines were generated from one parent GM tree pEM1-4-11 and the results therefore should not be extrapolated to reach general

conclusions about the biosafety of GM radiata pine. A single GM tree line was used in an effort to reduce the variability associated with the “plant” aspect of the experiment, so that root exudation and microbial communities could be more appropriately assessed when grown in soil conditions. However, the variability was still too high, especially for root exudates, to allow detection of any impact of the genetic modification on microbial communities. Because of the random insertion of transgenes into the plant chromosomes, GM plants created by different transgenic events may result in the integration of the foreign gene at different sites of the plant genome, and thus contribute to unexpected effects. This has been reported in several studies. Heuer et al. (2002) found that one GM line of T4 lysozyme-modified potato (DL4) showed some significantly different rhizosphere communities in comparison with control plants in several samplings, while no significant differences were found between the other GM line (DL5) and control lines using several analytical methods. Kaldorf et al. (2002) observed differences in the abundance and development of one ectomycorrhizal morphotype, which was rare and poorly developed on roots from one transgenic aspen line compared to the parental line. The authors supposed the effect was most likely the result of a somaclonal effect because the formation of this ectomycorrhiza type was not affected in the other transgenic line. In addition, only one type of soil and set environmental conditions were used in the growth room in the current experiment. Other studies have shown that differences between GM and WT plants sometimes only appear at a certain season or in particular field conditions (Dunfield and Germida 2001; Gyamfi et al. 2002; Heuer et al. 2002). Although three samplings were conducted in this experiment, radiata pine was grown in rhizotron units for only 10 months, which was a short time period within its life cycle (more than 100 years) or commercial rotation cycle (25 to 28 years) (C. Walter, pers. comm.). The tree may therefore still be “adapting” to the experimental soil and growth conditions in the growth room. Some potential impact of GM pine in comparison with control tree may not yet have been expressed. However, the focus of this experiment was more to develop and apply a novel approach to examine the OAs in root exudates and rhizosphere microbial communities together when the plants were grown in soil using both GM and WT radiata pines.

4.5 Conclusions

This Chapter described a novel experimental approach for studying soil microbial communities and OAs in exudates in corresponding samples of radiata pine roots under controlled conditions with minimal soil disturbance. This approach could be applied to any rhizosphere studies as well as study of potential impacts of GM plants. Although a high

level of natural variability was observed in OAs in exudate samples, the work is a significant step forward in advancing understanding of rhizosphere processes.

Significant differences in both OAs and microbial communities (with the exception of fungal communities) were detected between rhizosphere and non-rhizosphere samples indicating a rhizosphere effect from pine roots. Some significant shifts in microbial community by GM pines were observed when compared to WT trees. However, they were inconsistent and, consequently, may have limited biological significance to soil microbial communities. In addition, some significant but inconsistent differences in the microbial communities in the non-rhizosphere soils were also detected between the two tree lines, showing the natural spatial and temporal variability in soil which may be contributed by activities of roots and soil biota. Organic anions in root exudate samples, either collected *in situ* by AEM or extracted by sterile DI water, did not reveal any clear difference between GM and WT pines due to the large natural variability among replicates. Nevertheless, significantly greater amounts of OAs were detected in the rhizosphere compared to non-rhizosphere or bulk soil with certain OAs being predominant in the root-exudate samples. Although not statistically correlated, the transient differences in communities may be caused by changes in amounts and/or components of OAs in root exudates. Selected sugars and OAs in root exudates of radiata pine from this study were applied to soil to further investigate the relationship between exudates and microbial communities (Chapter 5).

Chapter 5 Influence of Root Exudate Components on Soil Microbial Communities

5.1 Introduction

Plants have been shown to select for the soil microorganisms which colonize their roots (Bais et al. 2006; Grayston et al. 1998; Hartmann et al. 2009). Plants release significant amounts of photosynthetic C into rhizosphere soils through root exudates which are considered the driving factor for the soil microbial growth and activities (Kapoor and Mukerji 2006; Lynch 1990). Thus it could be expected that root exudates are important in plants and microorganisms interactions and may promote mutualistic associations between them (Bais et al. 2006; Koo et al. 2005; Lynch and Whipps 1990). Low molecular weight C compounds present in root exudates, including sugars, OAs and amino acids, are readily assimilated by microorganisms and may regulate the microbial community structure in the rhizosphere (Chapter 1, Section 1.1.4; Bais et al. 2006; Weisskopf et al. 2008). Direct studies of the influence of low molecular weight C compounds in root exudates on soil microbial communities are technically challenging because of the extreme complexity of the rhizosphere environment (Biedrzycki and Bais 2009) and thus the link between exudates and rhizosphere microbial community has not been conclusively demonstrated (Micallef et al. 2009; Weisskopf et al. 2008). In the *in situ* approach used to study exudate-microbial interactions in the previous chapter, the high level of natural variability in OAs in the exudates of radiata pine made it difficult to draw clear relationships between OAs and rhizosphere microbial community structure and diversity (Chapter 4).

Accordingly, simplified microcosm experiments with artificial root exudates (ARE) composed of selected exudate compounds applied directly into soil have been used to study the impact of root exudates on soil microbial communities (Baudoin et al. 2003; Griffiths et al. 1999; Paterson et al. 2007). Although the experimental conditions cannot reproduce the complex rhizosphere environment, these studies offer an approach for studying the roles of exudates in shaping rhizosphere microbial community and provide information on potential plant mechanisms for control of soil microbial diversity and function (Baudoin et al. 2003). Several studies have previously shown that low molecular weight C compounds can affect general soil microbial communities (Baudoin et al. 2003; Griffiths et al. 1999; Landi et al. 2006; Paterson et al. 2007) or specific communities such as diazotrophs (Bürgmann et al. 2005) and nitrate reducers and denitrifiers (Henry et al. 2008).

Most of the studies investigating ARE on soil microbial communities have focussed on the exudate profiles of maize (Baudoin et al. 2003; Benizri et al. 2002; Henry et al. 2008) or perennial ryegrass (Paterson et al. 2007), in which sugars are the dominant component followed by OAs. Conversely, low molecular weight OAs are found in greater quantity than other components in root exudates of forest trees, typically present at 2 - 3 times the C content of sugars (Grayston et al. 1996; Smith 1976). Therefore, it would be expected that low molecular weight C compounds in root exudates of forest trees may have different impacts on soil microbial communities compared to those ARE based on maize or ryegrass, as soil microbial communities could be influenced by both the composition and concentrations of ARE compounds (Baudoin et al. 2003; Griffiths et al. 1999).

With the application of rRNA-DGGE technique, the structure of the active microbial communities in complex rhizosphere soils could be clearly demonstrated and compared (Chapters 2, 3 and 4). However, the identification of the specific microbial species that respond to the environmental stimulus (e.g. root exudates) could not be obtained without excising bands from gels and subsequent sequencing. The detailed analysis of bands on DGGE gels is labour intensive and expensive. Nucleic acid phylochip analysis is a more effective technique for detailed species analysis of complex communities. The technique has been successfully applied to study complex microbial communities in a range of habitats including rhizosphere soils (DeAngelis et al. 2008; Sanguin et al. 2006), soils (Brodie et al. 2006), composts (Franke-Whittle et al. 2009), aerosols (Brodie et al. 2007) and water (DeSantis et al. 2007). Rapid advances in genetic databases have facilitated the development of comprehensive phylochips encompassing the known range of bacterial diversity based on 16S gene sequences (DeSantis et al. 2006). The 16S phylochip (GeneChip, Affymetrix, CA, USA) used in this study allows for the simultaneous detection of 8432 bacterial taxa. In addition, it has the ability to identify individual taxa varying by over five orders of magnitude in abundance (Brodie et al. 2006). However, phylochips cannot detect taxa that have not been described previously in databases, as the design of phylochip platforms is highly dependent on information already known.

In this study, DI water or various ARE solutions composed of sugars and/or OAs based on the root exudates collected from radiata pine (Chapters 3 and 4) were applied daily to soil microcosms for 15 days. The main objective was to study the effects of two main components (i.e. sugars and OAs) in root exudates of radiata pine on activity and diversity of soil microbial communities.

5.2 Materials and Methods

5.2.1 Artificial Root Exudate Solution

Artificial root exudate solutions were prepared using a limited range of sugars and OAs. Three sugars (glucose, sucrose and fructose) were selected based on root exudate profiles of radiata pine (Appendix 3.1; Appendix 5.1) and information in the literature (Grayston et al. 1996; Smith 1976), and were applied to the soil as a single mixture. Although a diverse range of OAs was detected in the root exudates of radiata pine (Chapter 3 and 4), quinate, lactate and maleate were selected as representative OAs for this study. Lactate is a commonly present OA in root exudates as well as microbial metabolites, quinate was reported to attract *Rhizobia* previously (Parke et al. 1985; Tully 1988), while maleate is an OA which was not commonly reported in the plant root exudates in the literature. As it is unrealistic that root exudate components will be found in isolation in rhizosphere soil, and because of possible complex interactions between compounds in rhizosphere environments, ARE solutions were composed of various mixtures (sugars with individual OAs, sugars with all three OAs, three sugars alone, or three OAs alone) (Table 5.1).

Table 5.1 Composition and concentration of sugars and organic anions in ARE solutions.

Treatment code	Sugar (mg/g dry soil/day)	OA (mg/g dry soil/day)	Daily C input (mg C/g dry soil/day)
Con	0	0	0
S	Glucose 0.083 Sucrose 0.079 Fructose 0.083	0	0.1
SQLM	Glucose 0.083 Sucrose 0.079 Fructose 0.083	Quinate 0.152 Lactate 0.166 Maleate 0.161	0.3
SQ	Glucose 0.083 Sucrose 0.079 Fructose 0.083	Quinate 0.457	0.3
SL	Glucose 0.083 Sucrose 0.079 Fructose 0.083	Lactate 0.498	0.3
SM	Glucose 0.083 Sucrose 0.079 Fructose 0.083	Maleate 0.483	0.3
QLM	0	Quinate 0.152 Lactate 0.166 Maleate 0.161	0.2

Six ARE solutions were prepared with compositions and concentrations shown in Table 5.1. The ratio of C content from sugars and OAs was 1: 2 which was in the range of those typically found in tree exudates (Grayston et al. 1996; Smith 1976). The daily C input was

selected based on the previous experiment (Chapter 4) and fell within the range used in ARE experiments reported in the literature (Baudoin et al. 2003; Grayston et al. 1996; Griffiths et al. 1999; Henry et al. 2008; Paterson et al. 2007; Smith 1976). The pH values of all ARE solutions were adjusted to 5.5 using NaOH solution to avoid dropping of the soil pH due to the addition of ARE (Renella et al. 2006). The use of NaOH instead of buffer (e.g. phosphate buffer) to adjust solution pH was to eliminate the interference of other nutrients (e.g. P) in the experiment. After preparation, the ARE solutions and DI water were filter sterilised and stored, as aliquots of 1.5 ml for daily application, in sterile 25 ml Falcon tubes at -20°C.

5.2.2 Soil Microcosms

Horizon A of the Templeton silt loam soil which was used in rhizotron containers (top 26 cm) in the previous experiment (Chapter 4) was used in this experiment. The soil collection and preparation has been described in Chapter 4, Section 4.2.1, and the soil composition and characteristics are shown in Chapter 4, Section 4.2.1 and Appendix 4.2. The soil was moistened to 14% which was approximate 80% of field capacity and sieved through a 1.4 mm sieve before being packed into perspex containers (4.5 cm in diameter) (Figure 5.1). Containers held the equivalent weight of 50 g dry soil and were preincubated at 18°C in the dark for 14 days prior to the addition of ARE treatments to allow the microbial community to stabilise (Baudoin et al. 2003). During this period, soil moisture was maintained at approximate 80% of field capacity by weighing the soil microcosms regularly and adding sterile DI water to restore to the original weight. On the last day of preincubation, 0.45 mg nitrogen g⁻¹ dry soil of filter-sterilised ammonium nitrate solution was added to each microcosm and mixed well to ensure that nitrogen was not limited during the experiment incubation period. The ratio of total C to nitrogen applied into soils in different treatments ranged from 3-10 which was commonly found in plant root exudates (Grayston et al. 1996; Mench and Martin 1991).

To avoid a large and single organic C pulse which does not mimic natural release of exudates (Baudoin et al. 2003), ARE solutions were applied daily to the soils for a period of 15 days. DI water (control) or ARE solutions were applied to microcosms at a C input ranging from 0 to 0.3 mg C g⁻¹soil day⁻¹ and mixed well with the soil in each container using a sterile spatula (Table 5.1). As C input into each microcosm was calculated based on the total amount of soil in the container, the mixing was carried out to avoid accumulation of ARE solution on the top layer of soil. The working solution consisted of 1 ml of each ARE solution (which were thawed just before application) mixed with enough sterile water to maintain the soil moisture at 80% of field capacity throughout the entire experiment. The microcosms were incubated at 18°C in darkness for 15 days with four

replicates of each treatment randomly positioned in the incubator. The experiment duration used in this study was in the range of those reported in the literature (ranged from 7 to 28, with most at 14 days) (Benizri et al. 2002; Griffiths et al. 1999; Landi et al. 2006; Paterson et al. 2007; Renella et al. 2007).

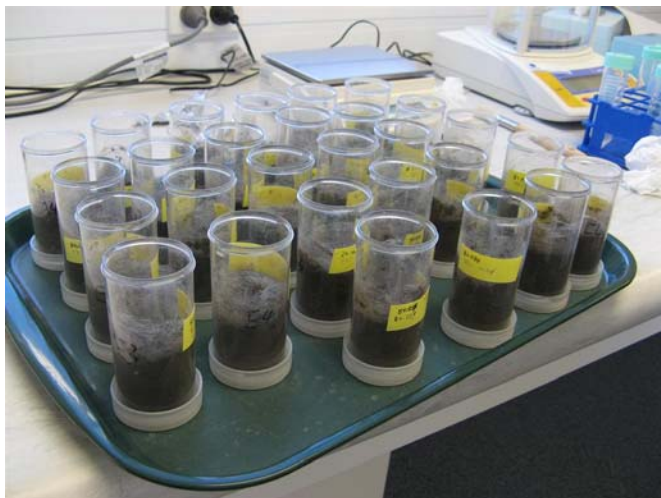


Figure 5.1 Soil microcosms used to study the impact of ARE solutions on soil microbial community.

At harvest, the soil in each microcosm was mixed well prior to sampling. Half gram of soil from each container was weighed into a sterile Eppendorf tube and stored at -80°C for subsequent RNA extraction and microbial community analysis. Soil moisture and pH in each microcosm were measured on the sampling day, while dehydrogenase activity was measured on the following day with soils stored in sealed bags at 4°C .

5.2.3 Soil Moisture and pH

Soil moisture in each microcosm was measured using the method described by Blakemore et al. (1987). Briefly, 10-20 g fresh soil was weighed accurately into a metal dish and the dish was placed in an oven at 105°C overnight. The following morning, immediately after removal from the oven the dish was placed in a desiccator with the lid on. When cool, the weight of the dish with soil inside was recorded. The % soil moisture was calculated as:

$$\% \text{ soil moisture} = (\text{moisture weight} - \text{dry weight}) / \text{dry soil weight} \times 100$$

For pH measurement, 25 ml of DI water was added to fresh soil (15 ± 0.05 g), stirred well and left overnight to stabilise. The pH of soil solution supernatant was measured the following day (Blakemore et al. 1987).

5.2.4 Soil Dehydrogenase Activity Analysis

Dehydrogenase activity in soil, as an indication of overall microbial activity, was assessed based on the use of triphenyltetrazolium (TTC) as an artificial electron acceptor according to the method described by Alef (1995). Basically, TTC solution (5 ml, 0.1% wt/vol) or Tris buffer (blank control) was added to 5 g of fresh soil and incubated for 24 h at 30°C . After incubation, 40 ml acetone was added to each tube followed by incubation for 2 h in the dark at room temperature with tubes shaken at intervals. The optical density of the

supernatant was measured against the blank at 546 nm using a UV visible spectrophotometer (Cary 50, Varian Australia Pty Ltd, Australia).

5.2.5 Molecular Analysis of Soil Microbial Communities

Microbial RNA was extracted from 0.5 g of soil samples and subsequent cDNA preparation as described in Chapter 2, Sections 2.2.2 and 2.2.3 using primers 1492R (5'-GGTTACCTTGTTACGACTT-3') (Brodie et al. 2007) for bacterial rRNA and FR1 (sequence see Chapter 2, Table 2.1) for fungal 18S rRNA. Three different PCR amplifications with primer sets (341F/534R-GC, NS1/FR1-GC and 27F.1/1492R) were used to check for the presence of DNA in RNA samples prior to the generation of cDNA according to the conditions described in Chapter 2, Sections 2.2.4 and 2.2.6 and Chapter 5, Section 5.2.5.2.

5.2.5.1 rRNA-DGGE microbial community analysis

The molecular analysis of general bacterial 16S and fungal 18S communities using rRNA-DGGE technique is described in Chapter 2, Sections 2.2.4, 2.2.6 and 2.2.7, although bacterial 16S rRNA in this study was amplified from cDNA converted by 1492R primer. Four replicates from each treatment were analysed for bacterial 16S communities. Because of the good reproducibility of the replicates (Figure 5.3), three replicates from each treatment (selected at random) were analysed for fungal 18S DGGE.

5.2.5.2 16S rRNA phylochip microbial community analysis

The analysis of bacterial communities by 16S phylochip was carried out at Lawrence Berkeley National Laboratory, California, USA. For practical reasons, 18 samples (six treatments; Con, S, SQLM, SQ, SL and SM with three randomly chosen replicates for each treatment) were selected for phylochip analysis. General bacterial 16S were amplified using the primer set 27F.1/1492R with the cDNA samples converted using 1492R primer as templates. The sequence for 27F.1 is 5'-AGRGTGGATCMTGGCTCAG-3' (Brodie et al. 2007). The PCR reaction was carried out in 100 µl volumes containing 4 µl cDNA (1:10 diluted after RT-PCR) or RT-PCR negative control, 1 x NH₄ buffer (Bioline, Australia), 0.2 mM dNTPs (Promega Corporation, WI, USA), 0.4 µM each primer, 2 mM MgCl₂, 0.4 mg ml⁻¹ BSA (Promega Corporation), and 4 U Biotaq DNA polymerase (Bioline). PCR amplifications were adapted from Brodie et al. (2007) and were performed in a thermal cycler (I Cycler, Bio-Rad, CA, USA) with an initial denaturing step at 95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 53°C for 30 s and 72°C for 1 min, before a final extension step at 72°C for 7 min. Amplified PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel with

ethidium bromide staining. Bacterial 16S PCR products were then cleaned using MinElute PCR purification kit (Qiagen Science, CA, USA) according to the manufacturer's instructions. The purified PCR products were lyophilized using a CentriVap® DNA Centrifugal Vacuum Concentrator (Labconco Corporation, Kansas City, USA) and sent to Berkeley.

In Berkeley, the lyophilized PCR product was dissolved by adding 10 µl of DNase/RNase-free sterile water (Invitrogen, CA, USA) into each tube prior to the PCR product quantitation. The quantitation was carried out using 2% E-Gel (Invitrogen) by loading a mixture of 1 µl of each PCR product and 19 µl of sterile water into each lane of the gel along with 10 µl low mass ladder (Invitrogen) and electrophoresis at 60 V for 15 min. Gels were then visualized and the amount of PCR products were calculated using a Fluor-S Multilmager (Bio-Rad).

Bacterial PCR products (304 ng) from each sample were spiked with known concentrations of amplicons derived from yeast and bacterial metabolic genes which acted as internal standards (Brodie et al. 2006). This mix was fragmented to 50-200 bp using DNase I (0.02 U µg⁻¹ DNA, Invitrogen) and One-Phor-All buffer (GE Healthcare, NJ, USA) following the manufacturer's protocols. The mixture was then incubated at 25°C for 25 min and then at 98°C for 10 min before biotin labelling with a GeneChip DNA labelling reagent kit (Affymetrix, CA, USA) according to the manufacturer's instructions. The labelled DNA was then denatured at 99°C for 5 min and hybridized to 16S rRNA GeneChip (Affymetrix) at 48°C and 60 rpm for 16 h. Phylochip washing and staining were performed according to the standard Affymetrix protocol described by Masuda et al. (2002).

Each phylochip was scanned using a GeneArray Scanner (Affymetrix) and recorded as a pixel image, and initial data acquisition and intensity determination were performed using standard Affymetrix software (GeneChip Microarray Analysis Suite, version 5.1). Background correction and data normalization between chips using internal standards were conducted according to criteria described by Brodie et al. (2006). The microbial community analysis was resolved as a subset of 58 designed phylum groups that cover 8432 bacterial taxa (Table 5.2). Each taxon is represented by a set of an average of 24 perfect match (PM)-mismatch (MM) probe pairs (minimum 11, maximum 30). Probe pairs scored as positive were those that met two conditions: i). the perfect match had an intensity of at least 1.3 times that of the corresponding mismatch, and ii). the difference in intensity, PM minus MM, was at least 500 times greater than the background level (Brodie et al. 2006). The positive fraction (pf) was calculated for each probe set as the number of positive probe pairs divided by the total number of probe pairs in a probe set. A taxa was

considered present in the sample when over 90% of its assigned probe pairs were positive (pf >0.9) (Brodie et al. 2006). A hybridization intensity score (HybScore) was calculated in arbitrary units for each probe set as the trimmed average (maximum and

Table 5.2 List of all the bacterial phyla and the number of taxa in each bacterial phylum available for hybridization in 16S rRNA Genephip (Affymetrix).

Phylum	Number of taxa	Phylum	Number of taxa
1959 group	1	NKB19	2
Acidobacteria	98	OD1	4
Actinobacteria	810	OD2	6
AD3	1	OP1	5
Aquificae	19	OP10	12
Bacteroidetes	880	OP11	20
BRC1	3	OP3	5
Caldithrix	2	OP5	3
Chlamydiae	27	OP8	8
Chlorobi	21	OP9/JS1	12
Chloroflexi	117	OS-K	2
Chrysiogenetes	1	OS-L	1
Coprothermobacteria	3	Planctomycetes	182
Cyanobacteria	202	Proteobacteria	3170
Deferribacteres	5	SPAM	2
Deinococcus-Thermus	18	Spirochaetes	150
Dictyoglomi	5	SR1	4
DSS1	2	Synergistes	19
EM3	2	Termite group 1	6
Fibrobacteres	4	Thermodesulfobacteria	4
Firmicutes	2012	Thermotogae	15
Fusobacteria	29	TM6	5
Gemmatimonadetes	15	TM7	45
LD1PA group	1	Unclassified	327
Lentisphaerae	8	Verrucomicrobia	78
marine group A	5	WS1	2
Natronoanaerobium	7	WS3	7
NC10	4	WS5	1
Nitrospira	29	WS6	4

minimum values removed before averaging) of the PM minus MM fluorescent intensity differences across the probe pairs in a given probe set (Brodie et al. 2006). HybScores were normalized to an average of 2500 arbitrary units based on internal standards. The HybScore values have been empirically shown to be linear with the log₁₀ of copy number of 16S gene products (Brodie et al. 2007), and were thus used as abundance of taxa populations. A taxon in each treatment was considered present if it occurred in at least two out of the three replicates and the HybScore values were used for taxon abundance

(DeAngelis et al. 2008). The HybScore values of taxa that were not present in treatments (did not fit the criteria with at least two replicates with $pf > 0.9$) were set to zero (since these taxa are scored as absent in treatments) (Yergeau et al. 2009). These HybScore values were used directly for a single taxon-level analysis and summed up to the class, phylum or domain (bacteria) for other analyses (Yergeau et al. 2009).

5.2.6 Statistical Analysis

Statistical analyses of soil pH, soil moisture and dehydrogenase activities across the treatments were conducted by ANOVA using Genstat™ 11 (VSN International Ltd, Rothamsted, UK). For multiple comparisons, treatment means were separated using Fisher's protected LSD at $P=0.05$ level. Statistical analysis of DGGE gels is described in Chapter 2, Section 2.2.8.

5.2.6.1 Phylochip analysis of soil bacterial community structures

The analysis of bacterial community structures using data obtained from phylochips were carried out in PRIMER 5 (Plymouth Routines in Multivariate Ecological Research, version 5.2.9, PRIMER-E Ltd, Plymouth, UK) which is a statistical software package for the analysis of ecological multivariate data.

Rank abundance curves

As a means of visualising diversity of microbial communities, rank abundance curves were produced, with taxa ranked in decreasing order of their importance in terms of abundance. The taxa richness is viewed as the number of different taxa on the chart (log transformed) and taxa evenness is derived from the slope of the relative abundance curve. A steep slope indicates low evenness, while a shallow slope indicates high evenness as the abundance of different taxa are similar (Magurran 1988). In this study, relative rank abundance curves from each treatment were produced using the mean of HybScore values in three replicates and visualised with the ordinary plotting method using Dominance Plot in PRIMER 5.

Cluster and non-metric multi-dimensional scaling (MDS) analyses

By taking HybScore values as taxa abundance, similarity matrices for the community profiles were constructed by calculating similarities between each pair of samples using the Bray-Curtis coefficient S :

$$S_{jk} = 100 \left\{ 1 - \frac{\sum_{i=1}^p |y_{ij} - y_{ik}|}{\sum_{i=1}^p (y_{ij} + y_{ik})} \right\}$$

where y_{ij} is the abundance value for the i th taxa in the j th sample. The Bray-Curtis coefficient is particularly suitable for biological community data as explained by Clarke and Warwick (2001) and has been used for molecular analysis of bacterial communities (Micallef et al. 2009; Rees et al. 2004; Wakelin et al. 2008). The similarity matrix was calculated based on square root transformed HybScore data. Square root transformation is carried out to reduce the emphasis from dominant species and take some consideration of rarer species (Clarke and Warwick 2001). This transformation has also been used previously for the analysis of phylochip data (Sanguin et al. 2006).

To visualise the relationship among samples within the same treatments and between treatments, the similarity matrices were analysed by hierarchical cluster analysis, a classification method that aims to group samples into discrete clusters based on similarity. The cluster analysis was performed by a weighted, group-average linkage agglomerative method and dendrograms were constructed from the ranked similarities using PRIMER 5. The similarity matrices were also used in non-metric MDS, an ordination method that condenses many independent variables, in this case the bacterial taxa, into a reduced space. Unlike other ordination methods such as principal component analysis, MDS analysis does not make any assumption about the distribution of the data and hence is particularly suitable for ecological multivariate data (Clarke and Warwick 2001). The MDS plots in two or three dimensions were plotted to represent the relationship among samples. In a MDS plot, the actual location of each data point in space is arbitrary, and the axes can be rotated freely. It is the relationship of the data points to each other that is important, with two near points representing more similarity to each other than to another point located at a distance (Clarke and Warwick 2001). The stress value assigned to the plot is a measure of how much distortion was introduced to allow the representation of the data in the specified dimensions. A stress value of <0.01 indicates a perfect representation with no prospect of misinterpretation, while a measure of stress <0.2 gives a potentially useful map. A measure >0.3 indicates that the level of distortion required to display the data on the map is too high for any reliable inferences to be made from the configuration (Clarke and Warwick 2001). In the analyses of phylochip data in this study, MDS plots were generated from the best possible ordination following 100 random restarts.

The non-parametric permutation procedure analysis of similarity (ANOSIM) was used to further analyse the statistical difference between treatments as plotted in cluster analysis and MDS ordination. This test applies ranks to similarity matrices used for cluster and MDS and combines this ranking similarity with Monte Carlo randomization to generate significance levels (P values). ANOSIM tests the null hypothesis that the average rank similarity between samples within a treatment is the same as the average rank similarity between samples across treatments and produces a test statistic R . An R value of 0

indicates the null hypothesis is true that all samples are the same. As R approaches 1, the null hypothesis is rejected and this indicates that replicates from one treatment are more similar to each other than to replicates from other treatments (Clarke and Warwick 2001).

5.2.6.2 Dynamic taxa in bacterial communities

To further investigate the response of taxa to different ARE solutions/compounds, two different comparisons were performed. The effects of various ARE solutions on soil bacterial communities were determined by comparison of the taxa in treatments S, SQLM, SQ, SL and SM to those in control treatment Con, respectively. To further examine the effects of selected OAs (i.e. quinate, lactate, maleate and a mixture of these OAs) on soil bacterial communities, taxa in bacterial communities in treatments SQ, SL, SM and SQLM were compared to those in treatment S, although the potential interaction of OAs with the sugars present in mixtures could not be simply eliminated in this way. Different “dynamic communities”, defined as comprising taxa that significantly responded to the presence of ARE solutions/compounds, were constructed. Each dynamic community contained those taxa which were only detected in one treatment and those taxa which differed significantly ($P < 0.05$) in abundance as measured by HybScore values between two treatments by paired t-test. All the HybScore were $\log_{10}(1+x)$ transformed and analysed by paired t-test using the statistical software R 2.8.1 (R Development Core Team, 2007).

5.2.6.3 Bacterial abundance (HybScore) analysis

The abundance of bacterial taxa as shown by HybScore values at different phylogenetic levels (domain, phylum or class) across treatments were analysed by ANOVA using Genstat™ 11 (VSN International Ltd). For multiple comparisons, treatment means were separated using Fisher’s protected LSD at $P=0.05$ level.

5.3 Results

5.3.1 Soil Moisture and pH

The soil moisture was maintained at approximately 80% of field capacity during the incubation period. At harvest, the soil moisture ranged from 12.8-13.4%, with no significant differences across treatments (Table 5.3). Interestingly, pH values in soils amended with ARE solutions containing OAs (SQLM, SQ, SL, SM and QLM) increased 1 to 3 units compared to that in the control soil and differed significantly ($P < 0.05$) between each other (Table 5.3). The soil pH in treatment S did not differ significantly ($P < 0.05$) from that in control soils.

Table 5.3 Soil moisture and pH in microcosms amended daily with either DI water (Con) or various ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. Values are presented as the mean \pm 1 standard error ($n=4$). Different letters indicated significant difference ($P<0.05$) between treatments.

Treatment	Soil moisture (%)	Soil pH
Con	12.8 \pm 0.2	4.68 \pm 0.01 a
S	13.0 \pm 0.2	4.66 \pm 0.01 a
SQLM	13.2 \pm 0.1	6.82 \pm 0.08 d
SQ	12.8 \pm 0.1	6.57 \pm 0.01 c
SL	13.4 \pm 0.1	7.61 \pm 0.03 f
SM	12.8 \pm 0.1	5.40 \pm 0.01 b
QLM	13.0 \pm 0.2	7.48 \pm 0.05 e
<i>P</i> -value	0.106	<0.001

5.3.2 Soil Dehydrogenase Activity

The dehydrogenase activities in soils after 15 days of daily addition of either DI water or ARE solutions ranged from 1.2 to 5.6 $\mu\text{g TTC dwt g soil}^{-1} \text{ h}^{-1}$ (Figure 5.2). There was a significant increase ($P<0.05$) in dehydrogenase activity in soils amended with all ARE solutions compared to the control, with the exception of treatment SM (Figure 5.2). The dehydrogenase activity in treatment SM decreased significantly ($P<0.05$) in comparison with the control soil. With the exception of treatments SQ and QLM, the activities of dehydrogenase in microcosms receiving different ARE solutions were significantly different across treatments, indicating an effect of the composition of the ARE solutions (Figure 5.2).

5.3.3 DGGE Analysis of Soil Microbial Communities

General bacterial 16S communities were analysed by rRNA-DGGE and clear differences in DGGE profiles were observed across the treatments (Figure 5.3). There were several common bands across all seven treatments. Unique dominant bands distinct to each treatment were also visible on gels. In general, more dominant bands appeared in the profiles of soils treated with ARE solutions than were seen in the control soil (Figure 5.3). The exception to this was treatment SM, in which profiles contained only a few dominant bands. Highly similar (almost identical) DGGE patterns were observed among the four replicates in each treatment, indicating good reproducibility among replicates (Figure 5.3). Discriminant analysis showed that the bacterial communities were highly significantly different ($P<0.05$) across all seven treatments (Table 5.4).

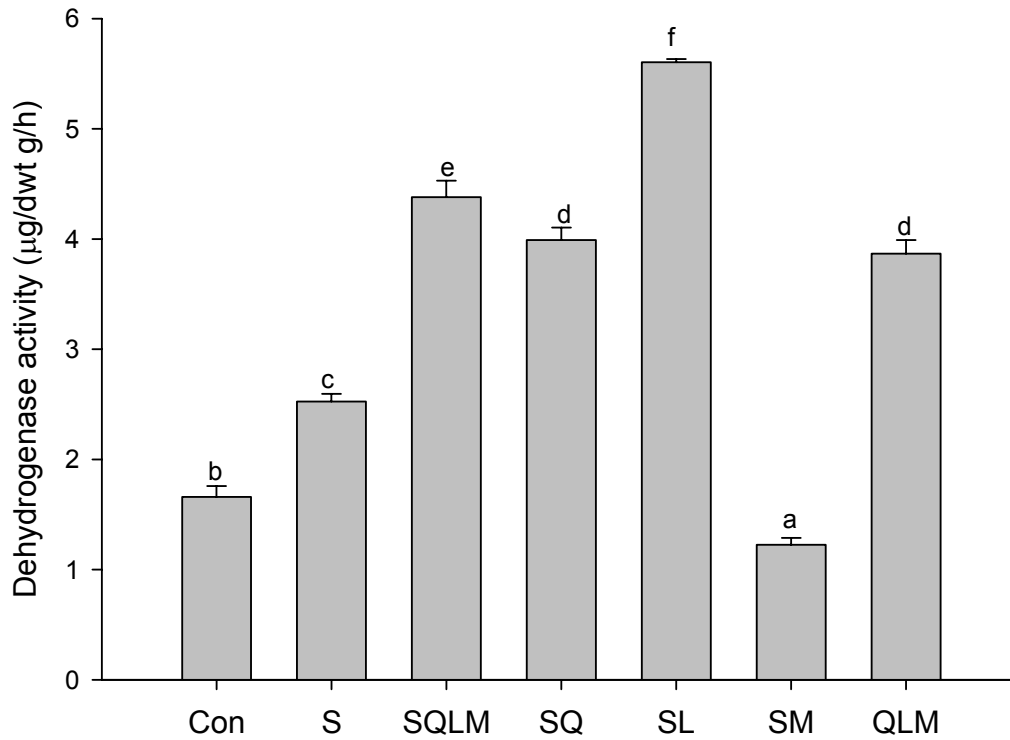


Figure 5.2 Dehydrogenase activity in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days in microcosms. Error bars show 1 standard error in each treatment, columns denoted with a different letter are significantly different ($P<0.05$) between treatments ($n=4$).

Table 5.4 Hotelling T^2 probabilities of the level of difference between microbial communities in soils amended with either DI water or ARE solutions, with each sample under the null hypothesis that all soil samples have the same mean ($n=4$ for bacterial 16S, $n=3$ for fungal 18S). The probability values where means were significantly different ($P<0.05$) are shown in bold.

Community	Treatment	Con	S	SQLM	SQ	SL	SM
Bacterial 16S	S	0.000	-				
	SQLM	0.000	0.000	-			
	SQ	0.000	0.000	0.000	-		
	SL	0.000	0.000	0.000	0.000	-	
	SM	0.000	0.000	0.000	0.000	0.000	-
	QLM	0.000	0.000	0.0343	0.000	0.000	0.000
Fungal 18S	S	0.0000	-				
	SQLM	0.0000	0.0110	-			
	SQ	0.0000	0.4321	0.0681	-		
	SL	0.0000	0.0001	0.0050	0.0002	-	
	SM	0.000	0.0001	0.0001	0.0001	0.0000	-
	QLM	0.0001	0.0027	0.0672	0.0068	0.0096	0.0000

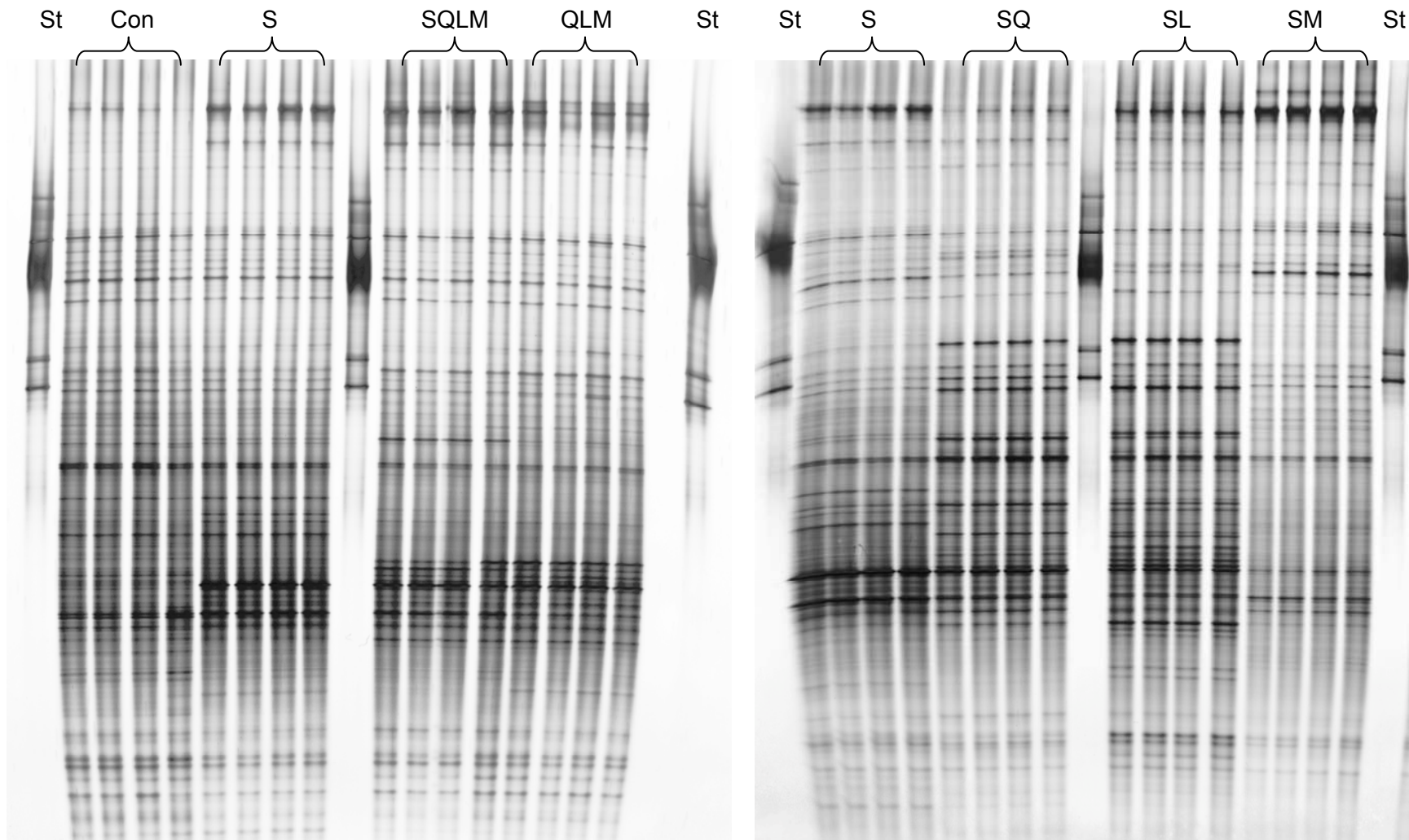


Figure 5.3 rRNA-DGGE profiles of bacteria in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. St: bacterial 16S DGGE marker consisted of bulked bacterial 16S PCR products from strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp..

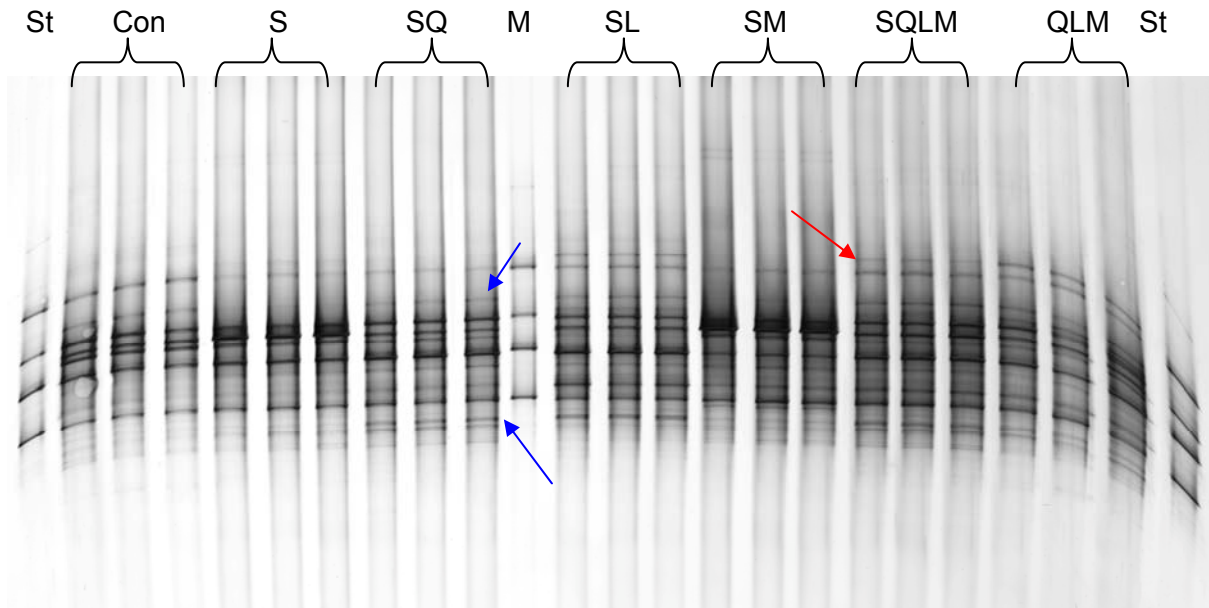


Figure 5.4 Fungal 18S rRNA-DGGE profiles in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) for 15 days. St: Fungal 18S DGGE marker consisted of bulked fungal 18S PCR products from strains *Umbelopsis ramanniana*, *Trichoderma reesei*, *Mortierella chlamydospora*, *Phoma cucurbitacearum*, *Clonostachys rosea*. Two bands that appeared in treatment SQ but not in treatment S are indicated by blue arrows. One band that appeared only in treatment SQLM and not in treatment SQ is indicated by a red arrow.

Fungal 18S communities in soils were also analysed by rRNA-DGGE, using three replicates randomly selected from each treatment (Figure 5.4). Compared to bacterial 16S, fungal DGGE profiles contained fewer bands. Discriminant analysis revealed that most of the treatments differed significantly from each other, with the exception of three pair comparisons (SQ with S, SQ with SQLM, and SQLM with QLM) (Table 5.4). However, minor differences in the presence/absence of bands between treatments SQ and S, SQ and SQLM are indicated by arrows on gel (Figure 5.4).

5.3.4 Phylochip Analysis of Soil Bacterial Communities

5.3.4.1 Bacterial community structures

Bacterial taxa richness

Of the possible 8432 resolvable bacterial taxa on the phylochip, 1595 taxa falling within 42 phyla were detected in at least one sample across the treatments. However, for community analysis, taxa which were present (i.e. had a pf value >0.9) in at least two out of three replicates in any treatments were examined. This criteria eliminated ~ 400 taxa and resulted in 1188 taxa from 38 phyla which comprised the total bacterial communities for further analysis.

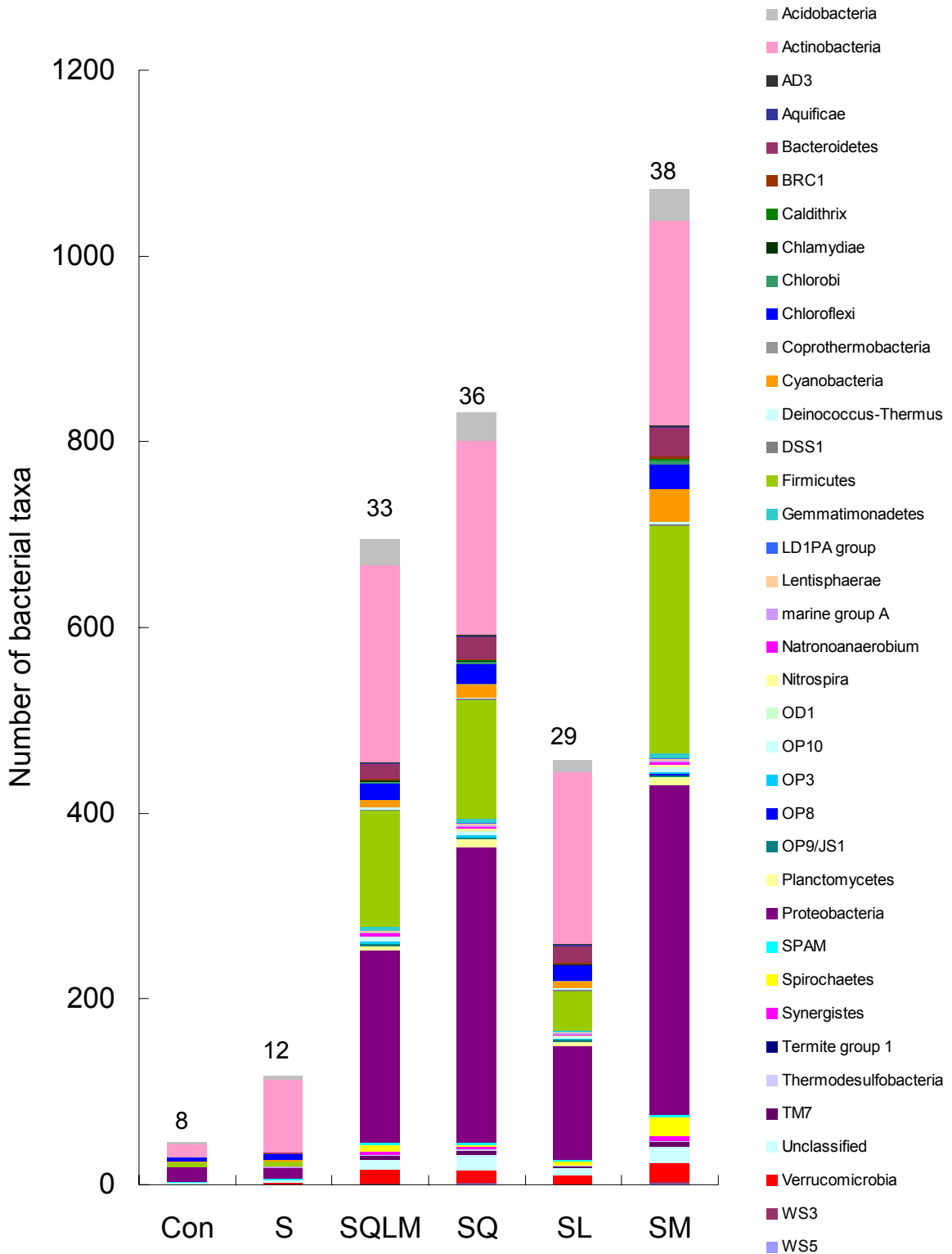


Figure 5.5 Number of bacterial taxa from various phyla detected in soils amended daily with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochip analysis. Taxa from different phyla are represented by different colours. A taxon was considered to be present if it occurred in two out of three replicates for each treatment. The number above each bar indicates the number of phyla detected in each treatment.

Only 46 bacterial taxa were detected in control soil communities analysed by rRNA 16S phylochips, while 2.5-fold more taxa were detected in bacterial communities in treatment S (Figure 5.5). With the addition of mixtures of OAs and sugars (SQLM, SQ, SL and SM), the number of bacterial taxa detected in soils reached over 450, which was approximately a 10-fold increase in the number of taxa found in the control soil. As many as 1072 bacterial taxa were detected in soil microcosms amended with ARE solution SM (Figure 5.5).

With the increase of taxa richness, the number of phyla to which taxa belonged also increased. Twelve phyla were detected in treatment S, while only eight were found in control soils. In other soil communities (treatments SQLM, SQ, SL and SM), the detected number of bacteria phyla ranged from 29 to 38 (Figure 5.5). Actinobacteria, Proteobacteria and Firmicutes were the three main phyla within each treatment (Figure 5.5). However, the numbers of taxa belonging to these three phyla, as well as Bacteroidetes, are also most abundant (over 800 taxa) on the phylochip (Table 5.2).

Rank abundance curves

The diversity of bacterial community, both taxa richness and individual abundance, is displayed in an ordered abundance curve in which the taxa are ranked in order of decreasing abundance (Figure 5.6). Community structures, as shown by rank curves, appeared to be generally equivalent for the treatments SQLM, SQ, SL and SM. The relative abundance curves of these communities were almost flat with the maximum relative abundance of a single taxon less than 0.5%, indicating a large number of bacterial taxa evenly distributed in these communities without dominant taxa (Figure 5.6). The abundance curves of the control soil and treatment S deviated from these flat curves. The bacterial community in treatment S was composed by one relatively dominant taxa, belonging to Chloroflexi (AJ306793), with just over 4% of total bacterial abundance and over 100 rare taxa (Figure 5.6). In the control soil, the three most abundant taxa comprised more than 20% of the total bacterial populations, with the most numerous taxon constituting over 9% of the total bacterial community. The most dominant taxon belonged to the phylum Chloroflexi (AJ306793) followed by another two taxa from the phylum Chloroflexi (AF507693) and Actinobacteria (AJ536866). The shape of the abundance curves indicates the bacterial community in the control soil was dominated by a few taxa and contained fewer total taxa (below 50) in comparison to other treatments (Figure 5.6).

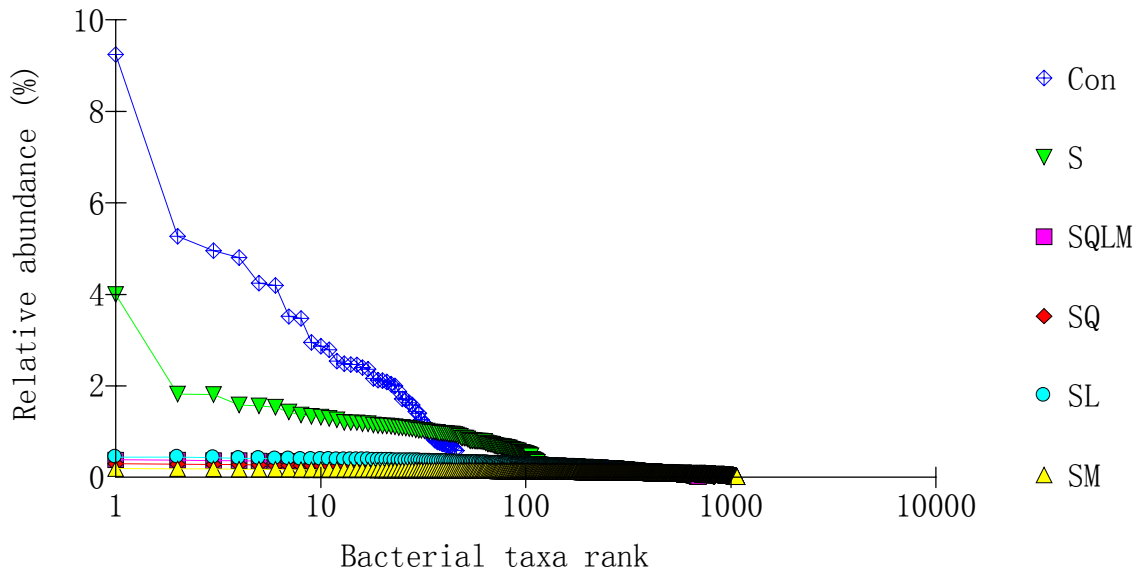


Figure 5.6 Bacterial rank abundance curves of communities in soil amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochips analysis.

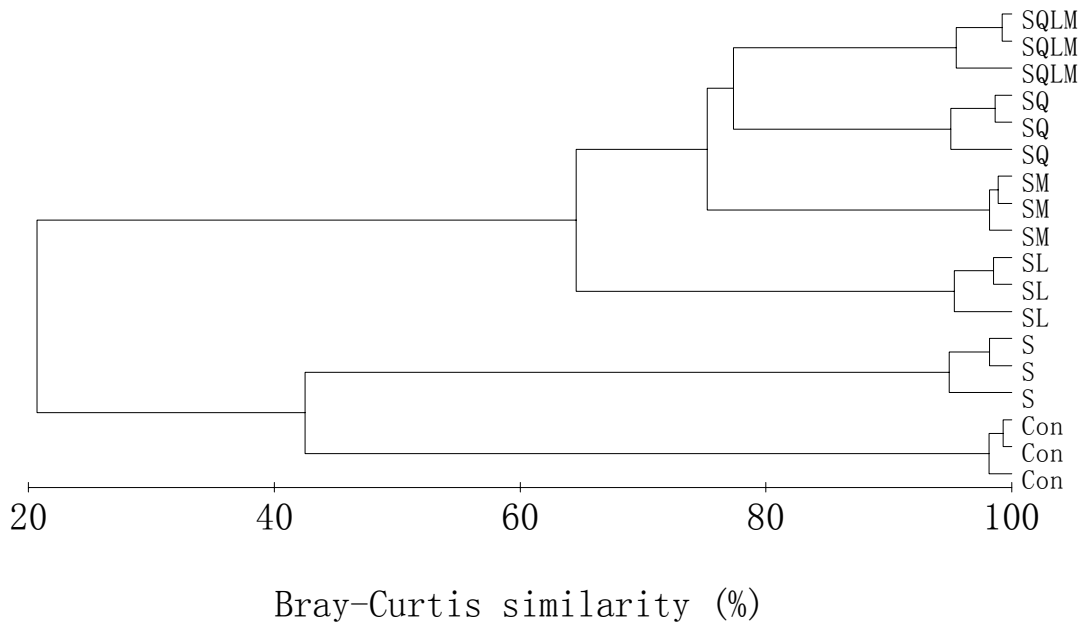


Figure 5.7 Hierarchical cluster dendrogram generated based on rank similarity of bacterial communities in soils amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data from phylochip analysis.

Cluster and MDS analysis

The relationship among active bacterial community structures based on abundance of different taxa across the various soil treatments were first assessed by hierarchical cluster analysis. This statistical method revealed distinct treatment-specific clusters of

communities (Figure 5.7). The cluster dendrogram showed that bacterial communities in soil amended with ARE solutions containing mixtures of sugars and OAs (SQLM, SQ, SL and SM) were more similar compared to the other treatments and thus grouped together at a similarity value of 64%. The soil bacterial communities in treatment S clustered with those in the control soil at a similarity level of 42% before clustering together with the other group at a low similarity value of 21%, indicating that the bacterial communities in treatment S were relatively more similar to those in control soil in comparison to those in treatments containing mixtures of sugars and OAs (Figure 5.7).

Clear separation of bacterial communities between treatments on the MDS plots further confirmed the robustness of different community structures across treatments observed by hierarchical cluster analysis (Figure 5.7, Figure 5.8). The stress value of MDS plots were 0, indicating perfect representations of the communities in two dimensional plots (Clarke and Warwick 2001). In addition, multiple restarts for two dimensional MDS ordination always generated the same configuration, exhibiting confidence in the output. The distance between bacterial communities in the control soil and treatment S was smaller than those between the control soil and other treatments, indicating bacterial community in the control soil was more similar to that in treatment S than it is to other treatments (Figure 5.8a). For a better visualization of the relationships among treatments SQLM, SQ, SL and SM, the ordination plot containing only these four treatments is shown in Figure 5.8b. Replicates from the same treatments were located closely together and showed clear treatment effects on the plot (Figure 5.8b).

Significant differences among the bacterial communities shown in cluster dendrogram and MDS plot across treatments were statistically analysed using the non-parametric ANOSIM test with the null hypothesis that there were no differences between communities. The resultant global R statistics of 1 with *P* value at 0.001 strongly indicated that there is a statistically significant difference between at least some community treatments. Pairwise comparisons revealed that all pairs of treatments were significant different with R value of 1, indicating that bacterial communities from the same treatment were more similar to each other than to communities of other treatments and bacterial communities from different treatments were significantly distinct.



Figure 5.8 Non-metric MDS ordination plots of bacterial communities in soils amended with either DI water (Con) or different ARE solutions (S = sugars, Q = quinate, L= lactate, M= maleate; see Table 5.1 for descriptions) using data obtained from phylochip analysis ($n=3$). a). Ordination of samples from all six treatments. b). Ordination of samples from four treatments SQLM, SQ, SL, SM.

5.3.4.2 Dynamic bacterial taxa response to ARE solutions

The dynamic bacterial communities containing the taxa which showed significant responses to the presence of various ARE solutions in soils in comparison to the control treatment are summarized in Table 5.5. The dynamic taxa included taxa which were significantly different ($P<0.05$) in HybScore values between treatments and that were only detected in one treatment (either positively or negatively) in pairwise comparisons.

The dynamic bacterial community with taxa that responded significantly to the presence of sugars, as analysed by comparing treatment S to treatment Con, was comprised of 101 taxa from 12 phyla. Actinomycetales in the phylum Actinobacteria were one of the groups

mostly influenced by the addition of sugars to the soil. Bacterial taxa from several other phyla including Bacteroidetes, Nitrospira and Verrucomicrobia were also stimulated by the addition of sugars and became detectable by rRNA 16S phylochips in treatment S. Most of these dynamic taxa responded positively to sugars added to the soil; however, 12 taxa from three phyla (Acidobacteria, Firmicutes, Proteobacteria) and one unclassified taxon either showed lower HybScore value than those in the control soil or became undetectable in treatment S (Table 5.5).

The activities of a very wide range of bacterial taxa were affected by ARE solutions SQLM, SQ, SL and SM in the experimental soil, as analysed by comparing relevant treatments to the control. The dynamic communities were comprised of 435 to 1067 taxa, which represented 37 to 90% of the total detected taxa across communities (Table 5.5). In the treatment SM, 1067 dynamic taxa were found from all of the detected 38 phyla. Although the size of the dynamic community influenced by ARE solution SL was relatively smaller (containing 435 taxa) compared to other dynamic communities responded to ARE solutions SQLM, SQ and SM, it was more than 4-times the size of dynamic bacterial community impacted by sugars in treatment S (Table 5.5). As with the effect of sugars in treatment S, only a minority of the taxa responded negatively to the presence of ARE solutions (Table 5.5). Actinobacteria, Firmicutes and Proteobacteria were three main phyla in the dynamic communities affected by ARE solutions SQLM, SQ, SL and SM (Table 5.5). A range of 11 to 33 taxa belonging to phyla of Acidobacteria and Bacteroidetes were also affected by the presence of ARE solutions SQLM, SQ, SL and SM in soil (Table 5.5).

To further analyse the impacts of OAs on soil microbial communities, the bacterial communities in treatments SQ, SL, SM and SQLM were compared to treatment S, respectively (Table 5.5). The influences of OAs (which may also result from the interactions between OAs and sugars) on bacterial communities were much greater than that of sugars, and this trend was consistent in each of the three individual OAs and the mixture of these three OAs. Lactate significantly affected 349 taxa from 27 phyla in soil bacterial communities. The largest impact on bacterial communities was detected with the presence of maleate in treatment SM. More than 1000 taxa were found in the dynamic community affected by maleate, which represented 85% of the total detected taxa in this study. Quinate also stimulated more than 700 taxa in the soil bacterial community. Over half of the total detected taxa responded significantly to the mixture of quinate, lactate and maleate in soil (Table 5.5). In contrast, the addition of sugars only (treatment S) affected 101 taxa from 12 phyla (Table 5.5). Among these dynamic communities impacted by OAs, only small numbers of taxa (less than 5%) responded negatively to the presence of OAs in

Table 5.5 Numbers of bacterial taxa in each of the phylogenetic groups found in the dynamic communities regulated by ARE solutions or compounds (S = sugars, Q = quinate, L= lactate, M= maleate). The influence of different ARE solutions were determined by comparing individual treatments to treatment Con, and the impact of OAs (individual and a mixture) were determined by comparing treatment SQ, SL, SM and SQLM to treatment S. Values indicate the number of taxa that responded positively to the presence of ARE solutions, whilst these in brackets indicate the number of taxa which responded negatively to the presence of ARE solutions or compounds. Total detected taxa indicate the number of the total detected taxa in each phylogenetic group across all six treatments. Taxa are shown mainly to phylum level, but where appropriate, some of the phyla are divided into class (indicated by *** and highlighted in light blue) or order (indicated by ## and highlighted in yellow) level.

Phylum/classes/orders	Compared to Con					Compared to S				Total detected taxa
	S	SQLM	SQ	SL	SM	SQLM	SQ	SL	SM	
Acidobacteria	3 (1)	27 (1)	29	11	32 (1)	25 (1)	26	10 (1)	31 (2)	39
Actinobacteria	69	202 (4)	198 (4)	172 (4)	212 (8)	138 (3)	133 (3)	108	147 (29)	247
***Actinobacteria	69	202 (4)	197 (4)	172 (4)	212 (7)	138 (3)	132 (3)	108	147 (28)	245
##Actinomycetales	63	180	174	156	186 (3)	121 (1)	114 (2)	98	127 (24)	211
##Bifidobacteriales	6	7	7	6	7	6	6	5	6	7
##Others	0	15 (4)	16 (4)	10 (4)	19 (4)	11 (2)	12 (1)	5	4 (4)	27
***Others	0	0	1	0	0 (1)	0	1	0	0 (1)	2
AD3	0	1	1	1	1	1	1	1	1	1
Aquificae	0	1	2	1	1	1	2	1	1	2
Bacteroidetes	1	15	23	28	32	14	22	17	31	37
***Bacteroidetes	0	7	8	7	10	7	8	7	10	14
***Sphingobacteria	0	7	13	7	16	7	13	7	16	17
***Others	1	1	2	4	6	0	1	3	5	6
BRC1	0	2	1	1	2	2	1	1	2	2
Caldithrix	0	1	1	0	2	1	1	0	2	2
Chlamydiae	0	1	1	2	1	1	1	2	1	2
Chlorobi	0	2	2	0	3	2	2	0	3	3
Chloroflexi	3	14 (3)	18 (3)	13 (3)	23 (4)	12 (1)	15 (1)	10 (1)	20 (6)	28
Coprothermobacteria	0	0	1	1	1	0	1	1	1	1
Cyanobacteria	1	8	13	6	35 (1)	7	12	5	34 (1)	36
Deinococcus-Thermus	0	2	2	2	2	2	2	2	2	2
DSS1	0	1	1	1	1	1	1	1	1	1

Table 5.5- continued

Firmicutes	0 (1)	120 (1)	124 (1)	38 (1)	241 (1)	120	123	38	241	250
***Bacilli	0	58	59	8	106	58	59	8	106	107
***Clostridia	0	49	49	23	112	49	49	23	112	117
***Others	0 (1)	13 (1)	16 (1)	7 (1)	23 (1)	13	15	7	23	26
Gemmatimonadetes	0	5	4	2	4	5	4	2	4	7
LD1PA group	0	0	1	0	1	0	1	0	1	1
Lentisphaerae	0	1	2	1	3	1	2	1	3	3
marine group A	1	1	1	1	1	0	0	0	0	1
Natronoanaerobium	0	3	3	1	3	3	3	0 (1)	3	3
Nitrospira	1	1	2	1	2	0	1	2	1	3
OD1	0	1	1	0	1	1	1	0	1	1
OP10	0	4	4	2	4	4	4	2	4	4
OP3	0	3	2	2	3	3	2	2	3	3
OP8	0	0	0	0	1	0	0	0	1	1
OP9_JS1	0	2	2	2	2	2	2	2	2	2
Planctomycetes	0	5	8	5	8	5	8	5	8	11
Proteobacteria	5 (10)	193 (8)	305 (3)	115 (4)	349 (8)	197 (1)	309 (1)	112 (1)	346 (3)	418
***α-	3 (2)	99 (5)	126 (1)	53 (2)	149 (5)	97	124	51	147 (1)	164
##Azospirillales	0	1	2	1	4	1	2	1	4	4
##Bradyrhizobiales	0	34	36	16	39	34	36	16	39	40
##Caulobacteriales	0	7	7	7	8	7	7	7	8	8
##Rhizobiales	0	15	20	0	31	15	18	1	31	32
##Sphingomonadales	1 (1)	18 (1)	27	15 (1)	25 (1)	18	27	15	25	30
##Others	2 (1)	24 (4)	34 (1)	14 (1)	42 (4)	22	34	11	40 (1)	50
***β-	0	20	51	18	50	20	51	18	50	62
##Burkholderiales	0	10	34	10	33	10	34	10	33	42
##Methylophilales	0	1	1	1	1	1	1	1	1	1
##Nitrosomonadeles	0	6	5	4	6	6	5	4	6	7
##Others	0	3	11	3	10	3	11	3	10	12

Table 5.5- continued

***γ-	0	12 (2)	58 (1)	14 (1)	54 (2)	12 (1)	58 (1)	14 (1)	54 (1)	83
###Pseudomonadale	0	0	5	1	2	0	5	1	2	7
###Others	0	14	54	14	54	13	54	14	53	66
***δ-	2 (1)	38 (1)	51 (1)	16 (1)	65 (1)	37	50	15	64 (1)	75
***ε-	0 (7)	22	18	13	30	29	25	13	30	32
##Campylobacterales	0 (7)	22	18	13	30	29	25	13	30	32
***Others	0	2	1	1	1	2	1	1	1	2
SPAM	1	1 (1)	1	1	1	0 (1)	0	0	0 (1)	2
Spirochaetes	0	7	2	4	21	7	2	4	21	21
Synergistes	0	4	3	0	4	4	3	0	4	4
Termite group 1	0	0	0	0	1	0	0	0	1	1
Thermodesulfobacteria	0	1	1	1	1	1	1	1	1	1
TM7	0	4	4	2	5	4	4	2	5	5
Unclassified	1 (1)	9	16	5	16 (1)	9 (1)	15	4	15 (2)	20
Verrucomicrobia	2	15	13	11	20	13	11	9	18	20
WS3	0	1	1	0	2	1	1	0	2	2
WS5	0	0	1	0	1	0	1	0	1	1
Total dynamic taxa	88 (13)	658 (18)	793 (12)	423 (12)	1043 (24)	587 (8)	718 (5)	345 (4)	963 (44)	1188
% total detected taxa	7.4 (1.1)	55.4 (1.5)	66.8 (1.0)	35.6 (1.0)	87.8 (2.0)	49.4 (0.7)	60.4 (0.4)	29.0 (0.3)	81.1 (3.7)	-

soil (i.e. either were not detected in OA treatments or their HybScore values were significantly reduced in OA treatments compared to those in treatment S) (Table 5.5).

Proteobacteria was one of the most impacted bacterial phyla by OAs in soils and a range of 112 (lactate) to 346 (maleate) taxa from this phylum responded positively to them. These taxa were within all five classes of Proteobacteria (α , β , γ , δ and ϵ) with the maximum number of affected taxa in the α -Proteobacteria. Within this phylum, taxa in Azospirillales, Bradyrhizobiales, Caulobacterales, Rhizobiales, Sphingomonadales from α -Proteobacteria; Burkholderiales and Nitrosomonadeles from β -Proteobacteria responded positively to OAs (especially quinate and maleate). Although only 7 Pseudomonadales taxa in the γ -Proteobacteria were detected across the treatments, they also responded to the presence of OAs in soils but not sugars, with the exception of treatment SQLM (Table 5.5). Firmicutes was another phylum which was significantly influenced by OAs but not by sugars (Table 5.5). The number of taxa in classes of Bacilli and Clostridia which responded positively to maleate were 106 and 112, respectively in the dynamic community comparing treatment SM to treatment S. Although greatly influenced by the presence of sugars in soil, more than 100 extra Actinobacteria taxa were affected by OAs (either individual or a mixture) in soils (Table 5.5). As with sugars, OAs mainly impacted the taxa from the order Actinomycetales. Apart from these three main phyla, some other taxa belonging to soil functionally important groups, such as Bacteroidetes and Sphingobacteria from Bacteroidetes, were also only found in the dynamic communities influenced by OAs but not sugars. The dynamic taxa which responded to OAs also covered some that are commonly present in soil but not well studied groups, such as Planctomycetes (Table 5.5).

Although the majority of bacterial phyla which were affected by all three selected OAs and a mixture of these OAs were common to four dynamic communities of OAs, a few phyla only responded to the presence of a particular OA. For example, taxa in phylum of Caldithrix, Chlorobi, LD1PA group, OD1, Synergistes, WS3 and WS5 responded positively to quinate and maleate but not lactate. One taxon in OP8 (AF419671) and one taxon in Termite group 1 (AB089050) only responded to maleate rather than other OAs or the mixture of three OAs. One taxon in SPAM (AJ519639) responded negatively to maleate in treatment SM but not to other OAs (Table 5.5).

5.3.4.3 Bacterial abundance (HybScore) response to ARE solutions

As much fewer bacterial taxa were detected in treatments Con and S in comparison to those in treatments SQLM, SQ, SL and SM (Figure 5.5), the comparison of abundance of a single bacterial taxon using HybScore value is not appropriate. However, the sums of

the relative overall HybScore values in bacterial domain and in the main phyla (including classes for Proteobacteria) are shown in Table 5.6. The average overall bacterial HybScore value in treatment S increased by 1.5-fold when compared to that of the control. However, in the communities with treatments containing mixtures of OAs and sugars (SQLM, SQ, SL and SM), the average bacterial HybScore values increased more than 5-fold with maximum 9-fold increase compared to that in control, implying greater effects on bacterial populations by the mixtures of sugars and OAs in soils. To eliminate the sugar effects and obtain “pure” OA impacts, the HybScore values in treatment SQLM, SQ, SL and SM were compared to treatment S, respectively. The average bacterial HybScore values increased 3.0-, 3.2-, 2.2- and 3.8-fold by the presence of a mixture of three OAs, quinate, lactate and maleate, respectively (Table 5.6). All of the increases in bacterial abundance induced by OAs were larger than that by sugars (1.5-fold) (Table 5.6).

With the exception of the sugar effects (Treatment S) in Cyanobacteria, Firmicutes and Proteobacteria, all of the examined phyla showed significant increases in HybScore values in treatments containing ARE solutions when compared to the control, and the increase was over 20-fold in the phylum Firmicutes and 28-fold in δ -Proteobacteria by the addition of ARE solution SM (Table 5.6). Both the HybScore values in Firmicutes and Proteobacteria in treatment S were significantly lower ($P < 0.001$) compared to the control, indicating the overall negative effect of sugars on the abundance of these two phyla. Further analysis of the Proteobacteria showed that the reductions of abundance in treatment S were mainly in the class γ -Proteobacteria. The influence of “pure” OAs on bacterial populations which were determined by comparing the HybScore values in treatments SQLM, SQ, SL and SM with those in treatment S, respectively are also shown in Table 5.6. The presence of OAs resulted in large increases of HybScore values in most of the examined phyla, especially Bacteroidetes (13 to 26-fold), Firmicutes (5 to 25-fold) and Proteobacteria (7 to 16-fold) (Table 5.6).

The abundance of Cyanobacteria did not differ significantly between treatment S and the control, but increased significantly (1.7 to 5.0-fold) in the treatments containing mixtures of sugars and OAs when compared to that in the control (Table 5.6). Beta-Proteobacteria were only detected in treatments containing mixtures of OAs and sugars, but not sugars or DI water. These results further indicated the much larger impact on the abundance of these main bacterial groups by OAs than by sugars. In addition, ϵ -proteobacteria were not detected in treatment S, but the HybScore values of this class were increased significantly in the treatments containing mixtures of sugars and OAs, indicating the taxa from this class were affected negatively by sugars but positively by OAs in this experiment (Table 5.6).

Table 5.6 Relative increases (fold) in the abundance of main phylogenetic groups detected by phylochips. The significant changes ($P<0.05$) in the abundance of phylogenetic group taxa between treatments are shown in bold. + indicates the phylogenetic groups which became detectable in the treatments but not in the control (either treatment Con or S); - indicate the phylogenetic groups that became undetectable in the treatments while detected in the control (either treatment Con or treatment S). nc indicates no significant change ($P<0.05$) in the presence or absence of a phylogenetic group. Proteobacteria phylum is divided into class (indicated by *** and highlighted).

Phylum/classes	Compared to treatment Con					Compared to treatment S			
	S	SQLM	SQ	SL	SM	SQLM	SQ	SL	SM
Bacteria	2.47	7.43	7.91	5.35	9.38	3.01	3.21	2.17	3.80
Acidobacteria	2.00	9.40	9.40	4.61	9.70	4.71	4.71	2.31	4.86
Actinobacteria	5.04	9.19	7.47	7.54	6.79	1.83	1.48	1.50	1.35
Bacteroidetes	+	+	+	+	+	12.89	16.83	14.68	26.24
Cyanobacteria	0.95	2.09	2.73	1.70	4.97	2.21	2.89	1.80	5.25
Firmicutes	0.83	9.74	9.44	3.91	20.38	11.79	11.43	4.74	24.66
Proteobacteria	0.79	7.91	12.64	5.66	12.89	9.97	15.93	7.14	16.25
***α-	0.99	7.28	9.84	5.30	10.82	7.38	9.98	5.37	10.98
***β-	nc	+	+	+	+	+	+	+	+
***δ-	1.74	18.81	25.13	9.25	28.46	10.79	14.41	5.31	16.32
***ε-	-	4.90	4.24	2.56	5.93	+	+	+	+
***γ-	0.77	3.20	15.04	4.32	11.89	4.17	19.60	5.63	15.50

5.4 Discussion

A simplified microcosm experiment was carried out to investigate the potential impact of radiata pine on rhizosphere microbial communities through low molecular weight C compounds (i.e. OAs and sugars) in root exudates. Although several microcosm studies have reported the influence of ARE solutions on soil microbial communities (Baudoin et al. 2003; Paterson et al. 2007), this is, to my knowledge, the first study to use ARE solutions based on root exudates from forest trees. Moreover, through analysis of extracted RNA the current study targeted the metabolically active microorganisms response to ARE solutions added to soils, while most other studies reported in the literature examined dominant microorganisms based on DNA samples (Baudoin et al. 2003; Landi et al. 2006) which may not substantially reflect the microbial community response to ARE solutions (Chapter 2, Section 2.1.4).

5.4.1 Impact of ARE Solutions on Soil Microbial Activity and Diversity

Clear effects of the various ARE solutions on soil dehydrogenase activity were observed in this study. With the exception of treatment SM, dehydrogenase activity increased in all the treatments. Increases were likely due to the enhanced microbial activity and microbial growth during the mineralization of these additional C compounds. Several other studies have also reported increases in various soil enzyme activities, including dehydrogenase,

fluorescein diacetate hydrolysis, nitrate reductase, denitrifying enzyme, phosphatase, urease and protease, after the addition of ARE solutions to soil (Dilly and Nannipieri 2001; Henry et al. 2008; Renella et al. 2007; Watkins et al. 2009; Zantua and Bremner 1976). Surprisingly, the dehydrogenase activity in soils amended with ARE solutions containing a mixture of sugars and maleate decreased significantly when compared to the control in the current study. Although Renella et al. (2007) reported that the stimulation of enzyme activity depended on the addition of substrates and soil types, the decrease of soil enzyme activity with the addition of C source has not been reported previously. As an indicator of general microbial activity, archaea, bacteria, fungi and algae in the soil could all contribute to the dehydrogenase activity (Herman and Maier 2000). In the current experiment, the reduced enzyme activity could be explained, at least in part, by the reduced archaeal population in treatment SM which was indicated by the failure to amplify archaeal 16S from cDNA samples derived from treatment SM, while samples from all other treatments showed positive amplification (Appendix 5.2). It would be useful to specifically test the relative contribution of archaea to the total dehydrogenase activity in soil. In addition, the effect of the ARE solution SM on other soil enzymes could also be tested.

Although microbial biomass was not measured in this study, an overall bacterial abundance indicated by HybScore values showed substantial increase in soils amended with different ARE solutions. The increases in abundance were also found in most of the main bacterial phyla in all the treatments compared to the control in the current study. Increases in populations of culturable bacteria in response to ARE have also been reported by Baudoin et al (2003), where culturable bacterial densities increased by 1.5 log CFU g⁻¹ dry soil with addition of ARE to soil. Kozdrój et al. (2000) also showed increases in culturable bacterial cell counts after addition of ARE into soil polluted with heavy metal. Renella et al (2007) reported increased double stranded DNA content in the rhizosphere layer of soil amended with low molecular weight organic compounds, indicating the induction of microbial growth. All of these results demonstrate the role of ARE in bacterial proliferation in soil.

A number of studies have reported that the addition of ARE solutions results in a restructuring of microbial communities in the soil (Baudoin et al. 2003; Henry et al. 2008; Landi et al. 2006; Paterson et al. 2007). For example, Kozdrój et al. (2000) showed that addition of an ARE solution based on the sugars, OAs and amino acids of maize exudates into the soil generated a different DGGE bacterial profile compared to that in the control soil. Griffiths et al. (1999) observed that the structure of soil microbial communities changed significantly when C compounds were loaded to soil at rates equal to or over 125

$\mu\text{g C g}^{-1} \text{ d}^{-1}$. Similarly, soils with the daily addition of ARE solutions for 15 days in the current study generated different bacterial and fungal communities in comparison to those in the control soil which were amended with sterile DI water.

Furthermore, ARE solutions with different compositions led to distinct bacterial profiles across treatments, indicating a differing effect of various ARE solutions on bacteria in soils. The different bacterial communities in each treatment observed by rRNA-DGGE gels was further confirmed by phylochip analysis, with the exception of treatment QLM which was not included in the phylochip analysis due to practical reasons. Although not as distinct as bacterial communities, most of the treatments also showed significant differences in fungal communities on DGGE, implying that the influences of the ARE solutions on fungal communities were also treatment-specific. Some of the differences in microbial communities could possibly be attributed to the different amounts of C (ranged from 100 to 300 $\mu\text{g C g}^{-1}$ dry soil) added in the treatments daily in the current experiment. Griffiths et al. (1999) showed that microbial community structures changed significantly when the same ARE solutions were added to soil at different rates. However, differences detected in the current study were more likely to be related to the composition of ARE solutions added into soils, since the microbial communities among the treatments SQLM, SQ, SL and SM with the same amount of C input also demonstrated different communities. Baudoin et al. (2003) amended soils with two types of ARE solutions which differed in C to nitrogen ratio and these resulted in distinct soil bacterial fingerprints. Henry et al. (2008) found that the soil nitrate reducer and denitrificater communities, as analysed by clone libraries, were similarly affected by the composition of applied ARE solutions. A clear separation of microbial phospholipid fatty acid profiles in soils amended with glucose, fumarate and glycine, respectively was also observed by Paterson et al. (2007).

A diverse range of bacterial taxa responded significantly to the presence of ARE solutions in soils, as revealed by phylochip analysis. Across all the treatments, up to 90% of the total detected bacterial taxa from 38 phyla were dynamic, either becoming detectable or undetectable by phylochip or by a significant change in the HybScore values in soil bacterial communities, after the addition of ARE solutions. DeAngelis et al. (2008) reported 147 dynamic bacteria taxa (7% of the total detected taxa) in rhizosphere soil significantly responded to the presence of wild oat (*Avena fatua*) roots. The difference in the proportion of dynamic taxa found in their study and the current study may be due to the different experiment design (i.e. *in vivo* wild oat rhizosphere environment and *in vitro* microcosm design with ARE solutions) with different experiment objectives. The higher proportion of dynamic taxa in the detected bacterial communities in the current microcosm study may also be due to the absence of plant root factors *in vitro*. The colonization by

rhizosphere microbes of plant roots may be restricted physically (limited root surface to be colonized on) and/or chemically (competition of nutrients with soil microbes) *in vivo* (Baudoin et al. 2003). The greater impact of ARE solutions on soil microbial communities *in vitro* than *in vivo* was supported by the study carried out by Baudoin et al. (2003) which showed that culturable bacterial populations were significantly increased *in situ* by addition of ARE solutions to maize seedlings, but to a lesser extent than had been found in an *in vitro* experiment where the same ARE solutions were added to soils.

It should be noted that while some bacterial taxa respond to the presence of ARE compounds in soil, this does not necessarily indicate that they are the primary utilizers of these compounds. By using stable isotope labelled plants, Haichar et al. (2008) provided evidence that some microorganisms assimilated root exudate compounds directly, whereas others benefited from refractory SOM partially solubilised by enzymes produced by specialised microorganisms (known as a “priming effect”). Some members of Caulobacterales and Rhizobiales in α -Proteobacteria (such as *Brevundimonas*), of Xanthomonadales in γ -Proteobacteria (such as *Rhodanobacter* and *Stenotrophomonas*) and of Actinomycetales in Actinobacteria, which were all detected in the current study, may use ARE compounds as energy sources and then produce SOM solubilising enzymes, enabling the growth of other microorganisms by using biologically available SOM (Haichar et al. 2008; Horwath 2007). Therefore, the diverse range of impacted bacterial taxa by the presence of ARE solutions may also be from the indirect effect of ARE compounds.

The majority of the bacterial taxa were positively stimulated by the addition of ARE solutions to soils. However, a small percentage of taxa were also impacted negatively. This negative response may result from direct inhibition of microorganisms by the presence of particular ARE compounds, but is more likely due to the competition for C source between soil microorganisms or changes in soil environmental factors. To effectively compete for the C source, active microorganisms in soil may use strategies such as production of antibiotics to inhibit growth of other microorganisms (Atlas and Bartha 1993). In addition, environmental factors, such as soil moisture and pH, could also affect microbial competition (Paterson et al. 2007). In the current study, soil moisture was maintained at constant levels throughout the experiment. In contrast, at harvest, the pH of the soils amended with ARE solutions containing OAs (either mixtures of OAs and sugars or a mixture of three OAs) increased significantly compared to the pH of the control soil. Conversely, the presence of sugars alone in treatment S did not change the pH compared with the control. Shifts in pH have been similarly reported in other studies where OAs have been applied to soil (Evangelou et al. 2008; Gramss et al. 2004). For example,

Evangelou et al. (2008) found that pH of their experimental soil amended with three OAs (citric, tartaric and oxalic acids) increased from 5.5 to 7.7 within 4 days and remained high over the 10 days of their experiment. Acidification of soil was only observed on the day of addition of OAs (soil pH at 6.8) (Evangelou et al. 2008). The increase in pH may have resulted from the microbial degradation of carboxylic acids where H^+ is consumed and OH^- and CO_2 liberated (Gramss et al. 2004; Gramss et al. 2003). The variable soil pH values observed at the end of the current experiment was likely due to microbial utilisation of the ARE compounds. This may indicate that various C compounds enriched for particular microbial communities and these microorganisms then generated favourable environments (such as optimal pH) to inhibit or depress the growth of other microorganisms.

5.4.2 Comparison effects of OAs and Sugars on Bacterial Communities

Although applied in mixtures, the effects of OAs on soil microbial communities were examined by comparing the communities in soils amended with ARE solutions containing mixtures of sugars and OAs to these in the treatment S to “remove” the effect caused by the sugars in the mixtures. However, the effect of OAs mentioned in this study may also result from the interactions between selected OAs and sugars. Nonetheless, the presence of OAs caused greater impacts on soil bacterial communities compared to sugars. This was reflected in both the number of affected bacterial taxa and the bacterial abundance as shown by HybScore values. This conclusion is strongly supported by the consistent trends obtained in each of the three individual OAs and the mixture of these three OAs, which may imply that the greater influence of OAs on bacterial communities was commonly present rather than related to a particular OA. In addition, these results may suggest that OAs are a biologically important component in root exudates of radiata pine trees, in addition to being the most quantitatively important component, as recognised by Smith (1976). The greater impacts of OAs in shaping soil bacterial communities in comparison to sugars have also been reported by others. Falchini et al. (2003) showed that DGGE profiles of bacterial communities in “rhizosphere soils” amended with oxalic and glutamic acids, respectively were changed compared to the control but not in the “rhizosphere soil” amended with glucose. Landi et al. (2006) also found that glucose induced fewer changes in the bacterial community than oxalic acid. The authors explained this might due to the fact that glucose could be used by a large proportion of soil microorganisms, whereas oxalic acid was decomposed by specialized microorganisms. However, in the current study with a 15-day experimental duration, a diverse range of microorganisms responded positively to the presence of ARE solutions SQ, SL, SM and SQLM and it did not appear that particular groups of bacteria were selected for (Figure 5.6).

In the current study, this result might be partly explained by the addition of higher amounts of C content in forms of the OAs compared to sugars, as Griffiths et al. (1999) have reported that the gradient of changes in microbial communities compared to control soils increased with greater C input. However, this was not the entire cause for the greater impact of OAs on bacterial communities, as three different OAs selected in the current study also showed a different extent of influence on the bacterial communities and impacted on different bacterial taxa. The application of OAs into soil could contribute to the solubilisation of SOM (Jones 1998; Kumar et al. 2006; Kuzyakov 2002) and as a consequence, a wide range of microorganisms may use the biologically available SOM for growth. This indirect impact of OAs on soil bacteria may further indicate the important role of OAs in the rhizosphere of radiata pines. In addition, the significant change in soil pH in treatments containing OAs compared to control may also have contributed to the larger shifts in the bacterial community caused by OAs than sugars, although as discussed above, this pH shift was likely the result of microbial utilization of OA compounds (Gramss et al. 2004; Gramss et al. 2003) rather than an initial driver of community change (see Section 5.4.1). Nonetheless, the pH could have played a role, particularly in maintaining the microbial communities in soil (Marschner et al. 2004; Wakelin et al. 2008).

5.4.3 Impact of Exudate Compounds on Specific Bacterial taxa

Particularly useful information provided by the phylochip analysis is the information on specific taxa within bacterial communities. In the current study, Proteobacteria, Actinobacteria and Firmicutes were three main phyla detected in bacterial communities in all the treatments. More than 60% of the taxa which responded positively to the addition of sugars in soil (Treatment S) belonged to Actinobacteria. Apart from Actinobacteria, Firmicutes and Proteobacteria were both highly responsive to the addition of OAs, followed by Acidobacteria and Verrucomicrobia. The common presence of these three phyla was consistent with numerous rhizosphere community studies with forest trees (Andreote et al. 2009; Chow et al. 2002; Filion et al. 2004) and other plants (DeAngelis et al. 2008; McCaig et al. 1999; Micallef et al. 2009; Sharma et al. 2005). The recently recognized group Acidobacteria was also frequently detected in rhizosphere soils (Chow et al. 2002; Filion et al. 2004; Sharma et al. 2005). Verrucomicrobia are not easily cultured in the laboratory (Sangwan et al. 2005), but culture-independent analyses revealed that this group is present in many soils as well as rhizosphere soils (Chow et al. 2002; DeAngelis et al. 2008; McCaig et al. 1999; Sharma et al. 2005; Ulrich and Becker 2006). Although knowledge of Acidobacteria and Verrucomicrobia groups is limited (Sanguin et al. 2006), the ubiquity of both groups in rhizosphere soils may indicate that they have functionally important roles in the ecology of rhizosphere processes. The high proportion

of Gram-positive bacteria (such as Actinobacteria and Firmicutes) detected in the dynamic communities in the current study further supports the suggestion by Smalla et al. (2001) that Gram-positive bacteria might be more dominant in the rhizosphere than previously supposed. Although the current study was conducted *in vitro*, the fact that the detected dominant bacteria phyla in the current experiment were highly similar to other rhizosphere studies increases the confidence of using the simplified microcosm experiment to study the impacts of these exudate compounds on rhizosphere microbial communities. Furthermore, it provides useful information on potential plant mechanisms for control of soil microbial diversity and function.

Pseudomonadales in γ -Proteobacteria have long been considered as important rhizosphere colonizers which respond quickly to exudates (Curl and Truelove 1986; Sørensen et al. 2001; Watt et al. 2006a). For example, 11 out of 67 clones from perennial ryegrass and white clover (*Trifolium repens*) rhizosphere soils were dominated by *Pseudomonas* (Marilley and Aragno 1999). However, only seven Pseudomonadales out of 1188 total bacterial taxa were detected by phylochip analysis in the current study. This result is in accordance with the results obtained by Chow et al. (2002), in which only six of the 709 clones constructed from a rhizosphere 16S rRNA library of lodgepole pine were *Pseudomonas*. Infrequent detection of Pseudomonadales was also reported in the rhizosphere of other plants, such as wild oat (DeAngelis et al. 2008), pasture (dominated by *Agrostis capillaris* and *Festuca ovina*) (McCaig et al. 1999) and *Arabidopsis* (Micallef et al. 2009). Taken together, these studies imply that the importance and abundance of Pseudomonadales in rhizosphere soils maybe overestimated by culture-based approaches and well-studied biocontrol and pathogenic pseudomonadales taxa.

Many of the bacterial taxa which responded positively to the ARE solutions (especially OAs) were related to bacterial genera and species known for their beneficial effects on direct plant growth (“biofertilizing” effect) and/or indirect protection against pathogens (“biocontrol” effect). Various groups of bacteria detected in the current study could contribute to the biogeochemical cycling of nitrogen. Those beneficial bacteria included nitrogen fixers (such as Azospirillales, Bradyrhizobiales and Rhizobiales in α -Proteobacteria (Mantelin and Touraine 2004), Burkholderia in β -Proteobacteria (Gillis et al. 1995; Santos et al. 2001), *Frankia* in Actinomycetales (Vergnaud et al. 1987), Cyanobacteria (Poly et al. 2001), Clostridia in Firmicutes (Sharma et al. 2005)), ammonia-oxidizing bacteria (Nitrosomonadeles in β -Proteobacteria, Nitrosococcus in γ -Proteobacteria (Purkhold et al. 2000)), and nitrite-oxidizing bacteria (Nitrospira (Ehrich et al. 1995; Marilley and Aragno 1999)). Some members of Bacilli, Rhizobiales and Actinomycetales have the ability to solubilise phosphate in soil (Richardson et al. 2009). In

addition, *Chlorobium limicola*, *Chlorobium ferrooxidans* in Chlorobi phylum and *Acidovorax* in Burkholderiales which were detected in the current study could contribute to the cycling of sulphur. Phytohormones produced by plant growth promoting microorganisms could enhance root growth and/or plant growth. For example, *Paenibacillus polymyxa* (within class of Bacilli) detected in this study has been shown to produce cytokinins which can stimulate plant cell division, control root meristem differentiation and promote root hair development (Richardson et al. 2009; Timmusk et al. 1999). Production of gibberellin, which can promote root elongation and lateral root extension and enhance the development of plant tissues, have been documented in several soil microorganisms, including *Bacillus pumilus*, *Bacillus licheniformis* and *Gluconobacter diazotrophicus* (Bottini et al. 2004; Gutiérrez-Mañero et al. 2001), all of which were detected in the current study. Some other microorganisms detected in the current study are related to species known to have antagonistic activities against plant phytopathogens. For instance, some strains of *Stenotrophomonas* sp. (belonging to γ -Proteobacteria) are able to produce antifungal compounds (Berg et al. 1996). Several members of Bacilli, such as *Bacillus cereus* and *Bacillus subtilis*, *Bacillus thuringiensis*, have been developed as biocontrol agents due to their antagonistic activities against soil-borne pathogens and pests (Emmert and Handelsman 1999). Some Actinomycetes are able to protect plants from fungal pathogen through various mechanisms, including the production of enzymes to degrade fungal pathogen cell walls (El-Tarabily and Sivasithamparam 2006; Sharma et al. 2005). For example, *Streptomyces* sp. GN 4-2 has been shown to suppress root and butt rot disease caused by *Heterobasidion* in Norway spruce seedlings (Lehr et al. 2008). The diversity and abundance of these potentially beneficial bacteria stimulated by the presence of ARE solutions (especially by OAs) in the soil strongly suggest the possibility of selection for beneficial bacteria by plants through root exudate compounds.

However, other bacteria previously known as pathogens of plants, such as some members of the Enterobacteriaceae within γ -Proteobacteria and *Stenotrophomonas maltophilia* in Xanthomonadales of γ -Proteobacteria, some species of *Burkholderia* in β -Proteobacteria and Clostridia in Firmicutes (Berg et al. 2005; Micallef et al. 2009; Raaijmakers et al. 2009), were also detected in the dynamic bacterial communities in soils amended with ARE solutions. It was not possible in the current study to determine whether their activity in soil was affected directly by the ARE compounds or indirectly through other interactions. The detection of potentially pathogenic bacterial species in the rhizosphere soils has also been reported by others (Haichar et al. 2008; Micallef et al. 2009; Raaijmakers et al. 2009). Collectively, the results indicate that root exudate

compounds in soils could stimulate both potentially beneficial and pathogenic bacteria in the rhizosphere soil.

5.5 Conclusions

Artificial root exudates composed of low molecular weight sugars (glucose, sucrose and fructose) and OAs (quinic, lactate and maleate), the selection of which was based on radiata pine root exudates, were shown to significantly influence the soil microbial activity (measured by dehydrogenase activity) and the structure of active soil microbial communities (both bacteria and fungi). Different ARE solutions resulted in distinct soil microbial community structures. The addition of ARE solutions containing OAs also resulted in the increase in the soil pH by 1 to 3 units. This change in soil pH may have resulted from microbial utilization of OA compounds and thus may further affect the composition of soil microbial communities. Such change may help to maintain the activities of microorganisms in a more favorable pH environment and through interactions and competitions inhibit the growth of other microorganisms.

As shown by phylochip analysis, wide ranges of bacterial taxa were affected by the addition of ARE compounds to soil, especially by OAs. Compared to sugars, OAs induced greater impacts on the soil bacterial communities, both in the number of affected bacterial taxa and in the abundance of bacteria as shown by HybScore values. All three selected OAs and a mixture of these three OAs greatly impacted soil bacterial communities. These results indicate the biological importance of OAs in root exudates of radiata pine. Detailed analysis of affected taxa revealed that both potentially beneficial and pathogenic bacteria responded to the ARE solutions (especially OAs) in soil. Further work to validate such changes in the rhizosphere of pine roots *in situ* is required.

Chapter 6 General Discussion and Future Research

6.1 General Discussion

This project was undertaken to investigate the influence of root exudates (particularly OAs) on rhizosphere soil microbial communities. Radiata pine was chosen as a model plant for this project and the impact of genetic modification of radiata pine was assessed. Due to the limitations of currently available techniques, a new approach was first developed for *in situ* collection of OAs in root exudates of radiata pine grown in a large-scale rhizotron system (Chapter 3). This approach was subsequently used in characterisation of the rhizosphere microbial communities associated with GM and unmodified *Pinus radiata* and to investigate possible links between root exudates and the structure of the active rhizosphere bacterial community (Chapter 4). Selected OAs and sugars shown to be present in radiata pine root exudates were further investigated for their impact on diversity and activity of soil microbial communities (Chapter 5). The main findings are discussed below.

6.1.1 Rhizotron System and *in situ* Sampling Technique

The large-scale rhizotron used in this study provides a novel system for collection of data on root exudates and rhizosphere microbial communities in a setting that closely approximates the field situation. The large volume of the rhizotron allows soil profiles (various horizons and bulk densities) to be packed to closely simulate natural conditions and gives plant roots a reasonable growth space. Unlike most of the small sized rhizobox systems, this system is also suitable for studying tree species with large root systems and for plants to be grown for long periods, so more mature plants can be sampled instead of seedlings. Access portals at various depths around the rhizotron enabled observation and study of root systems. This design overcomes the limitation of a field rhizotron (root window) in which only roots grown along the glass plate (window) on one surface could be studied (McMichael and Zak 2006). Compared to minirhizotron systems, the ability to remove tubes from the access portals provides improved access to the rhizosphere area and enables observation and sampling of roots and rhizosphere soil. In addition, the inflatable tubes effectively reduced the air gap at the soil-tube interface in the rhizotron, which can lead to unnatural root distribution (McMichael and Zak 2006; Taylor et al. 1990). Radiata pine trees grown in the rhizotron system in two experiments in this study (Chapters 3 and 4) had similar root morphology to field-grown pines.

This rhizotron system together with the AEM strip backed with moistened Whatman 3MM filter paper enabled *in situ* collection of OAs from the rhizosphere of plants grown in the

Biotron. A range of OAs (tartarate, quinate, formate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate) was collected by AEM strips from the exudates of radiata pine. The use of an endoscope system enabled precise collection of root exudates (Chapter 4). This *in situ* collection technique could also be used for temporal and spatial studies of OA exudation by plant roots (Appendix 4.3) which have been recognized as being important for understanding rhizosphere processes (Hinsinger et al. 2009).

The collection and analyses of OAs in root exudates and rhizosphere soil microbial communities in the corresponding samples *in situ* could be achieved using the techniques described in Chapter 4. Unlike a recent study conducted by Micallef et al. (2009) where exudates of *Arabidopsis thaliana* were collected from hydroponic-grown plants while bacterial communities associated with *A. thaliana* were analysed from soil-grown plants, the technique used in this study ensured that root exudate samples and rhizosphere soil microbial community samples were collected from the same sites in the heterogeneous rhizosphere environment. This sampling technique also minimises root damage compared to the one described by Weisskopf et al. (2008) which involved physically rubbing rhizosphere soil from extracted roots for both OA extraction and microbial analysis.

It proved to be very challenging to relate changes in amounts and composition of exudates to microbial communities in the rhizosphere due to the natural variability of root exudates (Chapter 4). This variability in rates and composition of exudates has also been noted in other studies that used *in situ* sampling techniques, as root exudates are influenced by numerous factors, both biological and environmental, interacting in the heterogeneous rhizosphere environment (Chapter 1, Section 1.1.2). This further illustrates that OAs, or more general root exudates, are an important regulator in which plants respond to the surrounding environment (e.g. nutrient availability, toxic metal, pH) and mediate accordingly to maintain their favourable rhizosphere environment (e.g. available of nutrients, colonization of beneficial microorganisms). That so many factors influence root exudates make rhizosphere studies very challenging and emphasises the importance of understanding the spatial and temporal variability in rhizosphere processes. These factors can be studied in the rhizotron system together with *in situ* sampling technique developed in this study.

The potential values of the rhizotron system and the *in situ* sampling technique to rhizosphere research have been demonstrated in this study. This approach could be used for a range of rhizosphere studies. For instance, the dynamics of root growth and changes in root morphology according to the environment could be examined through the access portals using a camera system. The spatial and temporal variability of many parameters

(OAs in root exudates, microbial communities, nutrients, soil pH, redox conditions, moisture, etc.) in the rhizosphere environment could also be investigated via the access portals using other techniques (*in situ* exudate collection techniques, visualization techniques (e.g. dye reactions with nutrients) and reporter gene techniques as recently reviewed by Neumann et al. (2009)). It is possible that multiple measurements (e.g. soil physicochemical properties, root morphology, root physiological condition, root exudates, microbial community) in the rhizosphere could be determined *in situ* using the rhizotron system with various techniques, and then dynamics in the rhizosphere could be modeled. The system used in this study provides experimental conditions that lie between artificial laboratory systems and natural complex conditions in the field. This enables the study of particular processes to be conducted in simplified (e.g. consistent temperature, light density) but realistic controlled conditions (large soil volume, vertical soil temperature profiles, etc.). The results obtained may provide useful information to link studies conducted in the laboratory to the research carried out in the field.

6.1.2 Characterisation of Soil Microbial Communities and Root Exudates Associated with GM and WT Radiata Pine

Some significant differences between active rhizosphere and non-rhizosphere microbial communities associated with GM and WT pine trees were detected using DGGE technique, although there were no consistent trends over the duration of the experiment (Chapter 4). There was no clear difference in OAs in root exudates of GM pines in comparison with WT pines due to the natural variability between replicates which were present in both the exudate samples from the two tree lines. Results suggest that this genetic modification had little impact on rhizosphere microbial communities. However, these results cannot be extrapolated to reach general conclusions about the biosafety of GM radiata pine as only one type of soil and one GM line were used and the experiment was conducted under one seasonal setting for only 10 months which was a short period within a pine life cycle.

6.1.3 Impact of Root exudate Components on Soil Microbial Communities

Because of the natural variability observed in root exudates, correlations between OAs in root exudates and microbial diversity could not be undertaken (Chapter 4). However, the transient differences in microbial communities may be caused by changes in OAs (composition and/or quantity) in root exudates of radiata pine. Accordingly, an *in vitro* microcosm experiment was designed to investigate selected root exudate components of radiata pine on diversity and activity of soil microbial community in a relatively stable system. As OAs are the predominant component in root exudates of forest trees, OAs

could be expected to play an important ecological role in the rhizosphere environment. In order to have a relative comparison, sugars, which are the most commonly present component in root exudates of plants and a widely studied exudate component, were also used in this study.

The large influence of selected low molecular weight OAs and sugars on soil microbial communities has been shown in the microcosm experiment (Chapter 5) with selected OAs (quininate, lactate and maleate) and sugars (glucose, sucrose and fructose) identified as components of root exudates of radiata pine trees. The addition of artificial root exudate solutions resulted in the stimulation of microbial activity (measured by dehydrogenase activity) to different extents (1.6 to 3.5-fold), with the exception of soils amended with a mixture containing sugars and maleate in which the dehydrogenase activity decreased significantly. It would be useful to test the effect of these ARE solutions on other soil enzymes, such as hydrolases. As hydrolytic enzymes (e.g. β -glucosidase, phosphatase, urease, arylsulfatase) involved in the cycling of principal nutrients (e.g. C, P, nitrogen and sulphur) in soil are crucial for plant nutrition (Nannipieri et al. 2003; Tejada et al. 2006), the measuring of these enzymes could provide further information in regard to the beneficial interactions of microorganisms in the rhizosphere. The structures of soil microbial communities (both general bacterial 16S and fungal 18S) in soils amended with ARE solutions were also significantly different from those in control soils, indicating that ARE have a strong influence on soil microbial communities. In addition, different ARE solutions resulted in development of distinct microbial communities, suggesting that different compounds could affect specific soil microorganisms.

The important biological role of OAs in shaping the rhizosphere soil microbial community of radiata pine was indicated by the results obtained in Chapter 5. Organic anions consistently demonstrated a greater influence on active soil microbial communities than sugars alone. This influence has been shown in both the numbers of bacteria taxa which were significantly affected by the presence of exudate compounds as well as the abundance of bacteria or specific phylogenetic groups of bacteria as indicated by HybScore values. The positive impacts on bacterial communities by root exudate compounds may be the result of direct utilisation of ARE compounds or bacteria may have benefited indirectly through solubilisation of soil organic matter by enzymes produced by primary utilizers of these compounds or by application of OAs into soils. On the other hand, a few taxa (up to 24) responded negatively to ARE solutions applied to soils. This may be due to the direct inhibition of particular taxa by the compounds, but it was more likely that these taxa were outcompeted by rapid growth of other microorganisms better able to access limited resources available in soil. Alternatively, taxa which responded

negatively to soil amendment may have been reacting to changes in environmental conditions, in particular pH which was shown to change significantly in most of treatments. Either directly or indirectly, ARE compounds, especially OAs, greatly influenced soil microbial communities.

A diverse range of microorganisms responded positively to the presence of ARE compounds (especially OAs) in soils, including various potentially beneficial bacteria and some potential plant pathogens. Some of these beneficial bacteria have important roles in the biogeochemical cycling of nutrients (e.g. nitrogen and P), or can produce phytohormones to enhance plant growth, or protect against soil-borne plant pathogens. The results may suggest that radiata pine is able to select a diverse range of potential beneficial bacteria through root exudates. Further study of the influence of root exudates on functional microbial groups or functional genes (e.g. relating to nutrient cycling, plant disease protection) would reveal the mechanisms of how plants regulate the rhizosphere microbial community for their benefit.

The microcosm experiment was carried out in the absence of plant roots, so it is difficult to predict how these exudate components would influence rhizosphere microbial communities *in situ*. For example, plants may compete for nutrients (i.e. P, nitrogen) with soil microbes and limited root surface is available for microbes to colonize in the narrow rhizosphere zone. Therefore, the findings will have to be verified *in situ* or in more complex experimental systems. However, the results obtained from microcosm system are still valuable. In fact, the dominant bacterial phyla detected in the microcosm experiment were in agreement with other *in situ* rhizosphere studies in the literature indicating the suitability of using this type of microcosm system for research. The changes of pH values in microcosm soils is consistent with the fact that the pH of rhizosphere soil can be significantly modified by activity of plant roots (Hinsinger et al. 2009). For example, when plants are grown in acid soils and depending on nitrogen source (i.e. NO_3^- , NH_4^+) a more alkaline rhizosphere has been observed (A. Richardson, pers. comm.; Marschner and Römheld 1983). This is in line with the increase in pH values in most of treatments using acid soil (original soil pH at 5.6) observed in the microcosm experiment. Based on these similarities between the *in vitro* microcosm and the *in situ* rhizosphere study (dominant bacteria species, pH), it can be expected that similar microbial community shifts will occur *in vivo* as observed *in vitro*, although this remains to be tested.

6.1.4 Organic Anions and Microbial Communities

Quinate, citrate and malonate were not detected in the exudate samples collected from radiata pines grown in the rhizotron unit in Chapter 3. However, all those anions were detected in the experiment as described in Chapter 4, although citrate and malonate were not detected in the root exudate samples collected on the firsting sampling period. The difference in detection of anions may due to the different soils used in the experiments, but more likely due to the spatial and temporal variabilities in the rhizosphere environemnt. The large natural varaibility of OAs in radiata pine exudates were detected in both rhizotron experiments (Chapter 3 and 4) further confirming that the commonly presence of the variability in the rhizosphere environment and it is not soil type-related. The direct comparison of DGGE profiles of soil microbial communities from experiments described in Chapter 3, 4 and 5 were not easy due to the use of different soils, trees and sampling techniques. However, it would be especially useful to further comparison the DGGE profiles and dominant species in the rhizosphere microbial communities *in situ* (Chapter 3 and 4) with the communities *in vitro* in the micrososome systems (Chapter 5). However, due to the numerous bands present in general bacterial and fungal DGGE profiles and the large variations between the replicates of *in situ* communities, the comparison was not conducted.

6.2 Future Research

Based on the findings of this study, herewith some specific recommendations for future research:

1. The significant impact of ARE compounds on active soil microbial communities should be further assessed *in situ* or in more complex systems. A possible experiment design could include application of artificial root exudate solutions into i). the soil with pine seedlings grown in pots, ii). soils in pots without seedlings, and the application of DI water as controls to soil with or without pine seedlings. The resulting analysis could include the structure of active microbial communities as well as detection of functional genes (e.g. functional gene microarray, nutrient cycling relating genes). In addition, microbial activity (e.g. hydrolase and oxidoreductase) analysis will also provide useful information on the overall microbial function on the cycling of principle nutrients.
2. In this study, the analysis of the fungal communities remained at the structural level (DGGE). Construction of a fungal clone library or sequencing of particular fungal bands on DGGE gels will reveal detailed information on species which are positively or negatively influenced by the presence of exudate compounds. In addition, further

study should also investigate the influence of exudates on ectomycorrhizal community/species which are important symbiotic partners of radiata pine.

3. Because of the highly complex rhizosphere environment, the analysis of microbial communities using the higher resolution phylochip technique could result in a better understanding of the *in situ* rhizosphere microbial communities of radiata pine grown in large-scale rhizotrons in comparison with DGGE technique. Further research could also focus on the influence of exudates on the functions of soil microbial communities, especially in the essential soil processes (e.g. nitrogen cycling, P solubilisation) and radiata pine disease protection.
4. Determination of spatial and temporal variability of OAs or root exudates is important in understanding rhizosphere processes and would provide information necessary for improved experimental design aimed at linking root exudates and rhizosphere soil microbial community *in situ*. This experiment could be conducted using the developed *in situ* sampling technique for frequent collection of OAs from various depths and root regions at regular time intervals. In addition, the measurement of other physicochemical conditions in the rhizosphere such as pH, redox conditions and nutrient (Al complexation, Fe³⁺ reduction, Fe²⁺ oxidation, Mn reduction, P solubilisation, etc.) availability should also be included, as these can impact the composition and quantity of root exudates in rhizosphere. Physicochemical parameters in specific areas of the rhizosphere could be measured by applying gels (agar, agarose, polyacrylamide) containing indicator reagents onto the surface of sampling sites (Neumann et al. 2009). The digital images of these colour gels could be captured using the endoscope system and quantitative data could be analysed using specific software. Reporter gene approaches could also be used to study spatial variability of particular exudate compounds in rhizotrons and their effect on specific groups of microorganisms. Collectively, this would lead to a better understanding of biochemical and biophysical interactions that occur in the rhizosphere and how plant roots interact with soil microorganisms and other soil biota in relation to different soil properties. In the longer term this knowledge would provide new opportunities for manipulating and managing the rhizosphere for maximum agronomic and ecological benefits leading to greater ecosystem sustainability.

References

- Agnihotri V P, Vaartaja O (1967) Root exudates from red pine seedlings and their effects on *Pythium ultimum*. Canadian Journal of Botany 45:1031-1040
- Ahonen-Jonnarh U, van Hees P A W, Lundström U S, Finlay R D (2000) Production of organic acids by mycorrhizal and non-mycorrhizal *Pinus sylvestris* L. seedlings exposed to elevated concentrations of aluminium and heavy metals. New Phytologist 146:557-567
- Alef K (1995) Dehydrogenase activity. In: Alef K, Nannipieri P (eds) Methods in Applied Soil Microbiology and Biochemistry, Academic Press, London. pp 228-231
- Amann R I, Ludwig W, Schleifer K H (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiological Reviews 59:143-169
- Anderson I C, Cairney J W G (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environmental Microbiology 6:769-779
- Anderson I C, Parkin P I (2007) Detection of active soil fungi by RT-PCR amplification of precursor rRNA molecules. Journal of Microbiological Methods 68:248-253
- Anderson I C, Parkin P I, Campbell C D (2008) DNA- and RNA-derived assessments of fungal community composition in soil amended with sewage sludge rich in cadmium, copper and zinc. Soil Biology and Biochemistry 40:2358-2365
- Andreote F D, Carneiro R T, Salles J F, Marcon J, Labate C A, Azevedo J L, Araújo W L (2009) Culture-independent assessment of rhizobiales-related alphaproteobacteria and the diversity of methylobacterium in the rhizosphere and rhizoplane of transgenic eucalyptus. Microbial Ecology 57:82-93
- Atlas R M, Bartha R (1993) Microbial ecology: fundamentals and applications. Benjamin/Cummings Publishing, Fonte, Canada
- Ayers W A, Thornton R H (1968) Exudation of amino acids by intact and damaged roots of wheat and peas. Plant and Soil 28:193-207
- Bååth E, Frostegård Å, Pennanen T, Fritze H (1995) Microbial community structure and pH response in relation to soil organic matter quality in wood-ash fertilized, clear-cut or burned coniferous forest soils. Soil Biology and Biochemistry 27:229-240
- Bais H P, Park S-W, Weir T L, Callaway R M, Vivanco J M (2004) How plants communicate using the underground information superhighway. Trends in Plant Science 9:26-32
- Bais H P, Walker T S, Schweizer H P, Vivanco J M (2002) Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of sweet basil (*Ocimum basilicum* L.). Plant physiology and biochemistry 40:983-995
- Bais H P, Weir T L, Perry L G, Gilroy S, 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
- Barber D A, Gunn K B (1974) The effect of mechanical forces on the exudation of organic substances by the roots of cereal plants grown under sterile conditions. New Phytologist 73:39-45
- Barber D A, Lynch J M (1977) Microbial growth in the rhizosphere. Soil Biology and Biochemistry 9:305-308
- Bastias B A, Anderson I C, Xu Z, Cairney J W G (2007) RNA- and DNA-based profiling of soil fungal communities in a native Australian eucalypt forest and adjacent *Pinus elliotti* plantation. Soil Biology and Biochemistry 39:3108-3114
- Baudoin E, Benizri E, Guckert A (2001) Metabolic structure of bacterial communities from district maize rhizosphere compartments. European Journal of Soil Biology 37:85-93
- Baudoin E, Benizri E, Guckert A (2002) Impact of growth stage on the bacterial community structure along maize roots, as determined by metabolic and genetic fingerprinting. Applied Soil Ecology 19:135-145
- Baudoin E, Benizri E, Guckert A (2003) Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. Soil Biology and Biochemistry 35:1183-1192
- Baziramakenga R, Simard R R, Leroux G D (1995) Determination of organic acids in soil extracts by ion chromatography. Soil Biology and Biochemistry 27:349-356
- Beard G, Piche Y (1989) Fungal growth stimulation by CO₂ and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Applied and Environmental Microbiology 55:2320-2325

- Bekkara F, Jay M, Viricel M R, Rome S (1998) Distribution of phenolic compounds within seed and seedlings of two *Vicia faba* cvs differing in their seed tannin content and study of their seed and root phenolic exudations. *Plant and Soil* 203:27-36
- Benizri E, Dedourge O, Dibattista-Leboeuf C, Piutti S, Nguyen C, Guckert A (2002) Effect of maize rhizodeposits on soil microbial community structure. *Applied Soil Ecology* 21:261-265
- Benizri E, Nguyen C, Piutti S, Slezack-Deschaumes S, Philippot L (2007) Additions of maize root mucilage to soil changed the structure of the bacterial community. *Soil Biology and Biochemistry* 39:1230-1233
- Berg G, Eberl L, Hartmann A (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environmental Microbiology* 7:1673-1685
- Berg G, Marten P, Ballin G (1996) *Stenotrophomonas maltophilia* in the rhizosphere of oilseed rape- occurrence, characterization and interaction with phytopathogenic fungi. *Microbiological Research* 151:19-27
- Berg G, 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
- Bertin C, Yang X H, Weston L A (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant and Soil* 256:67-83
- Biedrzycki M L, Bais H P (2009) Root secretions: from genes and molecules to microbial associations. *Journal of Experimental Botany* 60:1533-1534
- Blackwood C B, Buyer J S (2004) Soil microbial communities grown with Bt and non-Bt corn in three soils. *Journal of Environmental Quality* 33:832-836
- Blakemore L C, Searle P L, Daly B K (1987) Methods for chemical analysis of soils. NZ Soil Bureau Scientific Report 80
- Blumberg D D (1987) Creating a ribonuclease-free environment. *Methods in Enzymology* 152:20-24
- Boeuf-Tremblay V, Plantureux S, Guckert A (1995) Influence of mechanical impedance on root exudation of maize seedlings at two development stages. *Plant and Soil* 172:279-287
- Borneman J, Triplett E W (1997) Rapid and direct method for extraction of RNA from soil. *Soil Biology and Biochemistry* 29:1621-1624
- Bossio D A, Scow K M (1998) Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. *Microbial Ecology* 35:265-278
- Bottini R, Cassán F, Piccoli P (2004) Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Applied Microbiology and Biotechnology* 65:497-503
- Boudot J-P (1992) Relative efficiency of complexed aluminum noncrystalline Al hydroxide, allophane and imogolite in retarding the biodegradation of citric acid. *Geoderma* 52:29-39
- Bourne D, Iida Y, Uthicke S, Smith-Keune C (2008) Changes in coral-associated microbial communities during a bleaching event. *The ISME Journal* 2:350-363
- Bowen G D (1969) Nutrient status effects on loss of amides and amino acids from pine roots. *Plant and Soil* 30:139-142
- Bowen G D (1979) Integrated and experimental approaches to study the growth of organisms around root and seeds. In: Schippers B, Gams W (eds) *Soil-Borne Pathogens*, Academic Press, London. pp 209-227
- Bowen G D (1994) The ecology of ectomycorrhiza formation and functioning. *Plant and Soil* 159:61-67
- Brady N, Weil R (2002) *The nature and properties of soils*. Pearson Education, Inc, Upper Saddle River, NJ.
- Brimecombe M J, De Leij F A, Lynch J M (2007) Rhizodeposition and microbial population. In: Pinton R, Varanino Z, Nannipieri P (eds) *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*, CRC Press, New York. pp 73-110
- Bringhurst R M, Cardon Z G, Gage D J (2001) Galactosides in the rhizosphere: utilization by *Sinorhizobium meliloti* and development of a biosensor. *Proceedings of the National Academy of Sciences of the United States of America* 98:4540-4545
- Brodie E L, DeSantis T Z, Joyner D C, Baek S M, Larsen J T, Andersen G L, Hazen T C, Richardson P M, Herman D J, Tokunaga T K, Wan J M M, Firestone M K (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. *Applied and Environmental Microbiology* 72:6288-6298

- Brodie E L, DeSantis T Z, Parker J P M, Zubietta I X, Piceno Y M, Andersen G L (2007) Urban aerosols harbor diverse and dynamic bacterial populations. *Proceedings of the National Academy of Sciences* 104:299-304
- Broeckling C D, Broz A K, Bergelson J, Manter D K, Vivanco J M (2008) Root exudates regulate soil fungal community composition and diversity. *Applied and Environmental Microbiology* 74:738-744
- Bruinsma M, Kowalchuk G A, van Veen J A (2003) Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils* 37:329-337
- Brundrett M, Murase G, Kendrick B (1990) Comparative anatomy of roots and mycorrhizae of common Ontario trees. *Canadian Journal of Botany* 68:551-578
- Brunk C F, Eis N (1998) Quantitative measure of small-subunit rRNA gene sequences of the kingdom *Korarchaeota*. *Applied and Environmental Microbiology* 64:5064-5066
- Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E (2009) The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant and Soil* 321:189-212
- Buée M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. *Molecular Plant-Microbe Interactions* 13:693-698
- Bürgmann H, Meier S, Bunge M, Widmer F, Zeyer J (2005) Effects of model root exudates on structure and activity of a soil diazotroph community. *Environmental Microbiology* 7:1711-1724
- Canadell J, Jackson R B, Ehleringer J R, Mooney H A, Sala O E, Schulze E D (1996) Maximum rooting depth of vegetation types at the global scale. *Oecologia* 108:583-595
- Casarin V, Plassard C, Souche G, Arvieu J-C (2003) Quantification of oxalate ions and protons released by extromycorrhizal fungi in rhizosphere soil. *Agronomie* 23:461-469
- Castaldini M, Turrini A, Sbrana C, Benedetti A, Marchionni M, Mocali S, Fabiani A, Landi S, Santomassimo F, Pietrangeli B, Nuti M P, Miclaus N, Giovannetti M (2005) Impact of Bt corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. *Applied and Environmental Microbiology* 71:6719-6729
- Cawthray G R (2003) An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates. *Journal of Chromatography A* 1011:233-240
- Chen C R, Condrón L M, Davis M R, Sherlock R R (2002) Phosphorus dynamics in the rhizosphere of perennial ryegrass (*Lolium perenne* L.) and radiata pine (*Pinus radiata* D. Don.). *Soil Biology and Biochemistry* 34:487-499
- Cheneby D, Perrez S, Devroey C, Hallet S, Couton Y, Bizouard F, Iuretig G, Germon J C, Philippot L (2004) Denitrifying bacteria in bulk and maize-rhizospheric soil: diversity and N₂O-reducing abilities. *Canadian Journal of Microbiology* 50:469-474
- Chet I, Zilberstein Y, Henis Y (1973) Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. *Physiologia Plant Pathology* 3:473-479
- Chow M L, Radomski C C, McDermott J M, Davies J, Axelrood P E (2002) Molecular characterization of bacterial diversity in Lodgepole pine (*Pinus contorta*) rhizosphere soils from British Columbia forest soils differing in disturbance and geographic source. *FEMS Microbiology Ecology* 42:347-357
- Clarke K R, Warwick R M (2001) Change in marine communities: an approach to statistical analysis and interpretation. PRIMER-E, Plymouth, UK
- Clayton S J, Clegg C D, Murray P J, Gregory P J (2005) Determination of the impact of continuous defoliation of *Lolium perenne* and *Trifolium repens* on bacterial and fungal community structure in rhizosphere soil. *Biology and Fertility of Soils* 41:109-115
- Clough T J, Ray J L, Buckthought L E, Calder J, Baird D, O'Callaghan M, Sherlock R R, Condrón L M (2009) The mitigation potential of hippuric acid on N₂O emissions from urine patches: An in situ determination of its effect. *Soil Biology and Biochemistry* 41:2222-2229
- Costa R, Gomes N C M, Krögerrecklenfort E, Opelt K, Berg G, Smalla K (2007) *Pseudomonas* community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. *Environmental Microbiology* 9:2260-2273

- Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2006) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* 56:236-249
- Cromack K, Sollins P, Graustein W C (1979) Calcium oxalate accumulation and soil weathering in mats of the hypogeous fungus *Hyserangium crassum*. *Soil Biology and Biochemistry* 11:463-468
- Curl E A, Truelove B (1986) *The rhizosphere*. Springer, Berlin
- da Silva A K R, Salles J F, Seldin L, van Elsas J D (2003) Application of a novel *Paenibacillus*-specific PCR-DGGE method and sequence analysis to assess the diversity of *Paenibacillus* spp. in the maize rhizosphere. *Journal of Microbiological Methods* 54:213-231
- Darrah P R (1993) The rhizosphere and plant nutrition: a quantitative approach. *Plant and Soil* 155/156:1-20
- de Ridder-Duine A S, Kowalchuk G A, Klein Gunnewiek P J A, Smant W, van Veen J A, de Boer W (2005) Rhizosphere bacterial community composition in natural stands of *Carex arenaria* (sand sedge) is determined by bulk soil community composition. *Soil Biology and Biochemistry* 37:349-357
- DeAngelis K M, Brodie E L, DeSantis T Z, Andersen G L, Lindow S E, Firestone M K (2008) Selective progressive response of soil microbial community to wild oat roots. *The ISME Journal* 3:168-178
- Degens B P (1997) Macro-aggregation of soils by biological bonding and binding mechanisms and the factors affecting these: a review. *Australia Journal of Soil Research* 35:431-460
- Delhaize E, Ryan P R, Randall P J (1993) Aluminum tolerance in wheat (*Triticum aestivum* L.) 2. Aluminum stimulated excretion of malic acid from root apices. *Plant Physiology* 103:695-702
- Demanèche S, Jocteur-Monrozier L, Quiquampoix H, Simonet P (2001) Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. *Applied and Environmental Microbiology* 67:293-299
- DeSantis T Z, Brodie E L, Moberg J P, Zubietta I X, Piceno Y M, Andersen G L (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microbial Ecology* 53:371-383
- DeSantis T Z, Hugenholtz P, Larsen N, Rojas M, Brodie E L, Keller K, Huber T, Dalevi D, Hu P, Andersen G L (2006) Greengenes, a chimerachecked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* 72:5069-5072
- Dessureault-Rompere J, Nowack B, Schulin R, Luster J (2006) Modified micro suction cup/rhizobox approach for the *in-situ* detection of organic acids in rhizosphere soil solution. *Plant and Soil* 286:99-107
- Dessureault-Rompere J, Nowack B, Schulin R, Luster J (2007) Spatial and temporal variation in organic acid anion exudation and nutrient anion uptake in the rhizosphere of *Lupinus albus* L. *Plant and Soil* 301:123-134
- Di Cello F, Bevivino A, Chiarini L, Fani R, Paffetti D, Tavacchioni S, Dalmastrì C (1997) Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. *Applied and Environmental Microbiology* 63:4485-4493
- Di Giovanni G D, Watrud L S, Seidler R J, Widmer F (1999) Comparison of parental and transgenic alfalfa rhizosphere bacterial communities using biolog GN metabolic fingerprinting and enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). *Microbial Ecology* 37:129-139
- Dickie I A, Xu B, Koide R T (2002) Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist* 2002:527-535
- Dieffenbach A, Göttlein A, Matzner E (1997) *In-situ* soil solution chemistry in an acid forest soil as influenced by growing roots of Norway spruce (*Picea abies* [L.] Karst.). *Plant and Soil* 192:57-61
- Dijkstra F A, Cheng W (2007) Moisture modulates rhizosphere effects on C decomposition in two different soil types. *Soil Biology and Biochemistry* 39:2264-2274
- Dilly O, Nannipieri P (2001) Response of ATP content, respiration rate and enzyme activities in an arable and a forest soil to nutrient additions. *Biology and Fertility of Soils* 34:64-72
- Dinkelaker B, Hengeler C, Neumann G, Marschner H (1997) Root exudates and mobilization of nutrients. In: Rennenberg H, Eschrich W, Ziegler H (eds) *Trees: contributions to modern tree physiology* Backhuys Publishers, Leiden, The Netherlands. pp 441

- Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Sciences* 22:107-149
- Donegan K K, Palm C J, Fieland V J, Porteous L A, Ganio L M, Schaller D L, Bucaco L Q, Seidler R J (1995) Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. *Applied Soil Ecology* 2:111-124
- Donegan K K, Seidler R J, Doyle J D, Porteous L A, Di Giovanni G, Widmers F (1999) A field study with genetically engineered alfalfa inoculated with recombinant *Sinorhizobium meliloti*: effects on the soil ecosystem. *Journal of Applied Ecology* 36:920-936
- Donnelly D P, Boddy L, Leake J R (2004) Development, persistence and regeneration of foraging ectomycorrhizal mycelial systems in soil microcosms. *Mycorrhiza* 14:37-45
- Dormaar J F (1990) Effect of active roots on the decomposition of soil organic materials. *Biology and Fertility of Soils* 10:121-126
- Duarte G F, Rosado A S, Seldin L, Keijzer-Wolters A C, van Elsas J D (1998) Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *Journal of Microbiological Methods* 32:21-29
- Duineveld B M, Kowalchuk G A, Keijzer A, van Elsas J D, van Veen J A (2001) Analysis of bacterial communities in the rhizosphere of chrysanthemum via denaturing gradient gel electrophoresis of PCR amplified 16S rRNA as well as DNA fragments coding for 16S rRNA. *Applied and Environmental Microbiology* 67:172-178
- Duineveld B M, Rosado A S, van Elsa J D, van Veen J A (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. *Applied and Environment Microbiology* 64:4950-4957
- Duineveld B M, Van Veen J A (1999) The number of bacteria in the rhizosphere during plant development: relating colony-forming units to different reference units. *Biology and Fertility of Soils* 28:285
- Dunbar J, Ticknor L O, Kuske C R (2000) Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Applied and Environmental Microbiology* 66:2943-2950
- Dunfield K E, Germida J J (2001) Diversity of bacterial communities in the rhizosphere and root interior of field grown genetically modified *Brassica napus*. *FEMS Microbiology Ecology* 38:1-9
- Dunfield K E, Germida J J (2003) Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). *Applied and Environmental Microbiology* 69:7310-7318
- Dunfield K E, Germida J J (2004) Impact of genetically modified crops on soil and plant associated microbial communities. *Journal of Environmental Quality* 33:806-815
- Egerton-Warburton L M, Querejeta J I, Allen M F, Finkelman S L (2005) Mycorrhizal fungi. In: Hillel D, Hatfield J H, Powlson D S, et al. (eds) *Encyclopedia of Soils in the Environment*, Academic, London. pp 533-541
- Ehrich S, Behrens D, Lebedeva E, Ludwig W, Bock E (1995) A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Archives of Microbiology* 164:16-23
- El-Tarabily K A, Sivasithamparam K (2006) Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. *Soil Biology and Biochemistry* 38:1505-1520
- Emmert E A B, Handelsman J (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters* 171:1-9
- Engelen B, Meinken K, von Wintzingerode F, Heuer H, Malkomes H-P, Backhaus H (1998) Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Applied and Environmental Microbiology* 64:2814-2821
- Entry J A, Mills D, Jayachandran K, Moorman T B (2007) Symposium: molecular-based approaches to soil microbiology. *Soil Science Society of America Journal* 71:561
- Ettema C H, Wardle D A (2002) Spatial soil ecology. *Trends in Ecology & Evolution* 17:177-183
- Evangelou M W H, Ebel M, Hommes G, Schaeffer A (2008) Biodegradation: the reason for the inefficiency of small organic acids in chelant-assisted phytoextraction. *Water Air Soil Pollution* 195:177-188

- Falchini L, Naumova N, Kuikman P J, Bloem J, Nannipieri P (2003) CO₂ evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation. *Soil Biology and Biochemistry* 35:775-782
- Fan T W M, Lane A N, Pedler J, Crowley D, Higashi R M (1997) Comprehensive analysis of organic ligands in whole root exudates using nuclear magnetic resonance and gas chromatography-mass spectrometry. *Analytical Biochemistry* 251:57-68
- Fan T W M, Lane A N, Shenker M, Bartley J P, Crowley D, Higashi R M (2001) Comprehensive chemical profiling of gramineous plant root exudates using high-resolution NMR and MS. *Phytochemistry* 57:209-221
- Farrelly V, Rainey F A, Stackebrandt E (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* 61:2798-2801
- Faure D, Vereecke D, Leveau J H J (2009) Molecular communication in the rhizosphere. *Plant and Soil* 321:279-303
- Felske A, Akkermans A D L (1998) Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils. *Microbial Ecology* 36:31-36
- Felske A, Engelen B, Nubel U, Backhaus H (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Applied and Environmental Microbiology* 62:4162-4167
- Feris K P, Ramsey P W, Frazar C, Rillig M C, Gannon J E, Holben W E (2003) Structure and seasonal dynamics of hyporheic zone microbial communities in free-stone rivers of the western United States. *Microbial Ecology* 46:200-215
- Fierer N, Schimel J P, Holden P A (2003) Variations in microbial community composition through two soil depth profiles. *Soil Biology and Biochemistry* 35:167-176
- Filion M, Hamelin R C, Bernier L, St-Arnaud M (2004) Molecular profiling of rhizosphere microbial communities associated with healthy and diseased black spruce (*Picea mariana*) seedlings grown in a nursery. *Applied and Environmental Microbiology* 70:3541-3551
- Findlay R H (1996) The use of phospholipid fatty acids to determine the microbial community structure. In: Akkermans A D L, van Elsas J D, de Bruijn F J (eds) *Molecular Microbial Ecology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Fisher R F, Binkley D (2000) *Ecology and management of forest soils*. Wiley, New York
- Foster R C, Rovira A D, Cock T W (1983) The soil, root, and rhizosphere. In: Foster R C, Rovira A D, Cock T W (eds) *Ultrastructure of the root-soil interface*. The American photopathological society, Minnesota, USA.
- Fox T R, Comerford N B (1990) Low-molecular-weight organic acids in selected forest soils of the southeastern USA. *Soil Science Society of America Journal* 54:1139-1144
- Franke-Whittle I H, Knapp B A, Fuchs J, Kaufmann R, Insam H (2009) Application of COMPOCHIP microarray to investigate the bacterial communities of different composts. *Microbial Ecology* 57:510-521
- Fries N (1989) The influence of tree roots on spore germination of ectomycorrhizal fungi. *Agriculture, Ecosystems & Environment* 28:139-144
- Fries N, Bardet M, Serck-Hanssen K (1985) Growth of ectomycorrhizal fungi stimulated by lipids from a pine root exudate. *Plant and Soil* 86:287-290
- Fries N, Serck-Hanssen K, Dimberg L H, Theander O (1987) Abietic acid, an activation of basidiospore germination in ectomycorrhizal species of the genus *Suillus* (Boletaceae). *Experimental Mycology* 11:360-363
- Garbeva P, van Veen J A, van Elsas J D (2003) Predominant *Bacillus* spp. in agricultural soil under different management regimes detected via PCR-DGGE. *Microbial Ecology* 45:302-316
- Gardes M, Bruns T D (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2:113-118
- Gardner W K, Barrber D A, Parbery D G (1983) The acquisition of phosphorus by *Lupinus albus* L. III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. *Plant and Soil* 70:107-124
- George T S, Richardson A E, Hadobas P A, Simpson R J (2004) Characterization of transgenic *Trifolium subterraneum* L. which expresses *phyA* and releases extracellular phytase: growth and P nutrition in laboratory media and soil. *Plant, Cell and Environment* 27:1351-1361
- George T S, Richardson A E, Li S S, Gregory P J, Daniell T J (2009) Extracellular release of a heterologous phytase from roots of transgenic plants: does manipulation of rhizosphere

- biochemistry impact microbial community structure? *FEMS Microbiology Ecology* 70:433-445
- Gillis M, Tran Van V, Bardin R, Goor M, Hebbbar P, Willems A, Segers P, Kersters K, Heulin T, Fernandez M P (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N₂-fixing isolates from rice in Vietnam. *International Journal of Systematic Bacteriology* 45:274-289
- Giovannetti M, Sbraba C, Silvia A, Avio L (1996) Analysis of factors involved in fungal recognition response to host-derived signals by arbuscular mycorrhizal fungi. *New Phytologist* 133:65-71
- Girvan M S, Bullimore J, Ball A S, Pretty J N, Osborn A M (2004) Responses of active bacterial and fungal communities in soils under winter wheat to different fertilizer and pesticide regimens. *Applied and Environmental Microbiology* 70:2692-2701
- Girvan M S, Bullimore J, Pretty J N, Osborn A M, Ball A S (2003) Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils. *Applied and Environmental Microbiology* 69:1800-1809
- Glare T R, O'Callaghan M, Malone L A, Burgess E P, Philip B A (2004) Measuring environmental impacts of genetically modified crops in New Zealand. *Aspects of Applied Biology* 74:91-99
- Glasbey C A, Vali L, Gustafsson J S (2005) A statistical model for unwarping of 1-D electrophoresis gels. *Electrophoresis* 26:4237-4242
- Gobat J-M, Aragno M, Matthey W (2004) Life in action. In: Gobat J-M, Aragno M, Matthey W (eds) *The Living Soil*, Science Publishers Inc., NH, USA. pp 77-154
- Gochnauer M B, McCully M E, Labbe H (1989) Different populations of bacteria associated with sheathed and bare regions of roots of fieldgrown maize. *Plant and Soil* 114:107-120
- Gomes N C M, Heuer H, Schönfeld J, Costa R, Mendonça-Hagler L, Smalla K (2001) Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. *Plant and Soil* 232:167-180
- Gorissen A, Tietema A, Joosten N N, Estiarte M, Peñuelas J, Sowerby A, Emmett B A, Beier C (2004) Climate change affects carbon allocation to the soil in shrublands. *Ecosystems* 7:650-661
- Gower J C (1985) Measures of similarity, dissimilarity and distance. In: Kotz S, Johnson N L, Read C B (eds) *Encyclopaedia of Statistical Sciences*, Wiley, New York.
- Gramss G, Voigt K-D, Bergmann H (2004) Plant availability and leaching of (heavy) metals from ammonium-, calcium-, carbohydrate-, and citric acid-treated uranium-mine-dump soil. *Journal of Plant Nutrition and Soil Science* 167:417-427
- Gramss G, Voigt K-D, Bublitz F, Bergmann H (2003) Increased solubility of (heavy) metals in soil during microbial transformations of sucrose and casein amendments. *Journal of Basic Microbiology* 43:483-498
- Granse A, Wittenmayer L (2000) Qualitative and quantitative analysis of water-soluble root exudates in relation to plant species and development. *Journal of Plant Nutrition and Soil Science* 163:381-385
- Grayston S J, Campbell C D (1996) Functional biodiversity of microbial communities in the rhizosphere of Hybrid larch (*Larix eurolepis*) and Sitka spruce (*Picea sitchensis*). *Tree Physiology* 16:1031-1038
- Grayston S J, Griffith G S, Mawdsley J L, Campbell C D, Bardgett R D (2001) Accounting for variability in soil microbial communities of temperate upland grassland ecosystems. *Soil Biology and Biochemistry* 33:533-551
- Grayston S J, Vaughan D, Jones D (1996) Rhizosphere carbon flow in trees, in comparison with annual plants: The importance of root exudation and its impact on microbial activity and nutrient availability. *Applied Soil Ecology* 5:29-56
- Grayston S J, Wang S Q, Campbell C D, Edwards A C (1998) Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Biology and Biochemistry* 30:369-378
- Gregory P J (2006) Roots, rhizosphere and soil: the route to a better understanding of the soil science? *European Journal of Soil Science* 57:2-12
- Gremion F, Chatzinotas A, Harms H (2003) Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metalcontaminated bulk and rhizosphere soil. *Environmental Microbiology* 5:896-907
- Grierson P F (1992) Organic acids in the rhizosphere of *Banksia integrifolia* L.f. *Plant and Soil* 144:259-265

- Griffiths B S, Geoghegan I E, Robertson W M (2000a) Testing genetically engineered potato, producing the lectins GNA and ConA, on non-target soil organisms and processes. *Journal of Applied Ecology* 37:159-170
- Griffiths B S, Ritz K, Ebbelwhite N, Dobson G (1999) Soil microbial community structure: effects of substrate loading rates. *Soil Biology and Biochemistry* 31:145-153
- Griffiths R I, Whiteley A S, O'Donnell A G, Bailey M J (2000b) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environment Microbiology* 66:5488-5491
- Griffiths R I, Whiteley A S, O'Donnell A G, Bailey M J (2003a) Influence of depth and sampling time on bacterial community structure in an upland grassland soil. *FEMS Microbiology Ecology* 43:35-43
- Griffiths R I, Whiteley A S, O'Donnell A G, Bailey M J (2003b) Physiological and community responses of established grassland bacterial populations to water stress. *Applied and Environmental Microbiology* 69:6961-6968
- Griffiths R P, Baham J E, Caldwell B A (1994) Soil solution chemistry of ectomycorrhizal mats in forest soil. *Soil Biology and Biochemistry* 26:331-337
- Grineva G M (1963) Alcohol formation and excretion by plant roots under anaerobic conditions. *Soviet Plant Physiology* 10:361-369
- Groleau-Renaud V, Plantureux S, Guckert A (1998) Influence of plant morphology on root exudation of maize subjected to mechanical impedance in hydroponic conditions. *Plant and Soil* 201:231-239
- Grundmann G L, Debouzie D (2000) Geostatistical analysis of the distribution of NH_4^+ and NO_2^- oxidizing bacteria and serotypes at the millimeter scale along a soil transect. *FEMS Microbiology Ecology* 34:57-62
- Gutiérrez-Mañero F J, Ramos-Solano B, Probanza A, Mehrouachi J, Tadeo F R, Talon M (2001) The plant-growth promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. *Physiologia plantarum* 111:206-211
- Gyamfi S, Pfeifer U, Stierschneider M, Sessitsch A (2002) Effects of transgenic glufosinate-tolerant oilseed rape (*Brassica napus*) and the associated herbicide application on eubacterial and *Pseudomonas* communities in the rhizosphere. *FEMS Microbiology Ecology* 41:181-190
- Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* 3:307-319
- Haichar F e Z, Marol C, Berge O, Rangel-Castro J I, Prosser J I, Balesdent J, Heulin T, Achouak W (2008) Plant host habitat and root exudates shape soil bacterial community structure. *The ISME Journal* 2:1221-1230
- Hale M G, Moore L D, Griffin G J (1978) Root exudates and exudation. In: Dommergues Y R, Krupa S V (eds) *Interactions between Non-pathogenic Soil Microorganisms and Plants*, Elsevier, Amsterdam. pp 163-203
- Hamlen R A, Lukezic F L, Bloom J R (1972) Influence of clipping height on the neutral carbohydrate levels of root exudates of alfalfa plants grown under gnotobiotic conditions. *Canadian Journal of Plant Science* 52:643-649
- Hartmann A, Schmid M, van Tuinen D, Berg G (2009) Plant-driven selection of microbes. *Plant and Soil* 321:235-257
- Hawkes C V, DeAngelis K M, Firestone M K (2007) Root interactions with soil microbial communities and processes. In: Cardon Z, G. Whitbeck J, L. (eds) *The Rhizosphere*, Academic Press, Burlington. pp 1-29
- He X-Y, Wang K-L, Zhang W, Chen Z-H, Zhu Y-G, Chen H-S (2008) Positive correlation between soil bacterial metabolic and plant species diversity and bacterial and fungal diversity in a vegetation succession on Karst. *Plant and Soil* 307:123-134
- Head I M, Saunders J R, Pickup R W (1998) Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbial Ecology* 35:1-21
- Hedley M J, Kirk G J D, Santos M B (1994) Phosphorus efficiency and the forms of soil phosphorus utilized by upland rice cultivars. *Plant and Soil* 158:53-62
- Heim A, Brunner I, Frey B, Frossard E, Luster J (2001) Root spruce seedlings treated with aluminum in hydroponics. *Journal of Plant Nutrition and Soil Science* 164:519-526
- Heinrich D, Hess D (1985) Chemotactic attraction of *Azospirillum lipoferum* by wheat roots and characterisation of some attractants. *Canadian Journal of Microbiology* 31:26-31
- Henderson A R, Walter C (2006) Genetic engineering in Conifer Plantation Forestry. *Silvae Genetica* 55:253-262

- Hendrick R L, Pregitzer K S (1996) Temporal and depth-related patterns of fine root dynamics in northern hardwood forests. *Journal of Ecology* 84:167-176
- Henry S, Texier S, Hallet S, Bru D, Dambreville C, Chèneby D, Bizouard F, Germon J C, Philippot L (2008) Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. *Environmental Microbiology* 10:3082-3092
- Herman D C, Maier R M (2000) Physiological Methods. In: Maier R M, Pepper I L, Charles P G (eds) *Environmental microbiology*, Academic Press, San Diego, California. pp 235-265
- Herrmann K M, Weaver L M (1999) The shikimate pathway. *Annual Review of Plant Physiology and Plant Molecular Biology* 50:473-503
- Heuer H, Kroppenstedt R M, Lottmann J, Berg G, Smalla K (2002) Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. *Applied and Environmental Microbiology* 68:1325-1335
- Heuer H, Krsek M, Baker P, Smalla K, Wellington E M H (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environment Microbiology* 63:3233-3241
- Heuer H, Smalla K (1999) Bacterial phyllosphere communities of *Solanum tuberosum* L. and T4-lysozyme-producing transgenic variants. *FEMS Microbiology Ecology* 28:357-371
- Hillel D 1982 *Introduction to Soil Physics*. Academic Press, Inc, San Diego, CA, USA
- Hinsinger P, Bengough A G, Vetterlein D, Young I M (2009) Rhizosphere: biophysics, biogeochemistry and ecological relevance. *Plant and Soil* 321:117-152
- Hinsinger P, Gobran G R, Gregory P J, Wenzel W W (2005) Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical process. *New Phytologist* 168:293-303
- Hodge A, Paterson E, Thornton B, Millard P, Killham K (1997) Effects of photon flux density on carbon partitioning and rhizosphere carbon flow of *Lolium perenne*. *Journal of Experimental Botany* 48:1797-1805
- Hoffland E, Findenegg G R, Nelemans J A (1989) Solubilization of rockphosphate by rape. II. Local exudation of organic acids as a response to P-starvation. *Plant and Soil* 113:161-165
- Horwath W (2007) Carbon cycling and formation of soil organic matter. In: Paul E A (ed) *Soil Microbiology, Ecology, and Biochemistry*, Academic Press Burlington, MA, USA. pp 303-340
- Hoshino Y T, Matsumoto N (2007) DNA- versus RNA-based denaturing gradient gel electrophoresis profiles of a bacterial community during replenishment after soil fumigation. *Soil Biology and Biochemistry* 39:434-444
- Hotelling H (1947) *Multivariate Quality Control*. In: Eisenhart C, Hastay M W, Wallis W A (eds) *Techniques of Statistical Analysis*, McGraw-Hill, New York.
- Hu H-Y, Liu X-X, Zhao Z-W, Sun J-G, Zhang Q-W, Liu X-Z, Yu Y (2008) Effects of repeated cultivation of transgenic Bt cotton on functional bacterial populations in rhizosphere soil. *World Journal of Microbiology and Biotechnology* 25:357-366
- Hughes M, Donnelly C, Crozier A, Wheeler C T (1999) Effects of the exposure of roots *Alnus glutinosa* to light on flavonoid and nodulation. *Canadian Journal of Botany* 77:1311-1315
- Hurt R A, Qiu X, Wu L, Roh Y, Palumbo A V, Tiedje J M, Zhou J (2001) Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology* 67:4495-4503
- Ishii K, Fukui M (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. *Applied and Environmental Microbiology* 67:3753-3755
- Izumi H, Cairney J W G, Killham K, Moore E, Alexander I J, Anderson I C (2008) Bacteria associated with ectomycorrhizas of slash pine (*Pinus elliotii*) in south-eastern Queensland, Australia. *FEMS Microbiology Letters* 282:196-204
- Izumi H, Moore E R B, Killham K, Alexander I J, Anderson I C (2007) Characterisation of endobacterial communities in ectomycorrhizas by DNA- and RNA-based molecular methods. *Soil Biology and Biochemistry* 39:891-899
- Jackson C R, Liew K C, Yule C M (2009) Structural and functional changes with depth in microbial communities in a tropical Malaysian peat swamp forest. *Microbial Ecology* 57:402-412
- Jaeger I I C, Lindow S, Miller S, Clark E, Firestone M (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Applied and Environment Microbiology* 65:2685-2690
- James C (2003) *Preview: global status of commercialized transgenic crops*, ISAAA: Ithaca, NY.
- Jauregui M A, Reisenauer H M (1982) Dissolution of oxides of manganese and iron by root exudate components. *Soil Science Society of America Journal* 46:314-317

- Jones D L, Darrah P R (1995) Influx and efflux of organic acids across the soil-root interface of *Zea mays* L. and its implications in rhizosphere C flow. *Plant and Soil* 173:103-109
- Jones D L, Darrah P R, Kochian L V (1996a) Critical-evaluation of organic-acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake. *Plant and Soil* 180:57-66
- Jones D L, Prabowo A M, Kochian L V (1996b) Kinetics of malate transport and decomposition in acid soils and isolated bacterial populations - the effect of microorganisms on root exudation of malate under Al stress. *Plant and Soil* 182:239-247
- Jones D L (1998) Organic acids in the rhizosphere- a critical review. *Plant and Soil* 205:25-44
- Jones D L, Dennis P G, Owen A G, van Hees P A W (2003) Organic acid behavior in soils – misconceptions and knowledge gaps. *Plant and Soil* 248:31-41
- Jones D L, Edwards A C (1998) Influence of sorption on the biological utilization of two simple carbon substrates. *Soil Biology and Biochemistry* 30:1895-1902
- Joynt J, Bischoff M, Turco R, Konopka A, Nakatsu C H (2006) Microbial community analysis of soils contaminated with lead, chromium and petroleum hydrocarbons. *Microbial Ecology* 51:209-219
- Juo P, Stotzky G (1970) Electrophoretic separation of proteins from roots and exudates. *Canadian Journal of Botany* 48:713-718
- Kaldorf M, Fladung M, Muhs H J, Buscot F (2002) Mycorrhizal colonization of transgenic aspen in a field trial. *Planta* 214:653-660
- Kamh M, Horst W J, Amer F, Mostafa H, Maier P (1999) Mobilization of soil and fertilizer phosphate by cover crops. *Plant and Soil* 211:19-27
- Kamilova F, Kravchenko L V, Shaposhnikov A I, Azarova T, Makarova N, Lugtenberg B (2006) Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Molecular Plant-Microbe Interactions* 19:250-256
- Kape R, Wex K, Parniske M, Göрге E, Wetzell A, Werner D (1992) Legume root metabolites and VA-mycorrhiza development. *Journal of Plant Physiology* 141:54-60
- Kapoor R, Mukerji K G (2006) Rhizosphere microbial community dynamics. In: Mukerji K G, Manoharachary C, Singh J (eds) *Microbial Activity in the Rhizosphere*, Springer, Berlin. pp 55-66
- Ke X, Li P-J, Zhou Q-X, Zhang Y, Sun T-H (2006) Removal of heavy metals from a contaminated soil using tartaric acid. *Journal of Environmental Science* 18:727-733
- Keith H, Oades J M, Martin J K (1986) Input of carbon to soil from wheat plants. *Soil Biology and Biochemistry* 18:445-449
- Kennedy A C, de Luna L Z (2005) Rhizosphere. In: Hillel D, Hatfield J L, Powlson D S, et al. (eds) *Encyclopedia of Soils in the Environment*, Elsevier Academic Press, London. pp 399-406
- Kerem Z, Bravdo B-a, Shoseyov O, Tugendhaft Y (2004) Rapid liquid chromatography-ultraviolet determination of organic acids and phenolic compounds in red wine and must. *Journal of Chromatography A* 1052:211-215
- Kieft T, Amy P, Brockman F, Fredrickson J, Bjornstad B, Rosacker L (1993) Microbial abundance and activities in relation to water potential in the vadose zones of arid and semiarid sites. *Microbial Ecology* 26:59-78
- Kisand V, Wikner J (2003) Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *Journal of Microbiological Methods* 54:183-191
- Klamer M, Roberts M S, Levine L H, Drake B G, Garland J L (2002) Influence of elevated CO₂ on the fungal community in a coastal scrub oak forest soil investigated with terminal restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology* 68:4370-4376
- Koo B-J, Adriano D C, Bolan N S, Barton C D (2005) Root exudates and microorganisms. In: Hillel D, Hatfield J L, Powlson D S, et al. (eds) *Encyclopedia of Soils in the Environment*, Elsevier Academic Press, London. pp 421-428
- Koo B-J, Chang A C, Crowley D E (2006) Characterization of organic acids recovered from rhizosphere of corn grown on biosolids-treated medium. *Communications in Soil Science and Plant Analysis* 37:871-887
- Kowalchuk G A, Hol W H G, Van Veen J A (2006) Rhizosphere fungal communities are influenced by *Senecio jacobaea* pyrrolizidine alkaloid content and composition. *Soil Biology and Biochemistry* 38:2852-2859
- Kowalchuk G A, Smith E (2004) Fungal community analysis using PCR-denaturing gradient gel electrophoresis (DGGE). In: Kowalchuk G A, de Bruijn F J, Head I M, et al. (eds) *Molecular*

- Microbial Ecology Manual, Kluwer Academic Publishers, Dordrecht, The Netherlands. pp 771-788
- Kowalchuk G A, van Os G J, van Aartrijk J, van Veen J A (2003) Microbial community responses to disease management soil treatments used in flower bulb cultivation. *Biology and Fertility of Soils* 37:55-63
- Kozdrój J, van Elsas J D (2000) Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. *Soil Biology and Biochemistry* 32:1405-1417
- Krafczyk I, Trolldenier G, Beringer H (1984) Soluble root exudates of maize: Influence of potassium supply and rhizosphere microorganisms. *Soil Biology and Biochemistry* 16:315-322
- Kremer R J, Donald P A, Keaster A J, Minor H C (2000) Herbicide impact on *Fusarium spp.* and soybean cyst nematode in glyphosate-tolerant soybean. In 64th Annual Meeting of the Soil Science Society of America, Minneapolis
- Kropf S, Heuer H, Grüing M, Smalla K (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. *Journal of Microbiological Methods* 57:187-195
- Krupa S, Fries N (1971) Studies on ectomycorrhizae of pine. Production of organic volatile compounds. *Canadian Journal of Botany* 49:1425-1431
- Kuchenbuch R, Jung A (1982) A method for determining concentration profiles at the root-soil interface by thin slicing rhizospheric soil. *Plant and Soil* 68:391-394
- Kuiper I, Legendijk E L, Bloemberg G V, Lugtenberg B J J (2004) Rhizoremediation: a beneficial plant-microbe interaction. *Molecular Plant-Microbe Interactions* 17:6-15
- Kumar R, Pandey S, Pandey A (2006) Plant roots and carbon sequestration. *Current Science* 91:885-890
- Kuzyakov Y (2002) Review: Factors affecting rhizosphere priming effects. *Journal of Plant Nutrition and Soil Science* 165:382-396
- Lamarche J, Hamelin R C (2007) No Evidence of an impact on the rhizosphere diazotroph community by the expression of *Bacillus thuringiensis* Cry1Ab toxin by Bt white spruce. *Applied and Environmental Microbiology* 73:6577-6583
- Lambers H, Juniper D, Cawthray G R, Veneklaas E J, Martínez-Ferri E (2002) The pattern of carboxylate exudation in *Banksia grandis* (Proteaceae) is affected by the form of phosphate added to the soil. *Plant and Soil* 238:111-122
- Landeweert R, Leeflang P, Smit E, Kuyper T (2005) Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach. *Mycorrhiza* 15:1-6
- Landi L, Valori F, Ascher J, Renella G, Falchini L, Nannipieri P (2006) Root exudate effects on the bacterial communities, CO₂ evolution, nitrogen transformations and ATP content of rhizosphere and bulk soils. *Soil Biology and Biochemistry* 38:509-516
- LeBlanc P M, Hamelin R C, Filion M (2007) Alteration of soil rhizosphere communities following genetic transformation of white spruce. *Applied and Environmental Microbiology* 73:4128-4134
- Lehr N A, Schrey S D, Hampp R, Tarkka M T (2008) Root inoculation with a forest soil streptomycete leads to locally and systemically increased resistance against phytopathogens in Norway spruce. *New Phytologist* 177:965-976
- Levan M A, Ycas J M, Hummel J W (1987) Light leak effects on near surface soybean rooting observed in minirhizotrons. In: Taylor H M (ed) *Minirhizotron observation tubes: methods and applications for measuring rhizosphere dynamics*, American Society of Agronomy, Madison, Wis., USA pp 89-98
- Leyval C, Berthelin J (1993) Rhizodeposition and net release of soluble organic compounds by pine and beech seedlings inoculated with rhizobacteria and ectomycorrhizal fungi. *Biology and Fertility of Soils* 15:259-267
- Li M, Shinano T, Tadano T (1997) Distribution of exudates of lupin roots in the rhizosphere under phosphorus deficient conditions. *Soil Science and Plant Nutrition* 43:237-245
- Lilley A K, Bailey M J, Cartwright C, Turner S L, Hirsch P R (2006) Life in earth: the impact of GM plants on soil ecology? *Trends in Biotechnology* 24:9-14
- Linderman R G (1988) Mycorrhizal interactions with the rhizosphere microflora – the mycorrhizosphere effect. *Phytopathology* 78:366-371
- Lipton D S, Blanchar R W, Blevins D G (1987) Citrate, malate, succinate concentration in exudates from P-sufficient and P-stressed *Medicago sativa* L. seedlings. *Plant Physiology* 85:315-317

- López B, Sabaté S, Gracia C A (2001) Fine-root longevity of *Quercus ilex*. *New Phytologist* 151:437-441
- Lorenz M G, Wackernagel W (1987) Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and Environmental Microbiology* 53:2948-2952
- Lottmann J, Heuer H, Smalla K, Berg G (1999) Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. *FEMS Microbiology Ecology* 29:365-377
- Lundquist E J, Scow K M, Jackson L E, Uesugi S L, Johnson C R (1999) Rapid response of soil microbial communities from conventional, low input, and organic farming systems to a wet/dry cycle. *Soil Biology and Biochemistry* 31:1661-1675
- Lynch J M (1987) *The Rhizosphere*. Wiley Interscience, Chichester
- Lynch J M (1990) Introduction: some consequences of microbial rhizosphere competence for plant and soil. In: Lynch J M (ed) *The Rhizosphere*, John Wiley & Sons Ltd, West Sussex. pp 1-10
- Lynch J M, Whipps J M (1990) Substrate flow in the rhizosphere. *Plant and Soil* 129:1-10
- Lynch M R (1995) *The Rhizosphere*. Wiley, Chichester
- Magurran A E (1988) *Ecological Diversity and Its Measurement*. Princeton University Press, Princeton, New Jersey
- Mahmood S, Paton G I, Prosser J I (2005) Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environmental Microbiology* 7:1349-1360
- Malajczuk N, Cromack J K (1982) Accumulation of calcium oxalate in the mantle of ectomycorrhizal roots of *Pinus radiata* and *Eucalyptus marginata*. *New Phytologist* 92:527-531
- Malajczuk N, McComb A J (1977) Root exudates from *Eucalyptus calophylla* R.Br. and *Eucalyptus marginata* Donn. ex Sm. seedlings and their effect on *Phytophthora cinnamomi* rands. *Australian Journal of Botany* 25:501-514
- Manefield M, Whiteley A S, Griffiths R S, Bailey M J (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* 68:5367-5373
- Mantelin S, Touraine B (2004) Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. *Journal of Experimental Botany* 55:27-34
- Marilley L, Aragno M (1999) Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Applied Soil Ecology* 13:127-136
- Marilley L, Vogt G, Blanc M, Aragno M (1998) Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. *Plant and Soil* 198:219-224
- Mark G L, Dow J M, Kiely P D, Higgins H, Haynes J, Baysse C, Abbas A, Foley T, Franks A, Morrissey J, O'Gara F (2005) Transcriptome profiling of bacterial responses to root exudates identifies genes involved in microbe-plant interactions. *Proceedings of the National Academy of Sciences* 102:17454-17459
- Marschner H (1992) Nutrient dynamics at the soil-root interface (rhizosphere). In: Read D J, Lewis D H, Fitter A H, et al. (eds) *Mycorrhizas in Ecosystems*, CAB International, Wallingford, UK. pp 3-12
- Marschner H (1995) *Mineral nutrition of higher plants*. Academic Press, London
- Marschner H, Römheld V (1983) In vivo measurement of root-induced changes at the soil-root interface: Effect of plant species and nitrogen source. *Zeitschrift für Pflanzenphysiologie* 111:241-251
- Marschner H, Römheld V, Kissel M (1987) Localization of phytosiderophore release and of iron uptake along intact barley roots. *Physiologia Plantarum* 71:157-162
- Marschner P, Crowley D, Yang C H (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant and Soil* 261:199-208
- Marschner P, Grierson P F, Rengel Z (2005) Microbial community composition and functioning in the rhizosphere of three *Banksia* species in native woodland in Western Australia. *Applied and Environmental Microbiology* 28:191-201
- Marschner P, Neumann G, Kania A, Weisskopf L, Lieberei R (2002) Spatial and temporal dynamics of bacterial community composition in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant and Soil* 246:167-174
- Marschner P, Yang C H, Lieberei R, Crowley D (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry* 33:1437-1445

- Martin F, Duplessis S, Ditengou F, Lagrange H, C. V, Lapeyrie F (2001) Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. *New Phytologist* 151:145-154
- Masuda N, Church G M (2002) *Escherichia coli* gene expression responsive to levels of the response regulator EvgA. *Journal of bacteriology* 184:6225-6234
- Mathesius U, Weinman J J, Rolfe B G, Djordjevic M A (2000) Rhizobia can induce nodules in white clover by 'hijacking' mature cortical cells activated during lateral root development. *Molecular Plant-Microbe Interactions* 13:170-182
- Matilla M A, Espinosa-Urgel M, Rodríguez-Herva J J, Ramos J L, Ramos-González M I (2007) Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. *Genome Biology* 8:R179
- Maxwell C A, Phillips D A (1990) Concurrent synthesis and release of *nod*-gene-inducing flavonoids from alfalfa roots. *Plant Physiology* 93:1552-1558
- McCaig A E, Glover L A, Prosser J I (1999) Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Applied and Environmental Microbiology* 65:1721-1730
- McCalley D V (2005) Comparison of an organic polymeric column and a silica-based reversed-phase for the analysis of basic peptides by high-performance liquid chromatography. *Journal of Chromatography A* 1073:137-145
- McDougall B M (1970) Movement of ^{14}C -photosynthate into the roots of wheat seedlings and exudation of ^{14}C from intact roots. *New Phytologist* 69:37-46
- McKenzie B E, Peterson C A (1995a) Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 1. Anatomy and permeability of the white and tannin zones. *Botanica Acta* 108:127-137
- McKenzie B E, Peterson C A (1995b) Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm. 2. Anatomy and permeability of the cork zone. *Botanica Acta* 108:138-143
- McKew B A, Coulon F A, Osborn M, Timmis K N, McGenity T J (2007) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environmental Microbiology* 9:165-176
- McMichael B L, Zak J C (2006) The role of rhizotrons and minirhizotrons in evaluating the dynamics of rhizoplane-rhizosphere microflora. In: Mukerji K G, Manoharachary C, Singh J (eds) *Microbial Activity in the Rhizosphere*, Springer-Verlag, Berlin. pp 71-88
- Meharg A A (1994) A critical review of labelling techniques used to quantify rhizosphere carbon flow. *Plant and Soil* 166:55-62
- Meharg A A, Killham K (1989) Distribution of assimilated carbon within the plant and rhizosphere of *Lolium perenne* - influence of temperature. *Soil Biology and Biochemistry* 21:487-489
- Meharg A A, Killham K (1990) The effect of soil pH on rhizosphere carbon flow of *Lolium perenne*. *Plant and Soil* 123:1-7
- Meharg A A, Killham K (1991) A novel method of determining root exudates in the presence of soil microflora. *Plant and Soil* 133:111-116
- Meharg A A, Killham K (1995) Loss of exudates from the roots of perennial ryegrass inoculated with a range of microorganisms. *Plant and Soil* 170:345-349
- Mench M, Martin E (1991) Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. *Plant and Soil* 132:187-196
- Meyerhans A, Vartanian J-P, Wain-Hobson S (1990) DNA recombination during PCR. *Nucleic Acids Research* 18:1687-1691
- Micallef S A, Shiaris M P, Colón-Carmona A (2009) Influence of *Arabidopsis thaliana* accessions on rhizobacterial communities and natural variation in root exudates. *Journal of Experimental Botany* 60:1729-1742
- Miethling R, Wieland G, Backhaus H, Tebbe C C (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L.. *Microbiology Ecology* 40:43-56
- Milling A, Smalla K, Maidl F, Schloter M, Munch J C (2004) Effects of transgenic potatoes with an altered starch composition on the diversity of soil and rhizosphere bacteria and fungi. *Plant and Soil* 266:23-39
- Molina R, Massicotte H, Trappe J M (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen M (ed) *Mycorrhizal Functioning: an Integrative Plant-Fungal Process*, Chapman and Hall, London, UK. pp 357-423

- Moran M A, Torsvik V L, Torsvik T, Hodson R E (1993) Direct extraction and purification of rRNA for ecological studies. *Applied and Environmental Microbiology* 59:915-918
- Morgan C A, Hudson A, Konopka A, Nakatsu C H (2002) Analyses of microbial activity in biomass-recycle reactors using denaturing gradient gel electrophoresis of 16S rDNA and 16S rRNA PCR products. *Canadian Journal of Microbiology* 48:333-341
- Morgan J A W, Bending G D, White P J (2005) Biological costs and benefits to plant-microbe interactions in the rhizosphere. *Journal of Experimental Botany* 56:1729-1739
- Morris S J (1999) Spatial distribution of fungal and bacterial biomass in southern Ohio hardwood forest soils: fine scale variability and microscale patterns. *Soil Biology and Biochemistry* 31:1375-1386
- Mounier E, Hallet S, Cheneby D, Benizri E, Gruet Y, Nguyen C, Piutti S, Robin C, Slezack-Deschaumes S, Martin-Laurent F, Germon J C, Philippot L (2004) Influence of maize mulch on the diversity and activity of the denitrifying community. *Environmental Microbiology* 6:301-312
- Mucha A P, Almeida C M R, Bordalo A A, Vasconcelos M T S D (2005) Exudation of organic acids by a marsh plant and implications on trace metal availability in the rhizosphere of estuarine sediments. *Estuarine, Coastal and Shelf Science* 65:191-198
- Muyzer G, de Waal E C, Uitterlinden A G (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695-700
- Nakatsu C H (2007) Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Science Society of America Journal* 71:562-571
- Nannipieri P, Ascher J, Ceccherini M T, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. *European Journal of Soil Science* 54:655-670
- Nehl D B, Knox O G (2006) Significance of bacteria in the rhizosphere. In: Mukerji K G, Manoharachary C, Singh J (eds) *Microbial Activity in the Rhizosphere*, Springer, Berlin. pp 89-120
- Neumann G (2006) Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) *Handbook of methods used in rhizosphere research*, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.
- Neumann G, George T S, Plassard C (2009) Strategies and methods for studying the rhizosphere—the plant science toolbox. *Plant and Soil* 321:431-456
- Neumann G, Massonneau A, Martinoia E, Römheld V (1998) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* 208:373-382
- Neumann G, Römheld V (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil* 211:121-130
- Neumann G, Römheld V (2007) The release of root exudates as affected by the plant's physiological status. In: Pinton R, Varanino Z, Nannipieri P (eds) *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*, CRC Press, New York. pp 23-72
- Nicol G W, Glover L A, Prosser J I (2003) Spatial analysis of archaeal community structure in grassland soil. *Applied and Environmental Microbiology* 69:7420-7429
- Nikata T, Sumida K, Kato J, Ohtake H (1992) Rapid method for analyzing bacterial behavioral responses to chemical stimuli. *Applied and Environmental Microbiology* 58:2250-2254
- Nocker A, Burr M, Camper A K (2007) Genotypic microbial community profiling: a critical technical review. *Microbial Ecology* 54:276-289
- Noll M, Matthies D, Frenzel P, Derakshani M, Liesack W (2005) Succession of bacterial community structure and diversity in a paddy soil oxygen gradient. *Environmental Microbiology* 7:382-395
- Norby R J, O'Neill E G, Hood W G, Luxmoore R J (1987) Carbon allocation, root exudation and mycorrhizal colonization of *Pinus echinata* seedlings grown under CO₂ enrichment. *Tree Physiology* 3:203-210
- Normander B, Prosser J I (2000) Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing condition. *Applied and Environment Microbiology* 66:4372-4377
- Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann R I, Ludwig W, Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of bacteriology*:5636-5643
- NZ Soil Bureau (1968) Map 9. Soil map of the South Island. In *Soil Bureau Bulletin 27*. Soil Bureau, Lower Hutt, N. Z., DSIR

- O'Callaghan M, Gerard E M, Bell N L, Waipara N W, Aalders L T, Baird D B, Conner A J (2008) Microbial and nematode communities associated with potatoes genetically modified to express the antimicrobial peptide magainin and unmodified potato cultivars. *Soil Biology and Biochemistry* 40:1446-1459
- O'Callaghan M, Lorenz N, Gerard E M (2006) Characterization of phylloplane and rhizosphere microbial populations using PCR and denaturing gradient gel electrophoresis (DGGE). In: Cooper J E, Rao J R (eds) *Molecular Approaches to Soil, Rhizosphere and Plant Microorganism analysis*, CABI publishing, Wallingford, UK. pp 99-115
- Oburger E, Kirk G J D, Wenzel W W, Puschenreiter M, Jones D L (2009) Interactive effects of organic acids in the rhizosphere. *Soil Biology and Biochemistry* 41:449-457
- Oger P, Mansouri H, Dessaux Y (2000) Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. *Molecular Ecology* 3:145-151
- Ogram A, Sun W, Brockman F J, Fredrickson J K (1995) Isolation and characterization of RNA from low-biomass deep-subsurface sediments. *Applied and Environmental Microbiology* 61:763-768
- Olsen R, Bakken L (1987) Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. *Microbial Ecology* 13:59-74
- Oros-Sichler M, Gomes N C M, Neuber G, Smalla K (2006) A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. *Journal of Microbiological Methods* 65:63-75
- Pace N R, Stahl D A, Lane D J, Olson G (1986) The analysis of natural microbial populations by ribosomal RNA sequences. *Advances in Microbial Ecology* 9:1-55
- Paleologos E K, Kontominas M G (2005) Determination of acrylamide and methacrylamide by normal phase high performance liquid chromatography and UV detection. *Journal of Chromatography A* 1077:128-135
- Pallansch L, Beswick H, Talian J, Zelenka P (1990) Use of an RNA folding algorithm to choose regions for amplification by the polymerase chain reaction. *Analytical biochemistry* 185:57-62
- Parke D, Rivelli M, Ornston L N (1985) Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifolii*. *Journal of Bacteriology* 163:417-422
- Parkin T B (1993) Spatial variability of microbial process in soil- a review. *Journal of Environmental Quality* 22:409-417
- Pasonen H-L, Degefu Y, Brumós J, Lohtander K, Pappinen A, Timonen S, Seppänen S-K (2005) Transgenic *Betula pendula* expressing sugar beet chitinase IV forms normal ectomycorrhizae with *Paxillus involutus* in vitro. *Scandinavian Journal of Forest Research* 20:385-392
- Paterson E (2003) Importance of rhizodeposition in the coupling of plant and microbial productivity. *European Journal of Soil Science* 54:741-750
- Paterson E, Gebbing T, Abel C, Sim A, Telfer G (2007) Rhizodeposition shapes rhizosphere microbial community structure in organic soil. *New Phytologist* 173:600-610
- Payne R W, Harding S A, Murray D A, Soutar D M, Baird D B, Welham S J, Kane A F, Gilmour A R, Thompson R, Webster R, Tunnicliffe Wilson G (2007) *The guide to GenStat release 10, part 2: Statistics*. VSN International, Hempel Hempstead
- Pennanen T, Caul S, Daniell T J, Griffiths B S, Ritz K, Wheatley R E (2004) Community-level responses of metabolically-active soil microorganisms to the quantity and quality of substrate inputs. *Soil Biology and Biochemistry* 36:841-848
- Philippot L, Hallin S, Börjesson G, Baggs E M (2009) Biochemical cycling in the rhizosphere having an impact on global change. *Plant and Soil* 321:61-81
- Phillips R P, Ertlitz Y, Bier R, Bernhardt E (2008) New approach for capturing soluble root exudates in forest soils. *Functional Ecology* 22:990-999
- Poly F, Monrozier L J, Bally R (2001) Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Research in Microbiology* 152:95-103
- Polz M F, Cavanaugh C M (1998) Bias in template-to-product ratios in multitemplate PCR. *Applied and Environmental Microbiology* 64:3724-3730

- Prikryl Z, Vancure V (1980) Root exudates of plants VI. Wheat root exudation as dependent on growth, concentration gradient of exudates and the presence of bacteria. *Plant and Soil* 57:69-83
- Purdy K J, Embley T M, Takii S, Nedwell D B (1996) Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. *Applied and Environmental Microbiology* 62:3905-3907
- Purkhold U, Pommerening-Röser A, Juretschko S, Schmid M C, Koops H-P, Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Applied and Environmental Microbiology* 66:5368-5382
- R Development Core Team, 2007. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [http:// www.R-project.org](http://www.R-project.org)
- Raaijmakers J M, Paulitz T C, Steinberg C, Alabouvette C, Moëgne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* 321:341-361
- Ramessar K, Peremarti A, Gómez-Galera S, Naqvi S, Moralejo M, Munoz P, Capell T, Christou P (2007) Biosafety and risk assessment framework for selectable marker genes in transgenic crop plants. A case of the science not supporting the politics. *Transgenic Research* 16:261-280
- Rees G N, Baldwin D S, Watson G O, Perryman S, Nielsen D L (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. *Antonie van Leeuwenhoek* 86:339-347
- Reid C P P (1974) Assimilation, distribution and root exudation of ^{14}C by ponderosa pine seedlings under induced water stress. *Plant Physiology* 54:44-49
- Reinhold B, Hurek T, Fendrik I (1985) Strain-specific chemotaxis of *Azospirillum spp.*. *Journal of Bacteriology* 162:190-195
- Renella G, Egamberdiyeva D, Landia L, Mench M (2006) Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. *Soil Biology and Biochemistry* 38:702-708
- Renella G, Landi L, Valori F, Nannipieri P (2007) Microbial and hydrolase activity after release of low molecular weight organic compounds by a model root surface in a clayey and a sandy soil. *Applied Soil Ecology* 36:124-129
- Rengel Z (2002) Genetic control of root exudation. *Plant and Soil* 245:59-70
- Rengel Z, Römheld V, Marschner H (1998a) Uptake of zinc and iron by wheat genotypes differing in zinc efficiency. *Journal of Plant Physiology* 152:433-438
- Rengel Z, Ross G, Hirsch P (1998b) Plant genotype micro-nutrient influence colonization of wheat roots by soil bacteria. *Journal of Plant Nutrition* 21:99-113
- Richardson A E, Barea J-M, McNeill A M, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil* 321:305-339
- Ritz K, McNicol J W, Nunan N, Grayston S, Millard P, Atkinson e D, Gollotte A, Habeshaw D, Boag B, Clegg C D, Griffiths B S, Wheatley R E, Glover L A, McCaig A E, Prosser J I (2004) Spatial structure in soil chemical and microbiological properties in an upland grassland. *FEMS Microbiology Ecology* 49:191-205
- Rivoal J, Hanson A D (1994) Metabolic control of anaerobic glycolysis – overexpression of lactate dehydrogenase in transgenic tomato roots supports the Davies–Roberts hypothesis and points to a critical role for lactate secretion. *Plant Physiology* 106:1179-1185
- Roelofs R F R, Rengel Z, Cawthray G R, Dixon K W, Lambers H (2001) Exudation of carboxylates in Australian Proteaceae: chemical composition. *Plant, Cell and Environment* 24:891-904
- Ros M, Pascual J, Hernandez M, Garcia C (2009) Long-term effects of revegetation on composition and activities (including transcription) of fungal communities of a semi-arid soil. *Biology and Fertility of Soils* 45:435-441
- Rosling A, Landeweert R, Lindahl B D, Larsson K-H, Kuyper T W, Taylor A F S, Finlay R D (2003) Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytologist* 159:775-783
- Rosset R, Julien J, Monier R (1966) Ribonucleic acid composition of bacteria as a function of growth rate. *Journal of Molecular Biology* 18:308-320

- Rovira A D (1959) Root excretions in relation to the rhizosphere effect. IV. Influence of plant species, age of plant, light, temperature and calcium nutrition on exudation. *Plant and Soil* 11:53-64
- Rovira A D, Davey C B (1974) Biology of the rhizosphere. In: Carson E W (ed) *The Plant Root and its Environment*, Univ. Virginia Press, Charlottesville. pp 153-204
- Rózycki H, Strzelczyk E (1986) Organic acids production by *Streptomyces* spp. isolated from soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestrus* L.). *Plant and Soil* 96:337-345
- Ryan P R, Delhaize E, Jones D, L. (2001) Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* 52:527-560
- Sandnes A, Eldhuset T D, Wollebak G (2005) Organic acids in root exudates and soil solution of Norway spruce and silver birch. *Soil Biology and Biochemistry* 37:259-269
- Sanguin H, Remenant B, Dechesne A, Thioulouse J, Vogel T M, Nesme X, Moëgne-Loccoz Y, LGrundmann G L (2006) Potential of 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on *Agrobacterium* spp. and bacterial communities. *Applied and Environmental Microbiology* 72:4302-4312
- Sanguinety C J, Dias Neto E, Simpson A J G (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *Biotechniques* 17:915-919
- Sangwan P, Kovac S, Davis K E R, Sait M, Janssen P H (2005) Detection and cultivation of soil verrucomicrobia. *Applied and Environmental Microbiology* 71:8402-8410
- Santos P E-D L, Bustillos-Cristales R, Caballero-Mellado J (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Applied and Environmental Microbiology* 67:2790-2798
- Scales P F, Peterson R L (1991) Structure of ectomycorrhizae formed by *Wilcoxina mikolae* var. *mikolae* with *Picea mariana* and *Betula alleghaniensis*. *Canadian Journal of Botany* 69:2149-2157
- Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pleban T, Perez-Melis A, Bruedigam C, Kopka J, Willmitzer L, Zamir D, Fernie A R (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. *Nature Biotechnology* 24:447-454
- Scheffe C R, Watt M, Slattery W J, Mele P M (2008) Organic anions in the rhizosphere of Al-tolerant and Al-sensitive wheat lines grown in an acid soil in controlled and field environments. *Australian Journal of Soil Research* 46:257-264
- Schimel J P, Gullledge J M, Clein-Curley J S, Lindstrom J E, Braddock J F (1999) Moisture effects on microbial activity and community structure in decomposing birch litter in the Alaskan taiga. *Soil Biology and Biochemistry* 31:831-838
- Schmalenberger A, Tebbe C C (2002) Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar *Bosphore*. *FEMS Microbiology Ecology* 40:29-37
- Schonwitz R, Ziegler H (1982) Exudation of water soluble vitamins and some carbohydrates by intact roots of maize seedlings (*Zea mays* L.) into a mineral nutrient solution. *Zeitschrift fur Pflanzenphysiologie* 107
- Scott J T, Condrón L M (2004) Short term effects of radiata pine and selected pasture species on soil organic phosphorus mineralisation. *Plant and Soil* 266:153-163
- Sekiguchi H, Tomioka N, Nakahara T, Uchiyama H (2001) A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. *Biotechnology letters* 23:1205-1208
- Seppänen S-K, Pasonen H-L, Vauramo S, Vahala J, Toikka M, Kilpeläinen I, Setälä H, Teeri T H, Timonen S, Pappinen A (2007) Decomposition of the leaf litter and mycorrhiza forming ability of silver birch with a genetically modified lignin biosynthesis pathway. *Applied Soil Ecology* 36:100-106
- Sessitsch A, Kan F Y, Pfeifer U (2003) Diversity and community structure of culturable *Bacillus* spp. populations in the rhizospheres of transgenic potatoes expressing the lytic peptide cecropin B. *Applied Soil Ecology* 22:149-158
- Sharma S, Aneja M K, Mayer J, Munch J C, Schloter M (2005) Characterization of bacterial community structure in rhizosphere soil of grain legumes. *Microbial Ecology* 49:407-415
- Shen Y, Ström L, Jönsson J, Tyler G (1996) Low-molecular organic acids in the rhizosphere soil solution of beech forest (*Fagus sylvatica* L.) cambisols determined by ion chromatography using supported liquid membrane enrichment technique. *Soil Biology and Biochemistry* 28:1163-1169

- Sheppard S K, Lloyd D (2002) Direct mass spectrometric measurement of gases in soil monoliths. *Journal of Microbiological Methods* 50:175-188
- Siciliano S D, Theoret C M, deFreitas J R, Hucl P J, Germida J J (1998) Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Canadian Journal of Microbiology* 44
- Simms P J, Jeffries C T, Zhao X, Huang Y, Arrhenius T (2004) Gradient elution of organic acids on a [beta]-cyclodextrin column in the polar organic mode and its application to drug discovery. *Journal of Chromatography A* 1052:69-75
- Singh G, Mukerji K G (2006) Root exudates as determinant of rhizospheric microbial biodiversity. In: Mukerji K G, Manoharachary C, Singh J (eds) *Microbial Activity in the Rhizosphere*, Springer, Berlin. pp 39-54
- Slankis V, Runeckles V C, Krotkov G (1964) Metabolites liberated by roots of white pine (*Pinus strobus* L.) seedlings. *Physiologia Plantarum* 17:301-313
- Smalla K, Heuer H, Milling A, Götz M, Schloter M, Lottmann J, Berg G (2004) Are microbial communities affected by genetically modified plants? *Aspects of Applied Biology* 74:127-140
- Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* 67:4742-4751
- Smit E, Leeflang P, Glandorf B, van Elsas J D, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology* 65:2614-2621
- Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S, Wernars K (2001) Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular Methods. *Applied and Environmental Microbiology* 67:2284-2291
- Smith W H (1969) Release of organic materials from the roots of tree seedlings. *Forest Science* 15:138-143
- Smith W H (1970) Root exudates of seedling and mature sugar maple. *Phytopathology* 60:701-703
- Smith W H (1972) Influence of artificial defoliation on exudates of sugar maple. *Soil Biology and Biochemistry* 4:111-113
- Smith W H (1976) Character and significance of forest tree root exudates. *Ecology* 57:324-331
- Sørensen J, Jensen L E, Nybroe O (2001) Soil and rhizosphere as habitats for *Pseudomonas* inoculants: new knowledge on distribution, activity and physiological state derived from micro-scale and single-cell studies. *Plant and Soil* 232:97-108
- Steenwerth K L, Drenovsky R E, Lambert J J, Kluepfel D A, Scow K M, Smart D R (2008) Soil morphology, depth and grapevine root frequency influence microbial communities in a Pinot noir vineyard. *Soil Biology and Biochemistry* 40:1330-1340
- Stevenson F J (1967) Organic acids in soil. In: McLaren A D, Peterson G H (eds) *Soil Biochemistry*, Marcel Dekker Ltd. pp 119-146
- Strobel B W, Hansen H C B, Borggaard O K (1999) Low-molecular-weight aliphatic carboxylic acids in soil solutions under different vegetations determined by capillary zone electrophoresis. *Plant and Soil* 212:115-121
- Strobel B W, Hansen H C B, Borggaard O K, Andersen M K, Raulund-Rasmussen K (2001) Composition and reactivity of DOC in forest floor soil solutions in relation to tree species and soil type. *Biogeochemistry* 56:1-26
- Strzelczyk E, Pokojska-Burdziej A (1984) Production of auxins and gibberellin-like substances by mycorrhizal fungi, bacteria and actinomycetes isolated from soil and the mycorrhizosphere of pine (*Pinus sylvestris* L.). *Plant and Soil* 81:185-194
- Sun Y-P, Unestam T, Lucas S D, Johanson K J, Kenne L, Finlay R D (1999) Exudation-reabsorption in mycorrhizal fungi, the dynamic interface for interaction with soil and other microorganisms. *Mycorrhiza* 9:137-144
- Tamasloukht M, Waschke A, Franken P (2007) Root exudate-stimulated RNA accumulation in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Soil Biology and Biochemistry* 39:1824-1827
- Tang C-S, Young C-C (1982) Collection and identification of allelopathic compounds from the undisturbed root system of Bigalta limpgrass (*Hemarthria altissima*). *Plant Physiology* 69:155-160

- Taylor H M, Upchurch D R, McMichael B L (1990) Applications and limitations of rhizotrons and minirhizotrons for root studies. *Plant and Soil* 129:29-35
- Taylor J P, Wilson B, Mills M S, Burns R G (2002) Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques. *Soil Biology and Biochemistry* 34:387-401
- Tejada M, Hernandez M T, Garcia C (2006) Application of two organic amendments on soil restoration: effects on the soil biological properties. *Journal of Environmental Quality* 35:1010-1017
- Tesfaye M, Dufault N S, Dornbusch M R, Allan D L, Vance C P, Samac D A (2003) Influence of enhanced malate dehydrogenase expression by alfalfa on diversity of rhizobacteria and soil nutrient availability. *Soil Biology and Biochemistry* 35:1103-1113
- Tesfaye M, Temple S J, Allan D L, Vance C P, Samac D A (2001) Over-expression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. *Plant Physiology* 127:1836-1844
- Thomson J A (2006) GM crops: the impact and the potential. CSIRO Publishing, Collingwood VIC
- Thornton B, Paterson E, Midwood A J, Sim A, Pratt S M (2004) Contribution of current carbon assimilation in supplying root exudates of *Lolium perenne* measured using steady-state ¹³C labeling. *Physiologia Plantarum* 120:434-441
- Timmusk S, Nicander B, Granhall U, Tillberg E (1999) Cytokinin production by *Paenibacillus polymyxa*. *Soil Biology and Biochemistry* 31:1847-1852
- Toyota K, Ritz K, Kuninaga S, Kimura M (1999) Impact of fumigation with metam sodium upon soil microbial community structure in two Japanese soils. *Soil Science and Plant Nutrition* 45:207-223
- Trevors J T (1996) DNA in soil: adsorption, genetic transformation, molecular evolution and genetic microchip. *Antonie van Leeuwenhoek* 70:1-10
- Tsai S M, Phillips D A (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus spores in vitro*. *Applied and Environmental Microbiology* 57:1485-1488
- Tully R (1988) Synthesis of exopolysaccharide by *Brayrhizobium japonicum* during growth on hydroaromatic substrates. *Applied and Environmental Microbiology* 54:1624-1626
- Tzeneva V A, Salles J F, Naumova N, de Vos W M, Kuikman P J, Dolfin J, Smidt H (2009) Effect of soil sample preservation, compared to the effect of other environmental variables, on bacterial and eukaryotic diversity. *Research in Microbiology* 160:89-98
- Ulrich A, Becker R (2006) Soil parent material is a key determinant of the bacterial community structure in arable soils. *FEMS Microbiology Ecology* 56:430-443
- Uren N C (2007) Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In: Pinton R, Varanini Z, Nannipieri P (eds) *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*, CRC Press, New York. pp 1-22
- Vainio E J, Hantula J (2000) Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research* 104:927-936
- Valenzuela S, Balocchi C, Rodriguez J (2006) Transgenic trees and forest biosafety. *Electronic Journal of Biotechnology* 9:335-339
- van Frankenhuyzen K, Beardmore T (2004) Current status and environmental impact of transgenic forest trees. *Canadian Journal of Forest Research* 34:1163-1180
- van Hees P A, Rosling A, Essén S, Godbold D L, Finlay R D (2006) Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with *Pinus sylvestris*. *New Phytologist* 169:367-378
- van Hees P A W, Dahlén J, Lundström U S, Borén H, Allard B (1999) Determination of low molecular weight organic acids in soil solution by HPLC. *Talanta* 48:173-179
- van Hees P A W, Jones D L, Godbold D L (2002) Biodegradation of low molecular weight organic acids in coniferous forest podzolic soils. *Soil Biology and Biochemistry* 34:1261-1272
- van Hees P A W, Vinogradoff S I, Edwards A C, Godbold D L, Jones D L (2003) Low molecular weight organic acid adsorption in forest soils: effects on soil solution concentrations and biodegradation rates. *Soil Biology and Biochemistry* 35:1015-1026
- van Schöll L, Hoffland E, van Breemen N (2006) Organic anion exudation by ectomycorrhizal fungi and *Pinus sylvestris* in response to nutrient deficiencies. *New Phytologist* 170:153-163
- van Verseveld H W, Röling W F M (2004) Cluster analysis and statistical comparison of molecular community profile data. In: Kowalchuk G A, de Bruijn F J, Head I M, et al. (eds) *Molecular Microbial Ecology Manual*, Kluwer Academic Publishers, Dordrecht, The Netherlands. pp 1373-1396

- Vance G F, Stevenson F J, Sikora F J (1996) Environmental chemistry of aluminium-organic complexes. In: Sposito G (ed) *The Environmental Chemistry of Aluminum*, CRC Press. pp 169-220
- Vandenkoornhuysen P, Baldauf S L, Leyval C, Straczek J, Peter J, Young W (2002) Extensive fungal diversity in plant roots. *Science* 295:2051
- Vergnaud L, Chaboud A, Rougier M (1987) Preliminary analysis of root exudates of in vitro-micropropagated *Alnus glutinosa* clones. *Physiologia Plantarum* 70:319-326
- von der Weid I, Paiva E, Nobrega A, van Elsas J D, Seldin L (2000) Diversity of *Paenibacillus polymyxa* strains isolated from the rhizosphere of maize planted in Cerrado soil. *Research in Microbiology* 151:369-381
- von Wintzingerode F, Göbel U B, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews* 21:213-229
- Wagner M, Amann R, Lemmer H, Schleifer K H (1993) Probing activated sudge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology* 59:1520-1525
- Wagner R (1994) The regulation of ribosomal RNA synthesis and bacterial cell growth. *Archives of Microbiology* 161:100-109
- Wakelin S A, Macdonalda L M, Rogers S L, Gregga A L, Bolgerd T P, Baldock J A (2008) Habitat selective factors influencing the structural composition and functional capacity of microbial communities in agricultural soils. *Soil Biology and Biochemistry* 40:803-813
- Walbert K (2008) Ectomycorrhizal communities associated with a *Pinus radiata* plantation in the North Island, New Zealand. In Bioprotection and Ecology Division. Lincoln University, Canterbury
- Walker T S, Bais H P, Grotewold E, Vivanco J M (2003) Root exudation and rhizosphere biology. *Plant Physiology* 132:44-51
- Walter C (2004) Genetic engineering in conifer forestry: Technical and social considerations. In *Vitro Cellular and Developmental Biology* 40:434-441
- Wang G C-Y, Wang Y (1997) Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16S rRNA genes from mixed bacterial genomes. *Applied and Environmental Microbiology* 63:4645-4650
- Wang P, Zhou R (2006) Determination of organic acids exudated from plant roots by high performance liquid chromatography. *Chinese Journal of chromatography* 24:239-242
- Wang P, Zhou R, Cheng J, Bi S (2007) LC Determination of Trace Short-Chain Organic Acids in Wheat Root Exudates Under Aluminum Stress. *Chromatographia* 66:867-872
- Wang Z Y, Kelly J M, Kovar J L (2004) *In situ* dynamics of phosphorus in the rhizosphere solution of five species. *Journal of Environmental Quality* 33:1387-1392
- Ward D M, Weler R, Bateson M M (1990) 16S Ribosomal-RNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 345:63-65
- Wardle D A (2002) *Communities and ecosystems: linking the aboveground and belowground components*. Princeton University Press, Princeton, Oxford
- Watkins A J, Nicol G W, Shaw L J (2009) Use of an artificial root to examine the influence of 8-hydroxyquinoline on soil microbial activity and bacterial community structure. *Soil Biology and Biochemistry* 41:580-585
- Watt M, Evans J R (1999) Linking development and determinacy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO₂ concentration. *Plant Physiology* 120:705-716
- Watt M, Hugenholtz P, White R, Vinall K (2006a) Numbers and locations of native bacteria on field grown wheat roots quantified by fluorescence *in situ* hybridization (FISH). *Environmental Microbiology* 85:871-884
- Watt M, Silk W K, Passioura J B (2006b) Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. *Annals of Botany* 97:839-855
- Watts J E M (1999) Analysis of microbial diversity in polluted and nonpolluted soils: a comparison of genetic, functional and cultured based techniques. In University of Warwick. United Kingdom, Warwick
- Weigel D, Alvarez J, Smyth D R, Yanofsky M F, Meyerowitz E M (1992) LEAFY controls floral meristem identity in Arabidopsis. *Cell* 69:843-859
- Weisburg W G, Barns S M, Pelletier D A, Lane D J (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173:697-703

- Weisskopf L, Le Bayon R C, Kohler F, Page V, Jossi M, Gobat J M, Martinoia E, Aragno M (2008) Spatio-temporal dynamics of bacterial communities associated with two plant species differing in organic acid secretion: A one-year microcosm study on lupin and wheat. *Soil Biology and Biochemistry* 40:1772-1780
- Wells C E, Eissenstat D M (2001) Marked differences in survivorship among apple roots of different diameters. *Ecology* 82:882-892
- Whipps J M, Lynch J M (1986) The influence of the rhizosphere on crop productivity. *Advances in Microbial Ecology* 6:187-244
- White D C, Findlay R H (1988) Biochemical markers for measurement of predation effects on the biomass, community structure, nutritional-status, and metabolic-activity of microbial biofilms. *Hydrobiologia* 159:119-132
- White T J, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M A, Gelfand D H, Sninsky J J, et al. (eds) *PCR protocols: a guide to methods and applications*, Academic Press, New York. pp 315-322
- Wickham B, Watson M (1991) New Zealand: a good option for wood fibre processing. *New Zealand forestry Bulletin* Oct.:4-5
- Widmer F, Seidler R J, Gillevet P M, Watrud L S, Di Giovanni G D (1998) A highly selective PCR protocol for detecting 16S rRNA genes of the genus *Pseudomonas* (Sensu Stricto) in environmental samples. *Applied and Environmental Microbiology* 64:2545-2553
- Wirén N V, Römheld V, Shioiri T, Marschner H (1995) Competition between micro-organisms and roots of barley and sorghum for iron accumulated in the root apoplasm. *New Phytologist* 130:511-521
- Yan W D, Shi W M, Li B H, Zhang M (2007) Overexpression of a foreign Bt gene in cotton affects the low-molecular-weight components in root exudates. *Pedosphere* 17:324-330
- Yang C-H, Crowley D E (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied and Environmental Microbiology* 66:345-351
- Yergeau E, Schoondermark-Stolk S A, Brodie E L, Déjean S, DeSantis T Z, Gonçalves O, Piceno Y M, Andersen G L, Kowalchuk G A (2009) Environmental microarray analyses of Antarctic soil microbial communities. *The ISME Journal* 3:340-351
- Yuste J C, Baldocchi D D, Gershenson A, Goldstein A, Misson L, Wong S (2007) Microbial soil respiration and its dependency on carbon inputs, soil temperature and moisture. *Global Change Biology* 13:2018-2035
- Zantua M I, Bremner J M (1976) Production and persistence of urease activity in soils. *Soil Biology and Biochemistry* 8:369-374
- Zhang J, Neumann G, George E (2001) Organic acid exudation by roots of Norway spruce grown in hydroponic culture or in soil. In: Horst W J, Schenk M K, Bürkert A, et al. (eds) *Plant nutrition- food security and sustainability of agro-ecosystems*, Kluwer Academic Publishers. pp 564-565
- Zogg G, Zak D, Ringelberg D, Macdonald N, Pregitzer K, White D (1997) Compositional and functional shifts in microbial communities due to soil warming. *Soil Science Society of America Journal* 67:475-481

Personal Communication References

J. Lottmann, personal communication, February, 2007

D. Baird, personal communication, August 2008 to May 2009

J. Lottmann, personal communication, June 2009

C. Walter, personal communication, November, 2007

C. Walter, personal communication, July, 2008

C. Walter, Personal communication, June 2009

A. Richardson, personal communication, October 2009

Appendix 2.1 *Pinus radiata* Grown in Pot

Two radiata pine seedlings were grown in one pot (30 cm top diameter x 40 cm depth) filled with soil supplied by a local garden centre and sieved through a 5 mm sieve (Figure A2.1-1a). Pine seedlings were grown in a Conviron BDW120 growth room at the Biotron with the conditions described in Chapter 3, Section 3.2.3. After nine months growth, the radiata pine seedlings were harvested by removing them from the pot. The root systems were carefully removed and shaken out by hand (Figure A2.1-1b). The root samples with associated rhizosphere soils were sampled using an ethanol (70%) sterilised scissors and processed for rhizosphere soil according to the method described in Chapter 2, Section 2.2.1. Non-rhizosphere soils were also collected from the pot. The obtained soils were used for molecular method development as described in Chapter 2, Sections 2.2.2 to 2.2.7 (also see Appendix 2.2 and Appendix 2.5).



Figure A2.1-1 a). Young radiata pine trees grown in a pot in a growth room in the Biotron. **b).** Sampling pine trees by removing the trees from the pot.

Appendix 2.2 Selection of Nucleic Acid Extraction Method and Lysing Matrix Tubes

Objective

To select the most suitable RNA extraction method for this project.

Methods

Prior to RNA extraction, all solutions and glassware were rendered RNase free as described in Chapter 2, Section 2.2.2.1. The nucleic acid extraction method was developed using limited amounts of rhizosphere soil samples obtained from radiata pine grown in the pot (Appendix 2.1) by comparing two nucleic acid extraction methods and then four types of Lysing Matrix tubes using the selected method. The rhizosphere soils obtained from root samples ranged from 0.028 to 0.500 g.

1. Extraction method comparison

CTAB extraction method

The CTAB method was modified based on the method described by Griffiths et al. (2000). The detailed protocol is described in Chapter 2, Section 2.2.2.2

Sodium phosphate buffer (SPB) extraction method

This method was adapted from McKew et al. (2007). Briefly, soil (up to 0.5 g) and 0.5 ml of SPB extraction buffer (0.1 M; pH 8) were added into a Lysing Matrix B tube and mixed briefly by vortex prior to the addition of 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0) (Sigma-Aldrich Inc., USA). The tube was vortexed for 5 min, incubated on ice for 2 min then vortexed for another 5 min. The aqueous phase containing nucleic acids was separated by centrifugation at 13,000 g for 5 min at 4°C, and transferred to a new 2 ml tube, followed by the addition of an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich Inc.). The contents in the tube were mixed well prior to centrifugation at 13,000 g for 5 min at 4°C, after which the aqueous phase was transferred into a new 2 ml tube. The total nucleic acids were then precipitated by adding 2.5 volumes of ice-cold 100% ethanol and 1/10 volume of sodium acetate (3 M, pH 5.8) at -20°C overnight, followed by centrifugation at 13,000 g at 4°C for 30 min. The supernatant was carefully discarded and the pellet was washed with ice-cold 70% ethanol, followed by centrifugation for 10 min at 13,000 g at 4°C. This was repeated twice to clean the extracted nucleic acids. The resulting pellet was air dried for 5 min at room temperature prior to resuspension in 50 µl RNase free water (Applied Biosystems).

The main differences in these two methods were in the extraction buffer (CATB vs. NaPO₄), the precipitate solutions (PEG/NaCl vs. ethanol/sodium acetate), and precipitation times and conditions (2 h in room temperature vs. overnight at -20°C).

Four rhizosphere soil samples obtained from roots of radiata pine grown in a pot (Appendix 2.1) were extracted by both methods, and 5 µl of extracted nucleic acids were run on 1% agarose gel in 1x TAE buffer (0.04 M Tris-acetate, 1mM EDTA; pH 8.5) under 100 V for 50 min followed by ethidium bromide staining for detection.

2. Selection of Lysing Matrix tubes

Initially in the extraction method comparison experiment, Lysing Matrix B tubes were used according to the manufacturer’s description that the tube is suitable for isolating total RNA from Gram positive and Gram negative bacteria. Other Lysing Matrix tubes are available and four, including the Lysing Matrix tube B, were selected to determine the best nucleic acid extraction from bacteria as well as fungi in soil samples (Table A2.2-1).

Table A2.2-1 Details of Lysing Matrix tubes used and the manufacturers recommended isolation usage.

Lysing matrix tube	Beads inside the tubes	Isolation usage
Lysing Matrix B	0.1 mm silica spheres	Isolation of total RNA from Gram positive and Gram negative bacteria.
Lysing Matrix C	1.0 mm silica spheres	Isolation of total RNA from yeast and fungi.
Lysing Matrix D	1.4 mm ceramic spheres	Isolation of total RNA from plants and animals.
Lysing Matrix E	1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead	Isolation of any type of DNA found in soil or other environmental samples

Four subsamples of one non-rhizosphere soil and two rhizosphere soil samples associated with root samples collected from the top root zone were tested using four Lysing Matrix tubes B, C, D, and E (Q-Biogene, USA). Rhizosphere soil and non-rhizosphere soil were transferred into the tubes and were processed using the CTAB extraction method as described in Chapter 2, Section 2.2.2. Aliquots of 5 µl nucleic acid samples were detected by electrophoresis on 1% agarose gel and ethidium bromide staining. The quality of nucleic acid samples was determined by spectrophotometry using the Nanodrop with ratios of absorbance at 260 nm/230 nm and 260 nm/280 nm.

PCR was carried out using cDNA templates derived from RNA samples extracted using Lysing Matrix B and E for further comparison. The preparation of RNA and cDNA (bacterial and fungal) are described in Chapter 2, Section 2.2.3. Bacterial 16S and fungal

18S gene sequences were amplified according to the methods described in Chapter 2, Sections 2.2.4 and 2.2.6, respectively, and 5 μ l of the PCR products were visualised on a 1% agarose gel with ethidium bromide staining.

Results and discussion

1. Nucleic acid extraction method development

Even though limited amounts of rhizosphere soil were obtained from root samples (ranging from 0.028 to 0.50 g), both of the extraction methods were able to extract RNA and DNA from all the samples (Figure A2.2-1). Nucleic acids extracted by the CTAB method showed much sharper bands on the agarose gel in comparison to those extracted by the SPB method. The colour of nucleic acids extracted by the SPB method was brown, while those extracted by the CTAB method were clear. The ratios of absorbance at 260 nm/280 nm in both samples were similar, while the ratios of 260 nm/230 nm for samples extracted by the SPB method (0.8 in average) were much smaller compared to those extracted by the CTAB method (average of 2.2). These results indicate that a cleaning step will be needed to remove co-extracted contaminating compounds, such as humic acids, from nucleic acid samples extracted by the SPB method. Additionally, the overnight precipitation in the SPB method made the extraction process much longer in comparison with the CTAB method. Therefore, the CTAB extraction method was used in the subsequent experiments.

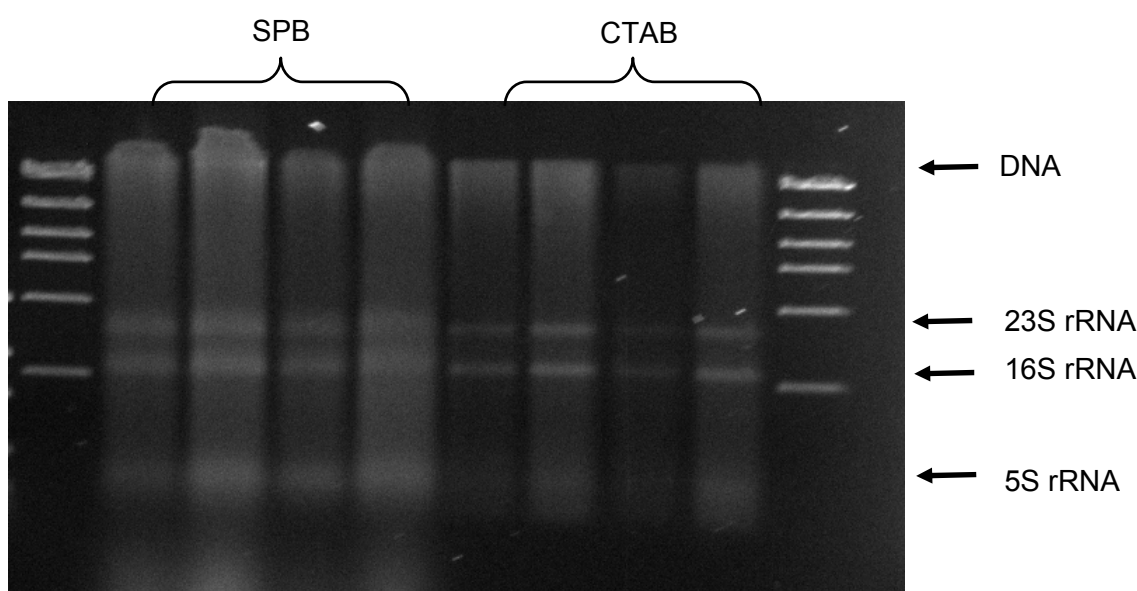


Figure A2.2-1 Nucleic acids extracted from rhizosphere soil samples using two different methods (CTAB vs. SPB). Left and right lanes were low DNA mass ladder (Invitrogen, USA) with band size (from top to bottom): 2000 bp, 1200 bp, 800 bp, 400 bp, 200 bp and 100 bp. DNA and RNA bands position in agarose gel are indicated by arrows.

2. Lysing Matrix tube selection

Four types of Lysing Matrix tubes were compared for the best nucleic acid extraction and higher amounts of nucleic acids were extracted by using Lysing Matrix B and E when compared to the other two types of tubes as quantified by both agarose gel (Figure A2.2-2) and Nanodrop (data not shown). The quality of RNA measured as ratios of absorbance at 260 nm/280 nm and 260 nm/230 nm by Nanodrop was similar in all the samples using either type of the Lysing Matrix tubes.

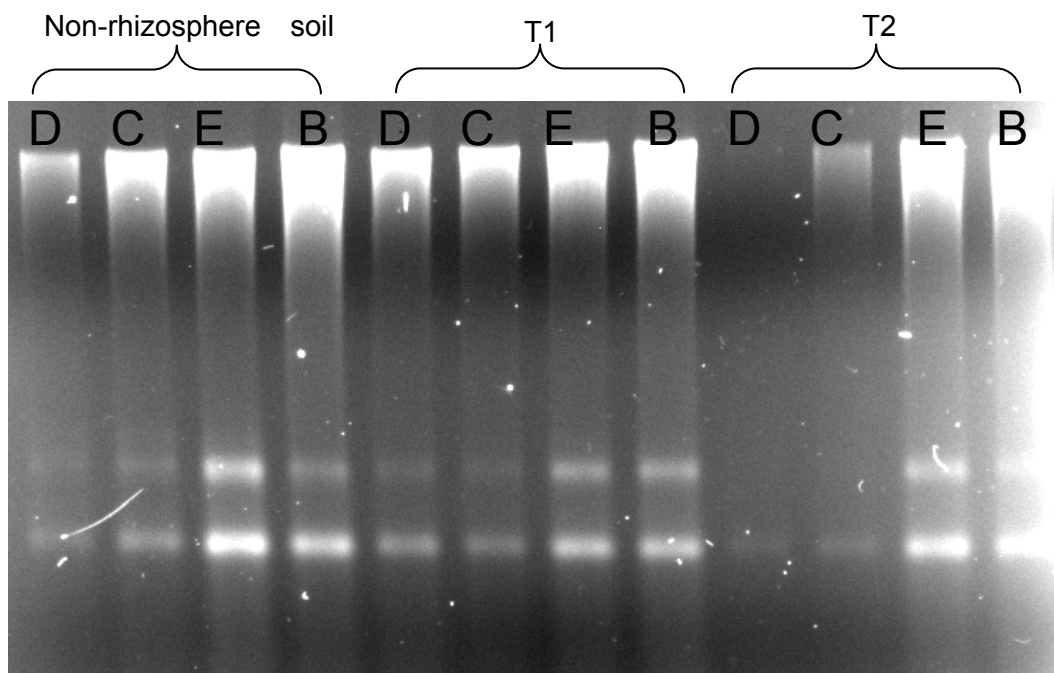


Figure A2.2-2 Nucleic acids extracted from three soil samples using four different types of Lysing Matrix tubes by the CTAB extraction method. D, C, E, B: Lysing Matrix tube types. T1, T2: rhizosphere soil samples associated with the root samples collected from the top root zone of radiata pine grown in the pot. Non-rhizosphere soil: composite non-rhizosphere soil collected from the area free of roots in the pot.

Further comparison was carried out in amplifications of bacterial 16S and fungal 18S sequences between cDNA samples derived from those extracted RNA samples using Lysing Matrix B and E tubes. All bacterial 16S PCR products with templates extracted from both types of extraction tubes showed strong bands on agarose gels (data not shown). Similarly, all the tested cDNA templates could be amplified by fungal 18S rRNA primer set (Figure A2.2-3). Consistent band densities of fungal 18S products were present in all three samples using Lysing Matrix B tube. One of the samples used Lysing matrix E showed very low density, while the other two showed much higher density (Figure A2.2-3).

As quality RNA samples could be obtained using the CTAB method with Lysing Matrix B tube, this type of tube was chosen for use in this project. Easily degraded RNA may have

better protection in Lysing Matrix B tubes which is designed for RNA isolation (Table A2.2-1).

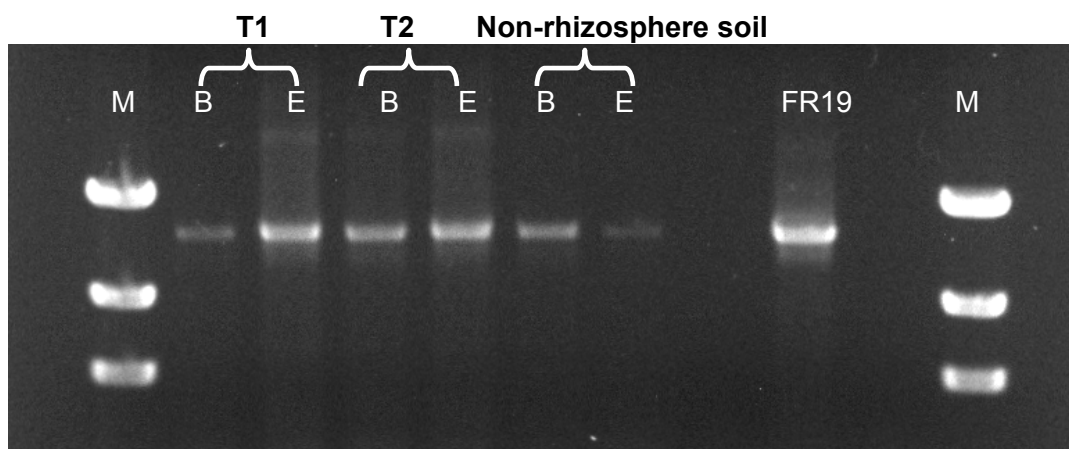


Figure A2.2-3 Fungal 18S PCR products on 1% agarose gel using cDNA templates derived from samples extracted using two different Lysing Matrix tubes (B or E) from soils. T1, T2: two rhizosphere soil samples associated with the root samples collected from the top root zone of radiata pine grown in the pot. Non-rhizosphere soil: composite non-rhizosphere soil collected from the area free of roots in the pot. M: low DNA mass ladder with band size (from top to bottom): 2000 bp, 1200 bp, 800 bp. FR19: *Trichoderma reesei* strain FR19 used as a positive control.

Conclusion

In summary, the CTAB extraction method adapted from Griffiths et al. (2000) with Lysing Matrix B tube was able to extract reasonable amounts of good quality RNA and DNA from limited amounts of soil samples (ranging from 0.028 to 0.5 g). No obvious PCR inhibition was observed. This nucleic acid extraction method was used for subsequent experiments throughout this project.

References

- Griffiths R I, Whiteley A S, O'Donnell A G, Bailey M J (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environment Microbiology* 66:5488-5491
- McKew B A, Coulon F A, Osborn M, Timmis K N, McGenity T J (2007) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environmental Microbiology* 9:165-176

Appendix 2.3 Solutions and Reagent Preparation

CTAB extraction buffer

0.7M NaCl: dissolve 4.09 g of NaCl in 100 ml DI water

10% CTAB: dissolve 10 g CTAB in 100 ml of 0.7M NaCl

240 mM potassium phosphate buffer (pH 8.0): dissolve 5.48 g $K_2HPO_4 \cdot 3H_2O$ in 100ml DI water. In another bottle, dissolve 3.266 g KH_2PO_4 in 100 ml DI water. Adjust pH of K_2HPO_4 solution using 240 mM KH_2PO_4 solution to 8.0.

CTAB extraction buffer: mix 100 ml of 10% CTAB with 100ml of 240mM potassium phosphate buffer (pH 8.0). Add 200 μ l DEPC and shake vigorously for a while and leave it in the shaker at 150 rpm at 22 °C overnight. Autoclave twice at 121°C for 20 min.

PEG 6000-1.6M NaCl

1.6 M NaCl: dissolve 9.35 g NaCl in 100 ml DI water.

PEG 6000-1.6M NaCl: dissolve 30 g PEG 6000 in 100 ml of 1.6 M NaCl. Mix well and add 100 μ l DEPC and shake vigorously for a while and leave it in the shaker at 150 rpm at 22°C overnight. Autoclave twice at 121°C for 20 min.

0.1M Sodium phosphate buffer (pH 8.0)

0.1M Na_2HPO_4 : dissolve 2.84 g Na_2HPO_4 in 200 ml DI water.

0.1M NaH_2PO_4 : dissolve 3.12 g $NaH_2PO_4 \cdot 2H_2O$ in 200 ml DI water.

0.1M sodium phosphate buffer: Adjust pH of Na_2HPO_4 solution using NaH_2PO_4 solution to 8.0. Take 200 ml of sodium phosphate buffer to a new bottle and add 200 μ l DEPC and shake vigorously for a while and leave it in the shaker at 150 rpm at 22 °C overnight. Autoclave twice at 121°C for 20 min.

Sodium Acetate (pH 5.8)

Dissolve 49.218 g sodium acetate in 200 ml DI water and adjust pH to 5.8 using HCl. Add 200 μ l DEPC and shake vigorously for a while and leave it in the shaker at 150 rpm at 22 °C overnight. Autoclave twice at 121°C for 20 min.

50 x Tris-Acetate EDTA (TAE) buffer

Dissolve 242 g Tris base, 18.6 g EDTA in 900 ml DI water. Add 57.1 ml glacial acetic acid into the solution and mix well. Adjust volume to 1 l with DI water. Autoclave. 1x TAE was prepared by diluting 50 times of this 50 x stock solution.

Appendix 2.4 Solution Preparation for DGGE and Silver Staining of Gels

- DGGE

Recipe for 0% and 100% denaturant solution

denaturing polyacrylamide	0%		100%	
Polyacrylamide	6%	8%	6%	8%
40% Acrylamide:Bisacrylamide (37:5:1) (Bio-Rad, USA)	15	20	15	20
Urea (g)	0	0	42	42
Formamide (ml)	0	0	40	40
50x TAE (ml)	1	1	1	1
Glycerol (ml)	2	2	2	2
DI water (Millipore) (ml)	to 100	to 100	to 100	to 100

Store in the dark at room temperature (20°C), low heat ($\leq 37^\circ\text{C}$) to dissolve urea.

2X DGGE Loading Dye

Reagent	Amount (ml)	Final concentration (%)
2 % Bromophenol blue	0.25	0.05
2 % Xylene cyanol	0.25	0.05
100 % Glycerol	7.0	70
DI water	2.5	-
Total volume	10.0	-

Store at room temperature.

- Solutions for Silver Staining of DGGE gels

Cairns' 8x fixation solution

200 ml 96 % ethanol

10 ml acetic acid

40 ml DI water

1x Cairns' = 50 ml 8xCairns' fixing solution to 350 ml distilled water

Silver staining solution

0.4 g AgNO_3 in 1x Cairns' fixing solution (200 ml)

Developer

A spatula tip of NaBH_4 (approx. 10 mg)

250 ml 1.5 % NaOH solution

750 μl formaldehyde

Cairn's preservation solution

250 ml 96 % ethanol

100 ml glycerol

650 ml distilled water

Appendix 2.5 rRNA-DGGE Gels

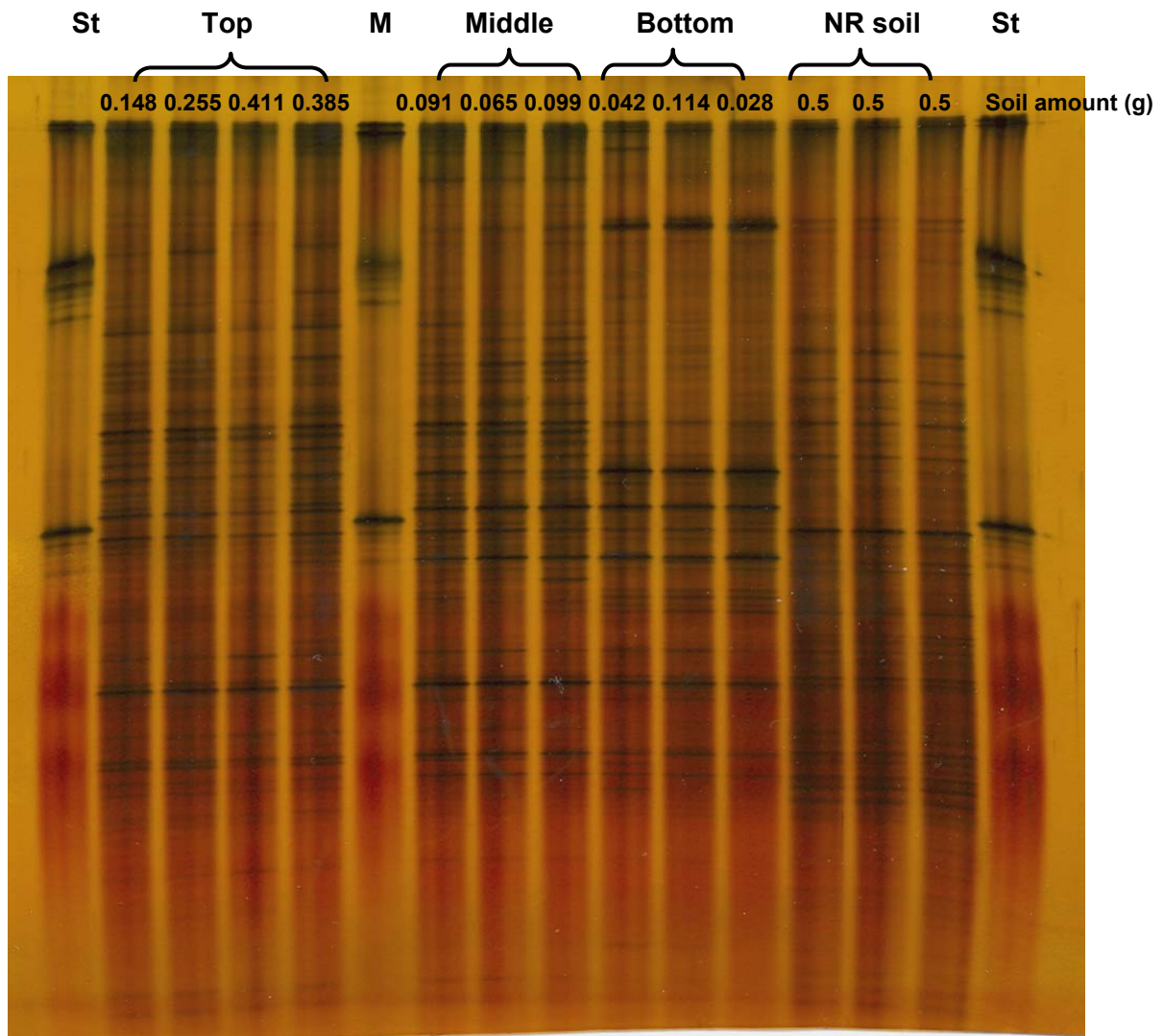


Figure A2.5-1 rRNA-DGGE profiles of general bacterial 16S in the non-rhizosphere soil and rhizosphere soil associated with the *Pinus radiata* root samples collected at various depths. St: bacterial 16S DGGE marker consisted of bulked bacterial 16S PCR products from strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp.. Top: rhizosphere soils associated with root samples collected at the depth of 0-10 cm ($n=4$); Middle: rhizosphere soils associated with root samples collected at the depth of 10-25 cm ($n=3$); Bottom: rhizosphere soils associated with root samples collected at the depth of 25-40 cm ($n=3$); NR soil: composite non-rhizosphere soil collected at area free of roots. The amounts of soils (g) used for nucleic acid extractions and subsequently for bacterial community analysis are indicated on top of gels.

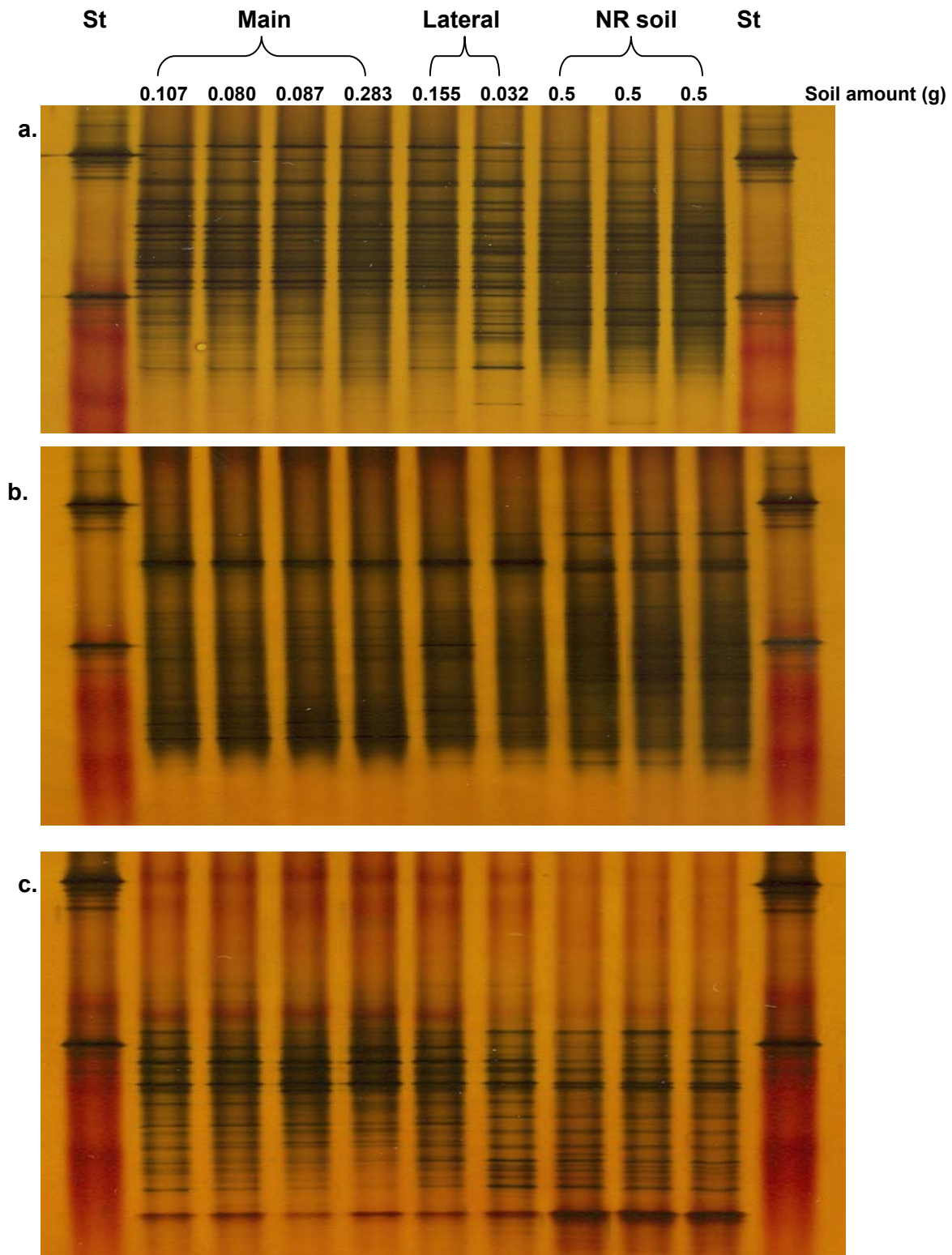


Figure A2.5-2 rRNA-DGGE profiles of taxon-specific 16S rRNA in the non-rhizosphere soil and rhizosphere soil associated with different root classes of *Pinus radiata* grown in a pot. a. α -proteobacterial 16S; b. β -proteobacterial 16S; c. pseudomonad 16S. St: bacterial 16S DGGE marker (see Figure A2.5-1). Main: rhizosphere soil associated with main roots ($n=4$); Lateral: rhizosphere soil associated with first lateral roots ($n=2$); NR soil: composite non-rhizosphere soil collected at area free of roots ($n=3$). The amounts of soils (g) used for nucleic acid extractions and subsequently for taxon-specific community analysis are indicated on top of gels.

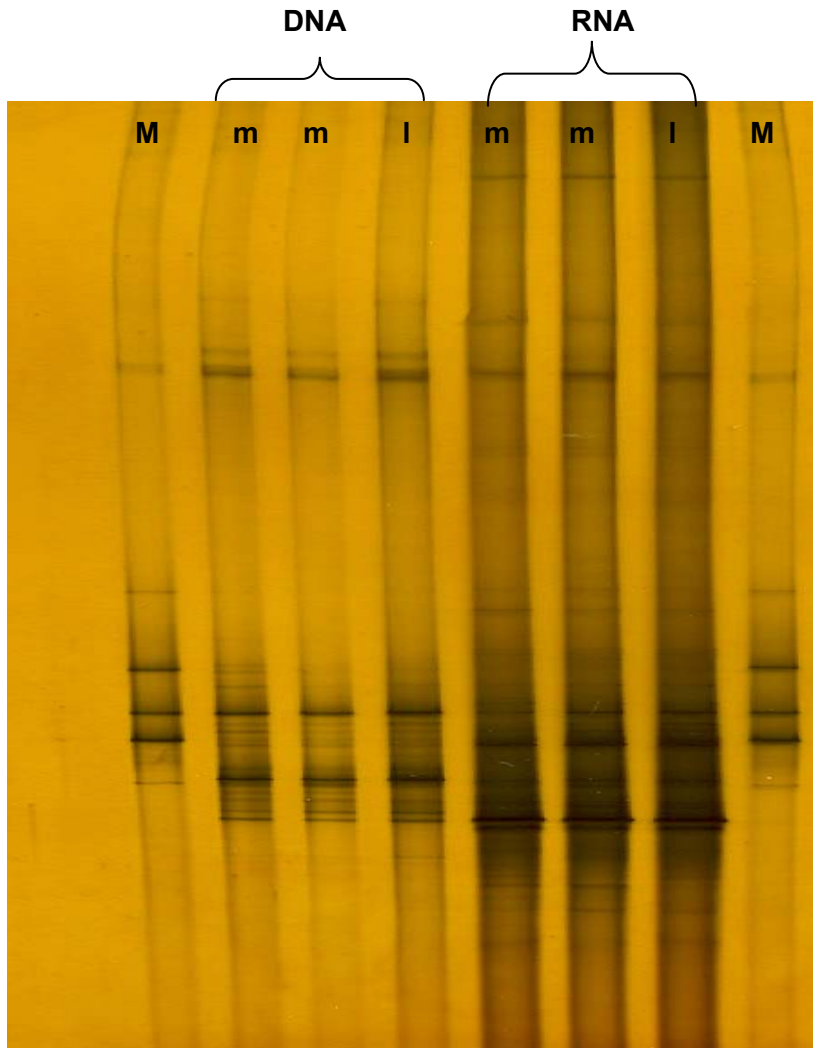


Figure A2.5-3 DGGE profiles of fungal 18S (amplified from DNA templates and cDNA samples derived from RNA templates) in the rhizosphere soil associated with different root classes of *Pinus radiata* grown in a pot. M: fungal 18S DGGE marker consisted of bulked fungal 18S PCR products from strain *Umbelopsis ramanniana*, *Trichoderma reesei*, *Mortierella chlamydospora*, *Phoma cucurbitacearum*, *Clonostachys rosea*. m: rhizosphere soil associated with main roots ($n=2$); l: rhizosphere soil associated with first lateral roots ($n=1$). DNA: samples derived from DNA templates; RNA: samples derived from RNA templates.

Appendix 3.1 Detection and Selection of Compounds for Radiata Pine Root Exudates Analysis

Objective

Due to the limited information on the composition of root exudates of radiata pine, a GC-MS scan of root exudate samples from *Pinus radiata* seedlings was carried out to identify exudate compounds. Based on this scan, a number of OAs were selected for root exudates analysis by HPLC at Lincoln University.

Methods

Ten radiata pine seedlings (approximate 25 cm tall) were grown in polyvinyl chloride pots (7.5 cm diameter x 14.5 cm depth) filled with clean river sand in a growth room at 20/12°C (day/night) in the Biotron (Figure A3.1-1a). Other conditions of the growth room were described in Chapter 3, Section 3.2.3. The seedlings were treated with Hoagland's nutrient solution (see Table A3.1-2) weekly until harvest. After three months growth, the seedlings were removed from the pots and the root systems were carefully washed with tap water followed by sterile water to remove sand particles. Each root system was then submerged in 200 ml of sterile 0.2 mM CaCl₂ in a sterile beaker (Figure A3.1-1b). The seedling was kept straight in a stand under the lights to allow for release of water-soluble exudates into the trap solution for 2 h. Immediately after collection, the trap solution containing root exudates was filtered through a sterile 0.45 µm filter (MFS-25, Micro Filtration Systems, USA) followed by freeze drying. Four root exudate samples were sent to Australian Centre for Plant Functional Genomics and Metabolomics (School of Botany, the University of Melbourne, Australia) for GC-MS scan using their standard database (Schauer et al. 2005).

Results and Discussion

GC-MS scan of root exudates of radiata pine

Five classes (carbohydrate, low molecular weight OAs, fatty acids, amino acids and derivatives and others) were identified in the root exudate samples of radiata pine seedlings (Table A3.1-1).



Figure A3.1-1 a). *Pinus radiata* seedlings grown in polyvinyl chloride pots filled with river sand in a growth room in the Biotron. b). Collection of root exudates from a radiata pine seedling by submerging its root system into sterile 0.2 mM CaCl₂.

Table A3.1-1. Compounds detected in root exudate solutions of *Pinus radiata* seedlings by GC-MS.

Class of Compounds	Compounds
Carbohydrates	glucose, fructose, maltose, raffinose, and sucrose
Low molecular weight OAs	oxalic acid, maleic acid, succinic acid, decanoic acid, malic acid, threonic acid, phenylpyruvic acid, malonic acid, tartaric acid, formic acid, citric acid, shikimic acid, quinic acid, lactic acid, acetic acid, gallic acid, and fumaric acid
Fatty acids	C16:0, C18:0 fatty acid, C18:1 fatty acid, and C20:0 fatty acid
Amino acids and derivatives	glycine, leucine, aspartic acid, serine, and 5-oxoproline
Others	phosphate, glycerol, , myo-inositol, , catechine, and galactinol

Selection of OAs for root exudate analysis

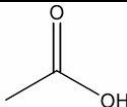
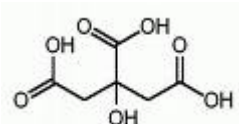
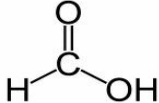
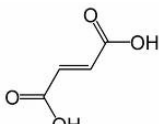
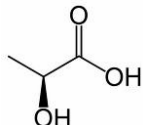
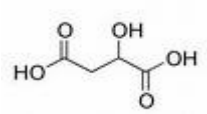
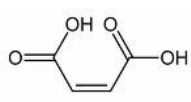
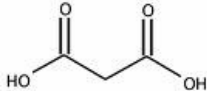
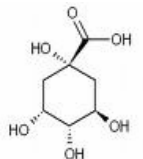
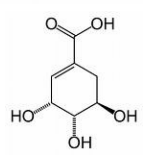
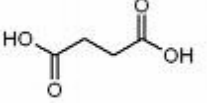
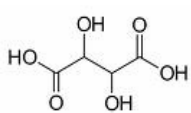
Based on this scan as well as the information about tree exudates in the literature (Fox and Comerford 1990; Grayston et al. 1996; Grierson 1992; Sandnes et al. 2005; Shen et al. 1996; Smith 1969; Smith 1976), 12 OAs, namely acetate, citrate, formate, fumarate, lactate, malate, maleate, malonate, quinate, shikimate, succinate and tartarate, were selected for radiata pine root exudate analysis. Oxalate which is commonly present in root exudates was also selected initially. However, due to the detection difficulties, oxalate was not included in this project (Appendix 3.6). The structure and properties of these OAs are outlined in Table A3.1-3.

Table A3.1-2 Recipe for Hoagland's nutrient solution used in this experiment.

Solution	Solution A	Solution B	Solution C
Recipe	280 mg H_3BO_3 340 mg $MnSO_4 \cdot H_2O$ 10 mg $CuSO_4 \cdot 5H_2O$ 22 mg $ZnSO_4 \cdot 7H_2O$ 10 mg $(NH_4)_6MO_7O_{24} \cdot 4H_2O$	0.5ml concentrated H_2SO_4	3.36 g Na_2EDTA 2.79 g $FeSO_4$
Preparation	Adjust volume to 100ml with DI water Store at 4 °C	Adjust volume to 100ml with DI water Store at 4 °C	Adjust volume to approximately 400 ml Heat the solution to 70°C while stirring until the colour turns yellow-brown Cool down, adjust the volume to 500 ml Store at 4°C

Solution	Hoagland's Stock Solution (10X)	Hoagland's Nutrient Solution (1x)
Recipe	4.7 g $Ca(NO_3)_2 \cdot 4H_2O$ 2.6 g $MgSO_4 \cdot 7H_2O$ 3.3 g KNO_3 0.6 g $NH_4H_2PO_4$ 5 ml solution A 0.5ml solution B	100 ml 10X stock 5 ml solution C
Preparation	Adjust volume to 500ml with DI water Store at 4 °C	Adjust volume to 1000ml with DI water Prepare just before use

Table A3.1-3 The structure and properties of the 12 selected organic anions.

Organic anion	Molecular formula	Molecular structure	Molecular weight	Carbon content %
Acetate	CH ₃ COOH		60.1	40.00
Citrate	C ₆ H ₈ O ₇		192.1	37.51
Formate	CH ₂ O ₂		46.0	26.10
Fumarate	C ₄ H ₄ O ₄		116.0	41.39
Lactate	C ₃ H ₆ O ₃		90.1	40.00
Malate	C ₄ H ₆ O ₅		134.1	35.83
Maleate	C ₄ H ₄ O ₄		116.1	41.39
Malonate	C ₃ H ₄ O ₄		104.1	34.63
Quinate	C ₇ H ₁₂ O ₆		192.2	43.75
Shikimate	C ₇ H ₁₀ O ₅		174.2	48.28
Succinate	C ₄ H ₆ O ₄		118.1	40.69
Tartarate	C ₄ H ₆ O ₆		150.1	32.01

References

- Fox T R, Comerford N B (1990) Low-molecular-weight organic acids in selected forest soils of the southeastern USA. *Soil Science Society of America Journal* 54:1139-1144
- Grayston S J, Vaughan D, Jones D (1996) Rhizosphere carbon flow in trees, in comparison with annual plants: The importance of root exudation and its impact on microbial activity and nutrient availability. *Applied Soil Ecology* 5:29-56
- Grierson P F (1992) Organic acids in the rhizosphere of *Banksia integrifolia* L.f. *Plant and Soil* 144:259-265
- Sandnes A, Eldhuset T D, Wollebak G (2005) Organic acids in root exudates and soil solution of Norway spruce and silver birch. *Soil Biology and Biochemistry* 37:259-269
- Schauer N, Steinhauser D, Strelkov S, Schomburg D, Allison G, Moritz T, Lundgren K, Roessner-Tunali U, Forbes M G, Willmitzer L, Fernie A R, Kopk J (2005) GC-MS libraries for the rapid identification of metabolites in complex biological samples. *FEMS Letters* 579:1332-1337
- Shen Y, Ström L, Jönsson J, Tyler G (1996) Low-molecular organic acids in the rhizosphere soil solution of beech forest (*Fagus sylvatica* L.) cambisols determined by ion chromatography using supported liquid membrane enrichment technique. *Soil Biology and Biochemistry* 28:1163-1169
- Smith W H (1969) Release of organic materials from the roots of tree seedlings. *Forest Science* 15:138-143
- Smith W H (1976) Character and significance of forest tree root exudates. *Ecology* 57:324-331

Appendix 3.2 Optimization of HPLC Method for Analysing Low Molecular Weight OAs in Root Exudates of Radiata Pine

Objective

To optimize a HPLC method for identification and quantification of 12 low molecular weight OAs in root exudates of radiata pine.

Methods

An OA mixture containing 12 OAs, prepared from tartaric, quinic, formic, malic, malonic, shikimic, lactic, acetic, maleic, citric, succinic and fumaric acids with each anion at 10 mg l⁻¹ was first tested for separation using C18 prevail™ organic acid column with 0.6 ml min⁻¹ of 25 mM KH₂PO₄ (pH 2.5) eluent according to the manufacturer's recommendation. However, two groups of OAs, tartarate, quinate and formate, and shikimate and lactate were co-eluted (Figure A3.2-1a). Different optimization methods were tested to improve the separation. These included:

1. Addition of methanol into the aqueous eluent (25 mM KH₂PO₄, pH 2.5) at the concentration of 2.5% and 7%.
2. Changing of the pH of the eluent (25 mM KH₂PO₄) from 2.5 to 2.0, 2.88 and 3.14.
3. Changing the column temperature from room temperature to 30, 40, 45 and 50°C when using 25 mM KH₂PO₄ (pH 2.5) as an eluent.

Results and discussion

1. Effect of methanol in the eluent on separation of OAs

Methanol was added into the aqueous eluent at the concentration of 2.5% and 7% for the possible better separation. However, the addition of methanol at either concentration resulted in the appearance of several negative peaks along the baseline. Some of the negative peaks appeared at the same retention times as several OAs, such as malate and citrate. In addition, the separation of OAs decreased with the increase of methanol level in the eluent, as the retention time for all the OAs reduced at different levels. Cawthray (2003) also found that the retention times for each OAs decreased by increasing the concentration of methanol in the eluent.

2. Effect of the pH of the eluent on separation of OAs

The pH of the eluent (25 mM KH₂PO₄) was adjusted to 2.0, 2.88 and 3.14 to test the effect on the separation of OAs in comparison to the original pH 2.5. Changing the pH of the

eluent to 2.0 resulted in tartarate, quinate and formate being eluted at the same time, as were malate and malonate. In addition, two negative peaks appeared at the beginning of the chromatogram. When the pH of eluent increased to 2.88, in addition to two negative peaks at the beginning, another negative peak appeared at the same retention time as malate and malonate. Adjusting the eluent to pH 3.14 resulted in the retention time for all OAs being shifted forward by at least 0.25 min, with the exception of succinate which was eluted out at a similar time to that seen with the eluent adjusted to pH 2.5. In the chromatogram, tartarate, quinate and formate anions were shown in one peak, as did malate and malonate, shikimate and lactate. Therefore, pH 2.5 was chosen for the following optimization as it gave the best separation among the four pH values examined.

3. Effect of column temperature on separation of OAs

Five column temperatures (room temperature, 30, 40, 45 and 50°C) were tested for their effect on the separation of OAs using isocratic HPLC. The chromatograms of OAs under different temperature are shown in Figure A3.2-1. With the increase of column temperature, the separation of tartarate, quinate and formate, shikimate and lactate improved. However, the peaks of malate and malonate moved closer as the temperature increased. At column temperatures of 45°C and 50°C, the peaks of maleate, acetate and citrate were much closer than seen with a column temperature of 40°C. A column temperature of 40°C was selected.

Pre-treatment of samples were suggested by Wang and Zhou (2006) and Neumann (2006) to be very important due to the complexity of root exudate compounds. The pH of samples was adjusted to 1, 2.5 and 7 to test any effect on the separation. Neither the peak areas nor the retention times of OAs were changed with the change of sample pH values. Therefore, no special pre-treatment of samples were used in this HPLC method. This resulted in a quicker and cheaper analysis.

Conclusions

Separation of 12 OAs, i.e. tartarate, quinate, formate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate, could be achieved by using a C18 prevailTM organic acid column with 0.6 ml min⁻¹ of 25 mM KH₂PO₄ (pH 2.5) eluent with column temperature at 40°C. No extra sample pre-treatment was needed for the analysis of OAs in root exudates using this improved HPLC analysis method. This method offers fast, cheap and simple analysis of 12 OAs in root exudate samples.

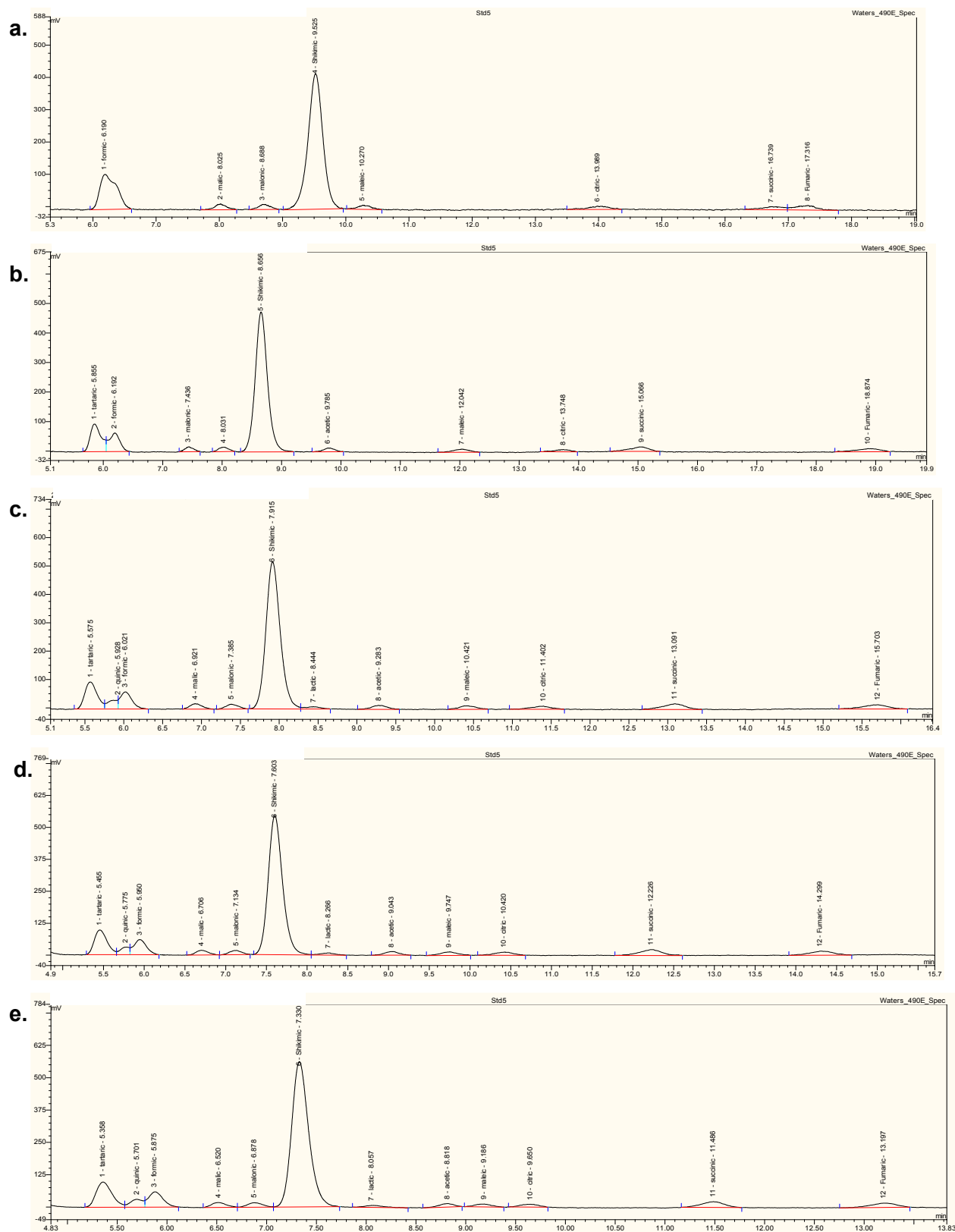


Figure A3.2-1 Separation of 12 OA standards by isocratic HPLC with 25 mM KH₂PO₄ (pH 2.5) at 0.6 ml min⁻¹ at five different column temperature. a). room temperature; b). 30°C; c). 40°C; d). 45°C; e). 50°C. The concentrations of anions shown in these chromatograms were: tartarate, quinate, formate and succinate 15 mg l⁻¹, malate and malonate 5 mg l⁻¹, shikimate 3 mg l⁻¹, lactate and citrate 4 mg l⁻¹, acetate 8 mg l⁻¹, maleate 0.08 mg l⁻¹, fumarate 0.1 mg l⁻¹.

References

- Baudoin E, Benizri E, Guckert A (2003) Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. *Soil Biology and Biochemistry* 35:1183-1192
- Neumann G (2006) Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) *Handbook of methods used in rhizosphere research*, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.
- Wang P, Zhou R (2006) Determination of organic acids exuded from plant roots by high performance liquid chromatography. *Chinese Journal of chromatography* 24:239-242

Appendix 3.3 Comparison of Efficiency of OA Recovery Using Filter Paper and Anion Exchange Membrane

Objective

Filter paper (Whatman 3MM) and anion exchange membrane (AEM) have both been used as root exudate collection media in previous studies (Neumann et al. 1998; Neumann and Römheld 1999; Schefe et al. 2008). This experiment was designed to compare the efficiency of recovery of OAs from soil using these two media.

Method

A silt loam soil (1500 g) was mixed with 250 ml of an OA mixture (12 OAs, 30 mg l⁻¹ each) to reach a soil moisture content of 16% which was similar to the moisture level maintained in the rhizotrons in Chapter 3. Whatman 3MM filter paper was cut into the same size as AEM strip (6 x 4 cm) and washed with methanol followed by sterile water before use. AEM strips were pre-charged with NaHCO₃ solution as described in Chapter 3 (Section 3.2.2.1). Four treatments of collection media were tested in this experiment (A= AEM strips; F= filter paper Whatman 3MM; FA= AEM covered with filter paper Whatman 3MM (to test whether filter paper can help absorbing any anion from soil and to assist in capturing anions by AEM); and FAF= AEM sandwiched between two pieces of Whatman 3MM filter paper (to test the combination of these two collection media and also to keep AEM strip moist during the collection period). A layer of soil (130 g) was placed evenly on top of the AEM strip/filter paper in each collection treatment and incubated in the dark at room temperature, with three replicates per treatment. After 3 h, the membrane/filter paper was removed from the soil. The AEM strips from treatment A, FA and FAF were rinsed with DI water before being placed into sterile flasks containing 10 ml of 0.5 M HCl solution. The captured OAs were eluted by shaking the flasks at 150 rpm at 4°C for several hours. The solutions were then stored at -20°C until analysis by HPLC. The filter paper from treatment F was placed in sterile centrifuge tubes filled with 3.6 ml of sterile DI water. The tubes were shaken vigorously for 10 min by hand before being centrifuged at 13,000 *g* for 10 min at 4°C. Due to the paper dissolving in water, a second centrifugation step under the same conditions was carried out and the supernatant was stored at -20°C until analysis by HPLC. The controls consisted of AEM and filter paper without incubation in soil were directly eluted to check for the presence of any anions.

Results

Because of the low amounts of OAs added into the soil, only two OAs (maleate and fumarate) at very low concentrations were detected by HPLC in samples collected using the four treatments of collection media (Figure A3.3-1). However, the results showed that AEM strips collected 10-fold more maleate in comparison with Whatman 3MM filter paper. A trace amount of fumarate ($0.002 \mu\text{g cm}^{-2}$) was detected in one of the three samples collected by Whatman 3MM filter paper. In contrast, fumarate was detected in all the samples collected using AEM strips with amounts ranging from 0.132 to $0.307 \mu\text{g cm}^{-2}$. The amounts of maleate and fumarate collected by AEM alone were significant higher than that collected by AEM in conjunction with Whatman 3MM (Figure A3.3-1).

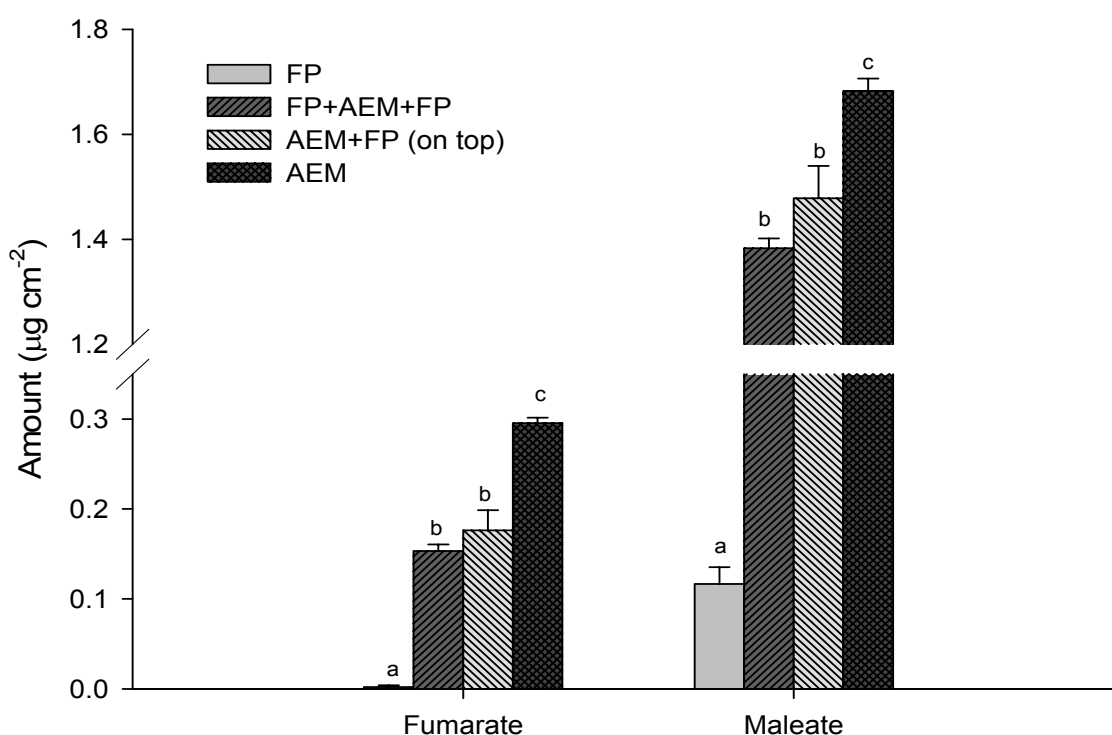


Figure A3.3-1 Comparison of the efficiency of recovery of OAs from soil amended with an OA mixture using four treatments of collection media. Shown is the amount of two OAs ($\mu\text{g cm}^{-2}$) collected over 3 h from soil (130 g) containing a mixture of OAs supplied at a concentration of $4.28 \mu\text{g}$ each anion per g soil. AEM: anion exchange membrane; FP: Whatman 3MM filter paper; AEM+FP (on top): AEM strips covered by Whatman 3MM filter paper on top; FP+AEM+FP: AEM sandwiched between two pieces of Whatman 3MM filter paper. Error bars show 1 standard errors and for each organic anion, columns denoted with a different letter are significantly different ($P < 0.05$) among the collection treatments ($n=3$).

Discussion

Although the designed experiment only collected two out of 12 OAs due to the low amounts of OAs added to the soil, AEM strips showed better efficiency of recovery of both of maleate and fumarate compared to filter paper Whatman 3MM. The difference in recovery can be explained by collection mechanisms of the two media. Filter paper Whatman 3MM can absorb solutions from soil or root surface until it reaches a moisture

equilibrium. The low collection ability of filter paper was also reported by Neumann (2006) who indicated that filter paper was only suitable for collection exudates from plants which produce large amount of root exudates. In contrast, AEM strips collect anions by exchanging them with pre-charged HCO_3^- on the surface. Therefore, AEM is a more suitable collection media for actively collecting anions from root exudates. The aim of using the filter paper together with AEM was to improve the absorption of any solution in the soil and, therefore, to bring anions to the surface of the AEM and assist their exchange with HCO_3^- on the AEM. However, this was not the case. Placing the filter paper on top or on both sides of the AEM strips resulted in a significant reduction in collected amounts of maleate and fumarate. This reduction may be due to the blockage of anions by the filter paper and possible absorbance of small amounts of OAs.

Conclusion

AEM is a better collection media for capturing OAs compared to Whatman 3MM filter paper and thus it was used in this project. Filter paper placed on top of AEM resulted in the reduction of amounts collected by AEM strips. Therefore, filter paper would be placed at the back of the AEM strip to keep AEM moist during the collection period as described in Chapter 3 (Section 3.2.2 and Section 3.2.4).

References

- Neumann G (2006) Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.
- Neumann G, Massonneau A, Martinoia E, Römheld V (1998) Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* 208:373-382
- Neumann G, Römheld V (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil* 211:121-130
- Scheffe C R, Watt M, Slattery W J, Mele P M (2008) Organic anions in the rhizosphere of Al-tolerant and Al-sensitive wheat lines grown in an acid soil in controlled and field environments. *Australian Journal of Soil Research* 46:257-264

Appendix 3.4 Chemical and Properties Analysis of the Soil Used in the Rhizotrons in Chapter 3

Analysis	Level Found	Medium Range	Low	Medium	High
pH	6.8	5.8 - 6.3			
Olsen P (mg/L)	18	20 - 30			
Potassium (me/100g)	0.23	0.50 - 0.80			
Calcium (me/100g)	8.2	6.0 - 12.0			
Magnesium (me/100g)	1.55	1.00 - 3.00			
Sodium (me/100g)	0.33	0.20 - 0.50			
CEC (me/100g)	11	12 - 25			
Base Saturation (%)	92	50 - 85			
Volume Weight (g/mL)	1.10	0.60 - 1.00			
Sulphate-S (mg/kg)	54	7 - 15			
Available N (15cm Depth) (kg/ha)	46	150 - 250			
Organic Matter (%)	2.4	7.0 - 17.0			
Total Carbon (%)	1.4				
Total Nitrogen (%)	0.15	0.30 - 0.60			
C/N Ratio	9.6				
AMN/TN Ratio (%)	1.9	3.0 - 5.0			
'Total' Sulphur (mg/kg)	224				
Total Phosphorus (ign) (mg/kg)	624				
Organic Phosphorus (mg/kg)	201				
Base Saturation	K 2.1 Ca 73 Mg 13.9 Na 2.9				
MAF Units	K 5 Ca 11 Mg 38 Na 17				
Anaerobically Mineralisable N	28 ug/g				

Appendix 3.5 Creation of the Horizontal Access Portals in a Rhizotron

The horizontal access portal in a rhizotron was created in four main steps as outlined in Figure A3.5-1. Briefly, the pre-existing opening (5.2 cm diameter) on the rhizotron wall was covered with metal plate before packing soil into the rhizotron (Figure A3.5-1a). After soil has been allowed to settle in the rhizotron, the plate was removed (Figure A3.5-1b) and the pre-existing opening with soil is shown in Figure A3.5-1c. A soil corer with 5 cm diameter (Chapter 3, Figure 3.2a) was then used to remove the soil core (45 cm depth x 5 cm diameter) from the rhizotron (Figure A3.5-1d). The physical and structural integrity of the access portal was maintained using a removable inflatable tube between samplings (Chapter 3, Figure 3.2b).



Figure A3.5-1 Creation of the horizontal access portal in a rhizotron. a). The rhizotron packed with soil. b). Removing the plate on the rhizotron wall. c). Shown is the pre-existing opening in the rhizotron with soil after removal of the plate. d). A soil corer is inserted into the rhizotron through the original opening to create an access portal.

Appendix 3.6 Causes of the Void Peak at the HPLC Chromatogram and Possible Solutions

Objective

The void peak appeared at the beginning of the HPLC chromatogram overlapped with the peak of oxalate. In some worse cases, the void peak may affect the quantification of earlier eluted anions (e.g. tartarate, quinate) (Chapter 3, Figure 3-11). Therefore, the possible causes of the void peak were analysed and potential solutions for this were tested.

Research

1. Sample injection

A small injection peak appeared in all the samples, including HPLC calibration standards and DI water (Figure A3.6-1). This is inherent with the HPLC system and cannot be avoided.

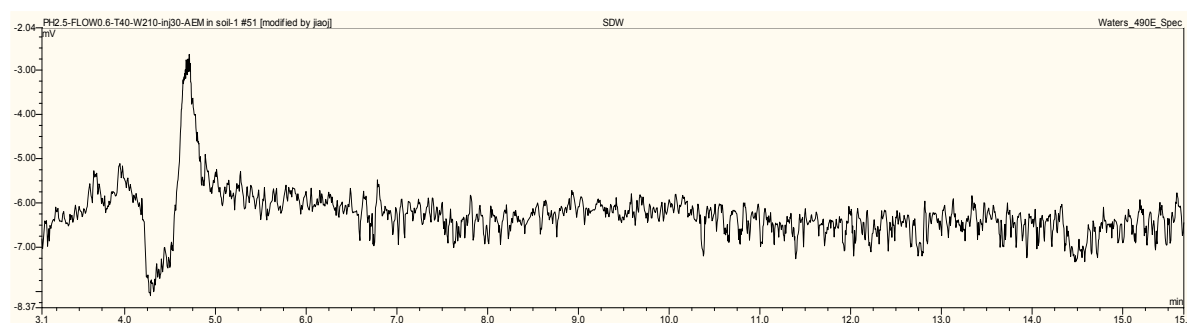


Figure A3.6-1 The HPLC chromatogram of a DI water sample.

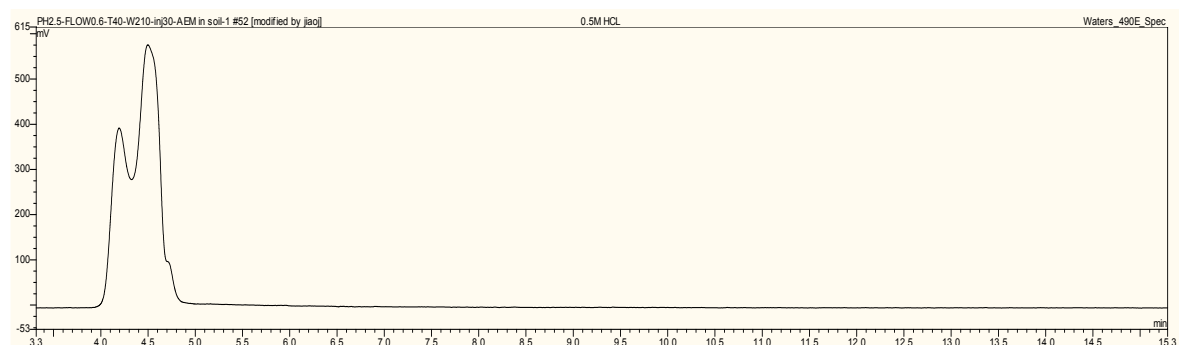


Figure A3.6-2 The HPLC chromatogram of 0.5 M HCl.

2. Inorganic ion Cl^- in AEM elution solution

The void peak was much bigger in OA samples collected by AEM strips compared to HPLC standards. The Cl^- in 0.5 M HCl elution solution mainly accounted for this void peak, which was confirmed by running HCl elution solution through HPLC (Figure A 3.6-

2). To try and reduce/avoid the void peak caused by Cl^- , other elution solution was tested. As KH_2PO_4 solution was used as the HPLC eluent, H_3PO_4 was then selected to elute anions on AEM strips.

The comparison of elution solution was set up as follows. AEM strips, which were pre-charged with HCO_3^- as described in Chapter 2, Section 3.2.2.1, were loaded with 500 μl of an OA mixture (tartarate, quinate, shikimate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate anions, 500 mg l^{-1} for each anion) on the surface. After 1 h, AEM strips were shaken briefly to remove excess solutions before being placed into sterile flasks containing three different elution solutions, namely 0.5 M H_3PO_4 , 0.2 M H_3PO_4 , and 0.5 M HCl. Three replicates were used for each elution solution. The flasks were shaken at 150 rpm at 4°C for 3 h to elute the anions.

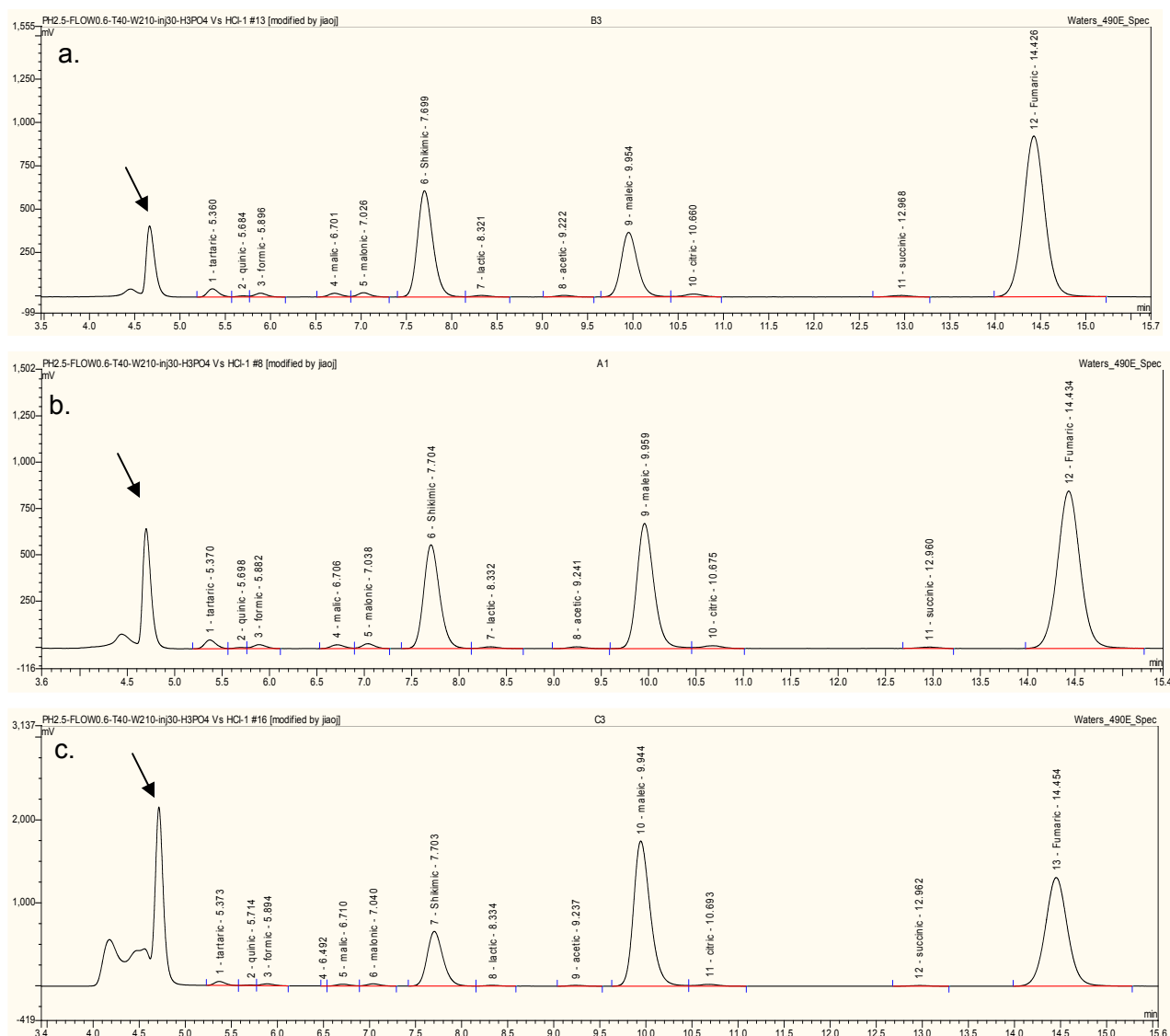


Figure A3.6-3 HPLC chromatograms of eluted OAs from impregnated AEM strips by three different elution solutions. a). 0.2 M H_3PO_4 , b). 0.5 M H_3PO_4 , c). 0.5 M HCl. The void peaks are indicated by arrows.

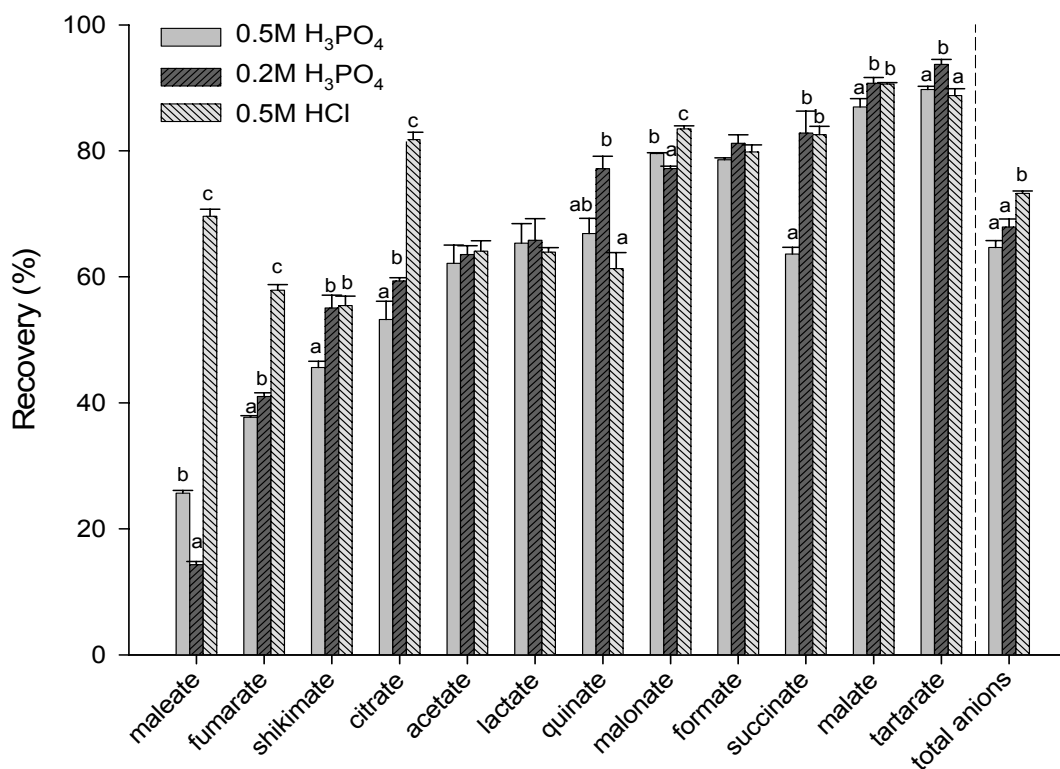


Figure A3.6-4 Effect of elution solution on recovery of OAs from AEM strips. Shown is the percent recovery (%) of individual OAs and the average total amount of anions recovered after eluting AEM strips that were loaded with 500 μl of an OA mixture (500 mg l^{-1} of each anion) in three different elution solutions (i.e. 0.5 M H_3PO_4 , 0.2 M H_3PO_4 and 0.5 M HCl). Error bars show 1 standard error and for each organic anion, columns denoted with different letters are significantly different ($P < 0.05$) among elution solutions ($n=3$).

The results showed that by using either concentration of H_3PO_4 as the elution solution, the void peak that appeared at the beginning of the chromatogram was much smaller, although still present, in comparison with HCl solution (Figure A3.6-3). However, the recovery of some anions, especially maleate, fumarate and citrate, reduced significantly compared to the samples which were eluted using HCl (Figure A3.6-4). Across all OAs, HCl elution demonstrated the significant higher recovery of total OAs compared to H_3PO_4 solution ($P < 0.05$).

Although the void peak could be partly reduced by using H_3PO_4 solution, this resulted in less efficient recovery of targeted OAs. Consequently, 0.5 M HCl was still selected as the elution solution to elute anions from the AEM strips in this project.

3. Inorganic ion collected from soil

The void peak was much bigger in samples collected from soils by AEM strips (Sections 3.3.2.4 to 3.3.2.5, 3.3.3.2) compared to the samples from solutions (Sections 3.3.2.1 to 3.3.2.3) (data not shown). The inorganic ions from soil may be the major contributor to the void peak compared to the other two factors (i.e. injection and Cl^- in the elution solution). A simple leaching experiment was set up by placing 20 g silt loam experimental soil in a funnel (75 mm diameter) lined with a piece of Whatman 40 filter paper. A volume of 40 ml

of DI water was applied slowly to the soil in the funnel. The leachate was collected for HPLC analysis and the chromatogram is shown in Figure A3.6-5. A void peak in the soil leachate sample (Figure A3.6-5) was more than 2 times higher than that caused by Cl^- in HCl as shown in Figure A3.6-2. The leachate was further analysed by ion exchange chromatography and revealed that it contained a reasonable number of Cl^- , NO_3^- , PO_4^- , SO_4^- . The results indicate that the main cause of the void peak is the excess of inorganic ions in soil.

In order to reduce the inorganic ions present in root exudate samples, several different types of solid phase extraction cartridges were tested for absorption of inorganic ions from samples according to manufacturer's instruction. However, all the tests resulted in incomplete absorption of inorganic ion and great reduction of OAs in the samples. Therefore, this pre-treatment was not employed in the project. The sample pH was adjusted to 1 and 2.5 by H_3PO_4 as suggested by Neumann (2006) prior to the HPLC analysis. However, no significant reduction of the void peak was observed.

These results indicate that the major contributor of the void peak in exudate samples collected from soil cannot be avoided. Consequently, the analysis of oxalate which was eluted at the same retention time as the void peak was not included in this project.

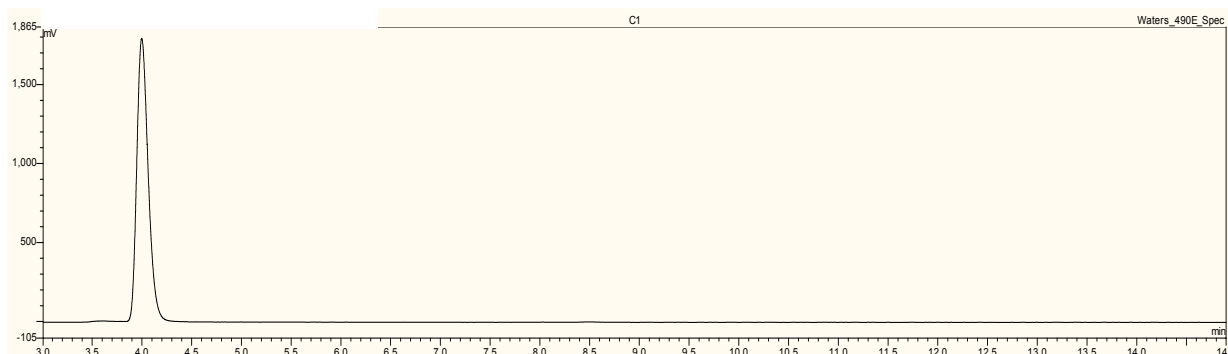


Figure A3.6-5 The HPLC chromatogram of the collected leachate from experimental soil when applying DI water.

Conclusion

In summary, various tests were carried out to identify the causes of the void peak and efforts were made to reduce it. However, there is no efficient way to reduce the void peak and maintain the efficient recovery of targeted OAs. Consequently, oxalate which was most affected was not included in this project. Caution must be used in interpreting the concentrations of early eluted anions in some samples.

Reference

Neumann G (2006) Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research, Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.

Appendix 3.7 Comparison of Rhizosphere Bacterial Communities Associated with Different Root Types of Radiata Pine

Objective

The aim of this experiment was to test the hypothesis that different types of predominant roots collected from three different depths in the rhizotron units could contribute to the variation of the rhizosphere bacterial communities observed along the depth.

Method

Different types of predominant radiata pine roots were collected from three different depths in soil (0-15 cm, 16-30 cm, 31-45 cm) when grown in the rhizotron units (Figure A3.7-1) as described in Chapter 3, Section 3.2.5. Root samples from the same depths from two rhizotron units were bulked together to obtain enough soil samples for analysis. Fine roots with numerous root tips or ectomycorrhizal colonized roots were commonly collected from the top 15 cm of the soil (Figure A3.7-1). The predominant roots at the soil depth of 16-30 cm were similar to those in the top 15 cm, but slight lower frequency of ECM was observed and the roots were slightly thicker. Here they are referred to as middle roots. Large and thick roots were often found in the bottom soil layer with the depth of 31-45 cm, although some root tips or young roots could also be observed (Figure A3.7-1). Here, they are referred to as big roots. Due to the limited root samples, only two replicates of rhizosphere soils from fine roots and middle roots and one sample of the rhizosphere soil from big roots were available for analysis. Two replicates of the non-rhizosphere soil were also included in the analysis (Chapter 3, Section 3.2.5). On the same day as sampling, the rhizosphere soils were collected from root samples and stored at -80°C until RNA extraction according to the method described in Chapter 2, Section 2.2.2.2. The molecular analysis of general bacterial 16S communities was described in Chapter 2, Sections 2.2.3 and 2.2.4. The DGGE gel were analysed according to the statistical method described in Chapter 2, Section 2.2.8; however, no discrimination analysis was carried out due to the limited replicates in this experiment.



Figure A3.7-1 Images of the predominant radiata pine root samples collected from three soil depths (0-15 cm, 16-30 cm, 31-45 cm) in the rhizotron units. a). Fine roots frequently colonized by ECM collected from the depth of 0-15 cm. b). Middle roots with some colonized by ECM collected from the depth of 16-30 cm. c). Big roots collected from the depth of 31-45 cm.

Results

The bacterial communities associated with these three types of roots as well as the non-rhizosphere communities were shown in rRNA-DGGE gel (Figure A3.7-2). The bacterial community profiles in the non-rhizosphere clearly differed from those in the rhizosphere soils from any root type. The differences between the rhizosphere bacterial 16S communities associated with the fine roots and the middle roots were minor and only two different bands were observed by eyes. Several different bands could be seen between the DGGE profiles associated with the big roots and the fine/middle roots.

Diversity software identified 17 bands which were different across the gel (data not shown). Three bands which were only appeared in the bacterial community of the middle roots but not of the fine roots were identified by the software. And two bands which were only appeared in fine roots but not in middle roots were indicated by the software. The analysis showed that 12 bands were different in the bacterial community of big roots compared to either the fine roots or the middle roots.

Discussion

Although only a few replicates were collected in this experiment, the DGGE gel clearly indicated that different bacterial communities were present in the rhizosphere soils associated with different types of roots. This finding agrees with others (Clayton et al. 2005; Marschner et al. 2001; Yang and Crowley 2000). These three types of roots were typical roots collected from three depths in the rhizotron units. This experiment suggests that different types of roots presented at various depths in soil is one of the factors causing the differences in rhizosphere bacterial communities associated with depth.

M FR FR MR MR BR NR NR M

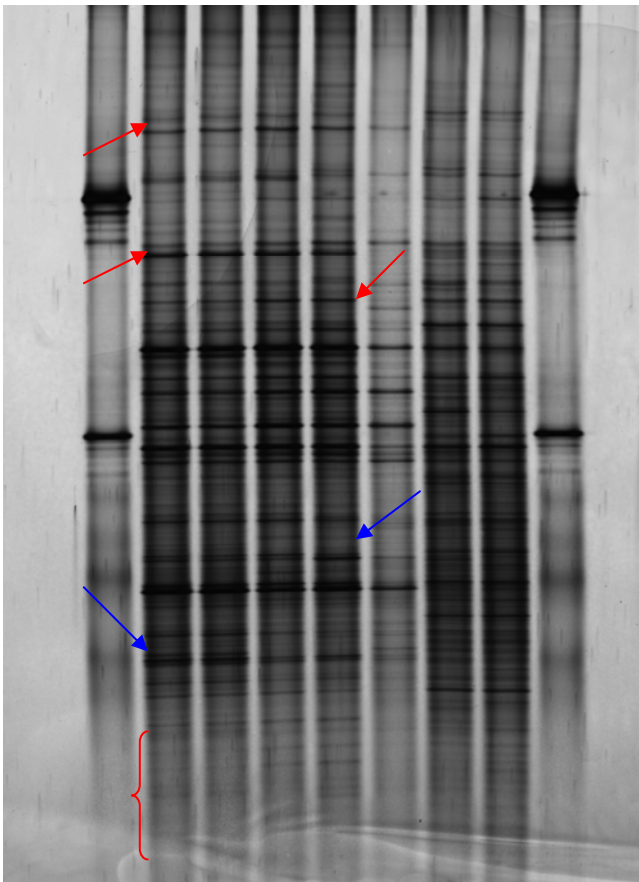


Figure A3.7-2 rRNA-DGGE profiles of bacterial 16S rRNA in the non-rhizosphere soil and the rhizosphere soil associated with three different root types of *Pinus radiata* collected from three depths when grown in the rhizotron units. M: bacterial 16S DGGE standard (See Appendix A2.5, Figure A2.5-1). FR: fine roots ($n=2$); MR: middle roots ($n=2$); BR: big roots ($n=1$); NR: composite non-rhizosphere soil collected at area free of roots ($n=2$). The bands appeared in the fine/middle roots but not in the big roots are indicated by red arrows and bracket. The different bands between fine roots and middle roots are indicated by blue arrows.

References

- Clayton S J, Clegg C D, Murray P J, Gregory P J (2005) Determination of the impact of continuous defoliation of *Lolium perenne* and *Trifolium repens* on bacterial and fungal community structure in rhizosphere soil. *Biology and Fertility of Soils* 41:109-115
- Marschner P, Yang C H, Lieberei R, Crowley D (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry* 33:1437-1445
- Yang C-H, Crowley D E (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Applied and Environmental Microbiology* 66:345-351

Appendix 4.1 Measurement of Npt II protein in root samples

Objective

The GM *Pinus radiata* trees used in the rhizotron experiment in Chapter 4 was transformed with *leafy* gene from *Arabidopsis thaliana* together with *nptII* marker gene. The expression of NptII protein in root samples was examined in this experiment.

Method

The analyses of NptII protein in root samples from all three samplings were carried out using enzyme-linked immunosorbent assay (ELISA) in Scion.

Results

The NptII protein was detected in all the root samples from GM trees in all three samplings, although the amounts varied (Table A4.9-1). Different expression levels were expected and can result from different integration characteristics and individual growth conditions. Significant low ($P<0.05$) amounts of protein was also detected in few root samples of WT pine tree at the first and second samplings; however, such low level was generally considered as the background. There was no NptII protein detected in any of the root samples from WT trees at the 3rd sampling.

Table A4.9-1 Mean of NptII protein (ng mg⁻¹ protein) detected in root samples of GM and WT pine trees collected at the three samplings in rhizotron units. Values are presented as the mean \pm 1 standard error for samples in each treatment if they could be detected ($n=4$ for the first and second samplings and $n=4$ for the third sampling). The probability values with significant difference ($P<0.05$) are shown in bold. Different letters indicate significantly different ($P<0.05$) between the treatments.

Sampling	GM 10cm/ D1*	WT 10cm/D1	GM 20cm/D2	WT 20cm/D2	GM D3	WT D3	p-value
1 st sampling	5.17 \pm 0.782 b	0.05 \pm 0.039 a	8.03 \pm 1.379 b	0.10 \pm 0.060 a	NA [#]	NA	<0.001
2 nd sampling	11.73 \pm 3.182 b	0.38 \pm 0.251 a	14.70 \pm 2.031 b	0.12 \pm 0.072 a	NA	NA	<0.001
3 rd sampling	14.78 \pm 1.519 b	0.00 a	10.56 \pm 3.574 b	0.00 a	11.43 \pm 4.326 b	0.00 a	<0.001

*D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm. [#]NA = not applicable

Appendix 4.2 Chemical and Properties Analysis of the Soil Used in the Rhizotron Experiments in Chapter 4

Horizon A soil.

Analysis	Level Found	Medium Range	Low	Medium	High
pH	5.6	5.8 - 6.3			
Olsen P (mg/L)	41	20 - 30			
Potassium (me/100g)	0.63	0.50 - 0.70			
Calcium (me/100g)	3.5	6.0 - 12.0			
Magnesium (me/100g)	0.92	1.00 - 3.00			
Sodium (me/100g)	0.09	0.20 - 0.40			
CEC (me/100g)	11	12 - 25			
Base Saturation (%)	48	55 - 75			
Volume Weight (g/mL)	1.15	0.60 - 1.00			
Sulphate-S (mg/kg)	5	7 - 15			
Available N (15cm Depth) (kg/ha)	97	150 - 250			
Organic Matter (%)	4.4	7.0 - 17.0			
Total Carbon (%)	2.5				
Total Nitrogen (%)	0.24	0.30 - 0.60			
C/N Ratio	10.6				
AMN/TN Ratio (%)	2.4				
'Total' Phosphorus (mg/kg)	542				
'Total' Sulphur (mg/kg)	246	600 - 1000			
Base Saturation	K 5.9 Ca 32 Mg 8.6 Na 0.9				
MAF Units	K 15 Ca 5 Mg 24 Na 5				
Anaerobically Mineralisable N	56 ug/g				

Horizon B soil.

Analysis	Level Found	Medium Range	Low	Medium	High
pH	5.6	5.8 - 6.3			
Olsen P (mg/L)	32	20 - 30			
Potassium (me/100g)	0.57	0.50 - 0.70			
Calcium (me/100g)	3.1	6.0 - 12.0			
Magnesium (me/100g)	0.86	1.00 - 3.00			
Sodium (me/100g)	0.09	0.20 - 0.40			
CEC (me/100g)	9	12 - 25			
Base Saturation (%)	51	55 - 75			
Volume Weight (g/mL)	1.24	0.60 - 1.00			
Sulphate-S (mg/kg)	5	7 - 15			
Available N (15cm Depth) (kg/ha)	73	150 - 250			
Organic Matter (%)	2.7	7.0 - 17.0			
Total Carbon (%)	1.5				
Total Nitrogen (%)	0.15	0.30 - 0.60			
C/N Ratio	10.0				
AMN/TN Ratio (%)	2.6				
'Total' Phosphorus (mg/kg)	466				
'Total' Sulphur (mg/kg)	184	600 - 1000			
Base Saturation	K 6.3 Ca 34 Mg 9.5 Na 1.0				
MAF Units	K 14 Ca 5 Mg 24 Na 5				
Anaerobically Mineralisable N	39 ug/g				

Appendix 4.3 Radiata Pine Exude Different Amounts of Organic Anions into the Rhizosphere at Various Periods within a Day

Objective

The composition and quantities of root exudates can be affected by numerous environmental factors, including plant species, root location, light density, nutrient availability, soil pH, soil moisture, etc. (Chapter 1, Section 1.1.2). Therefore, it is very likely that plants, in response to the environment changes (e.g. temperature, light intensity) and to the different plant physiological needs, would release different amounts or composition of OAs in root exudates at different time periods within a day. The aim of this experiment was to investigate the variability in OAs (in quantity and composition) exuded by radiata pine at five root regions across different time periods in a day. This information was necessary in planning the sampling period for the rhizotron experiment (Chapter 4).

Material and Methods

After eight months growth in rhizotron units as described in Chapter 4 (Section 4.2.2), two *Pinus radiata* (one GM and one WT tree) were sampled for root exudates *in situ* in this experiment. Two root regions from WT (at a depth of 10 cm) (labelled as W1 and W3, respectively) and three root regions from GM pine (two at a depth of 20 cm and one at a depth of 10 cm) (labelled as G1, G2 and G3, respectively) in access portals were sampled for OAs using AEM in conjunction with Whatman 3MM filter paper (size: 1 x 2 cm). A wide range of roots, such as elongation root area, root tips, a mature root with several lateral roots, as well as roots colonized by ectomycorrhizal fungi, were selected for this study to have an overall understanding of exudation by pine roots. The *in situ* sampling of exudates is described in Chapter 4, Section 4.2.4.2. Briefly, the AEM strips backed with moist Whatman 3MM were placed on top of the selected root regions using thumbtacks. The inflatable tubes were then inserted back to the access portals and pumped to make sure AEM strips were in close contact with roots. After 2 h, the AEM strips were removed and new AEM strips backed with Whatman 3MM were placed in the same regions for next collection. This step was repeated every 2 h for eight times for the collection from 6 am to 10 pm. After each collection, the AEM strips were rinsed with DI water briefly before being placed into 1.5 ml of 0.5 M HCl for eluting the collected anions in sterile tubes in a shaker at 150 rpm at 4°C for 3 h. The anions were stored at -20°C immediately after elution from AEM until analysis by HPLC with the method described in Chapter 3, Section 3.2.1. The amounts of collected OAs were calculated as detected concentrations per cm² of root area

which was calculated based on the image using developed software (see Chapter 4, Section 4.2.4.2) over 2 h collection period.

Results

Eleven OAs, including tartarate, formate, malate, malonate, shikimate, lactate, acetate, maleate, citrate, succinate and fumarate, were detected consistently in all the root exudate samples collected *in situ* from five sites across eight collection periods, although some of them (e.g. shikimate, maleate and fumarate) were at trace amounts. The concentrations of all 11 OAs changed consistently across the collection periods at all five sampling regions, although the level of shifts in concentrations between individual OAs varied greatly (Figure A4.2-1). For instance, lactate showed great changes in amounts in exudate samples from all five regions. In one sampling region of WT pine roots (Figure A4.2-1e), the amount of lactate collected during the period of 2-4 pm was 120-fold more than that collected in the early morning (6-8 am). Although the concentrations of fumarate and maleate were low (ranging from 0.011 to 0.51 $\mu\text{g cm}^{-2}$ of root surface and 0.011 to 0.278 $\mu\text{g cm}^{-2}$ of root surface, respectively), levels of differences in amounts of both anions collected at different periods in the same region reached to 25-fold. When considering the exudates from different root regions in both GM and WT as replicates ($n=3$ and 2, respectively), the temporal variation in the amounts of each OAs could still be clearly seen among the eight collection periods (Figure A4.2-1 f and g).

The amounts of some OAs released at different root regions at the same time period were also very different, especially for these anions present in greater amounts. For instance, lactate collected from GM root regions at 10 cm (G1) and 20 cm (G3) showed very low amount (less than 10 $\mu\text{g cm}^{-2}$ of root surface) at the period of 2-4 pm. However, the amount of lactate was over 100 $\mu\text{g cm}^{-2}$ of root surface in the exudate samples collected from WT root region at 10 cm (W3) at that time period (Figure A4.2-1a, c and e). In the exudates collected from other two root regions (G2 and W1), the highest amount of lactate appeared between 10-12 pm and 8-10 am, respectively (Figure A4.2-1b and d).

Discussion

Temporal variability

The release of root exudates can be affected by plant physiological conditions and soil environment, especially by temperature and photoperiod (Koo et al. 2005). In this experiment, the results indicate that the amounts of each OA in root exudates collected at the same regions from radiata pine at different time periods were clearly different. The air temperature, humidity and light density in the growth room were regulated at different levels (Chapter 4, Section 4.2.2), especially at light (6 am to 8 pm) and dark period (8 pm

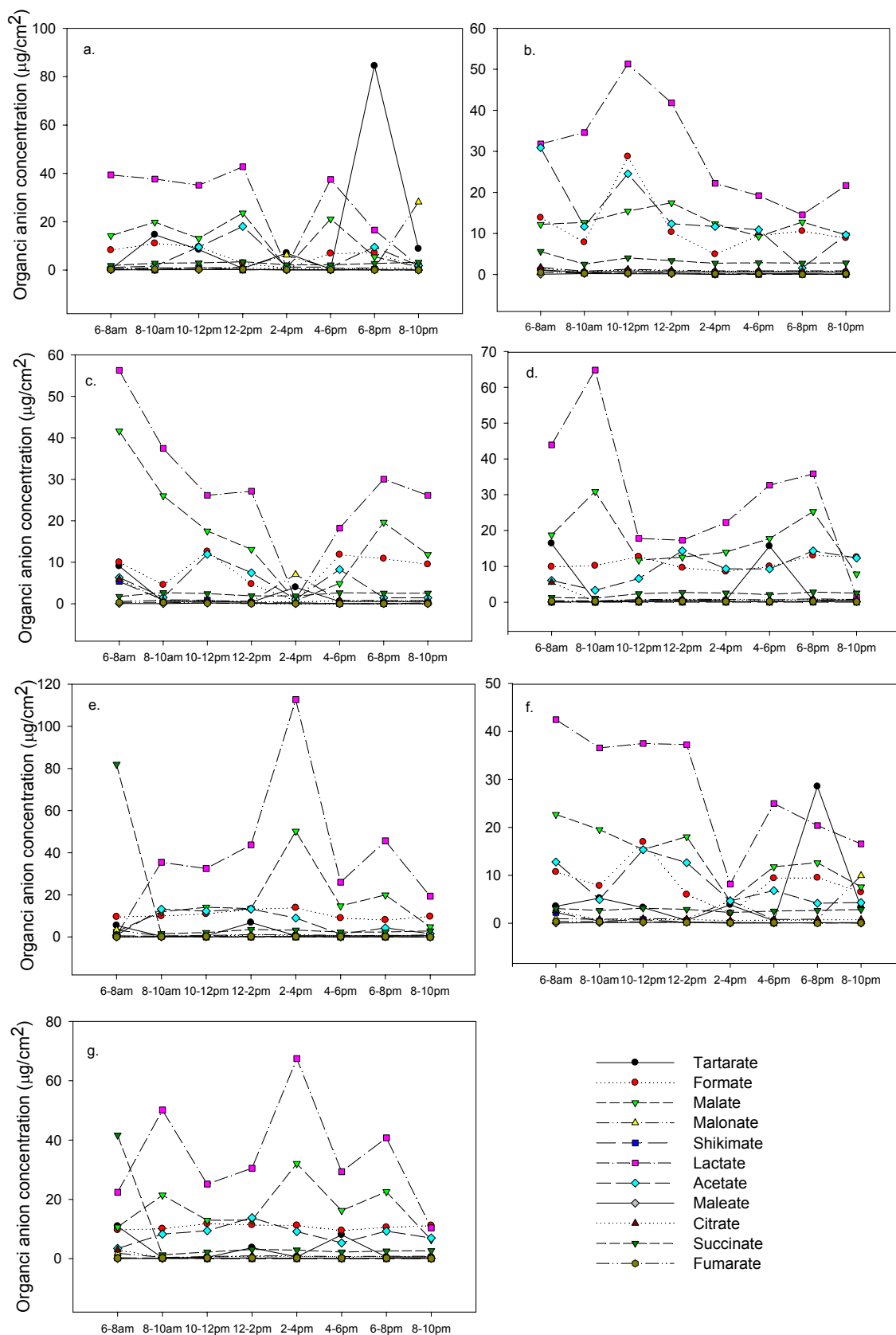


Figure A4.2-1 Organic anions collected from five root regions of radiata pine from 6am to 10pm in 2 h intervals *in situ* in the rhizotrons. Roots from the GM radiata pine line at a depth of a). 20 cm, G1; b). 20 cm, G2; c). 10 cm, G3. Root from the WT radiata pine line at a depth of d). 10 cm, W1; e). 10 cm, W3. The average of OAs in exudates samples from f). GM pine roots ($n=3$); g). WT pine roots ($n=2$).

to 6 am). These parameters could affect the photosynthesis rate of the plants and affect the plant cell metabolism, permeability and nutrient uptake (Rovira 1959; Smith 1972). Exudates are primarily from recent-assimilate photosynthesis (Neumann and Römheld 2007). Consequently, photosynthetic rate could strongly affect the release of root exudate compounds and exudate rates. In 1959, Rovira studied the directly link of light density to the root exudates of tomato and clover and found that the amounts of serine, glutamic acid and α -alanine in clover exudates decreased with the reduction of light density; however, in tomato exudates, aspartic acid, glutamic acid, phenylalanine and leucine decreased with decreased light density but serine and asparagine increased. In the same study, the author also reported that high temperature could increase the amounts of amino acids exuded by both tomato and clover (Rovira 1959). Bekkara et al. (1998) reported that the exudation of tannins and phenolic compounds in *Vicia faba* was greatly reduced at 4°C compared to the amounts exuded at 30°C. Citrate efflux was studied by Watt and Evans (1999) in white lupin and they found that higher efflux rate were detected during the daytime in comparison to that at night time. Several other studies also reported that different concentrations and/or composition of root exudates were released by plants during the day and night time (Kuzyakov and Siniakina 2001; Melnitchouck et al. 2005). In addition, Walker et al. (2003) noticed that the majority of compounds did not persist continually in the root exudates and were not detected at every harvest day in their study of metabolic profiling of root exudates in *Arabidopsis thaliana*. The release of some compounds in exudates, such as phyto siderophore, was influenced by light or other physiochemical parameters (such as iron deficiency) and consequently only appeared at certain time of the day (Reichman and Parker 2007). However, in the current study, no clear trend of OAs was found according to the time period (Figure A4.2-1). This may be due to the collection of exudates in specific root areas (*in situ*) which were influenced greatly by microscale environment (soil pH, moisture, etc.), while in all the cited research, the exudates from whole root system in trapping solution were analysed.

Apart from the individual root areas chosen for this study, the variability in the detected amount may be caused by other factors. Some of the OAs showed high concentrations when collected early in the morning at the period of 6 to 8 am, with the concentration dropping down in the second collection. This may be due to the accumulation of these OAs on the surface of root samples before collection. Even though the *in situ* sampling used here in rhizotrons reduced the risk of damaging plant roots to a minimum level, some roots may be stressed by thumbtacks or samplers during the operation, especially at the later collection periods. Damaged roots were also observed in root region W3 in the last

three collections and the sampling region was moved down a little bit to avoid the damaged roots.

Because the root samples in different regions were different (types and tree lines), the samples were analysed individually as well as groups (GM and WT trees). No consistent patterns of exudation of individual OA over time in five regions were observed in both analyses.

Spatial variability

Spatial variability in OAs collected at the same periods across five sampling regions may be explained by different root types selected. In addition, the surrounding soil environment and root depths may also contribute to the observed differences. A diverse range of roots (types: root tips, root elongation zone, root colonized by ECM, depths: 10 and 20 cm, tree lines: GM and WT) were selected in the current experiment with the aim to investigate the commonly present temporal variability of OAs in the exudate samples of pine trees. Due to the large amount of work and limited equipment, only five regions (no replicates per region) could be processed within a day.

Conclusions

The composition of OAs exuded by radiata pine roots was relatively stable, but the quantities of each OA changed greatly over time. No consistent pattern of exudation of OAs in five examined root regions was found. In order to compare the root exudates exuded by radiata pine roots in Chapter 4, the time period between 10 am to 12 pm was selected for exudate collection from radiata pine trees in the rhizotrons based on the observation that most of OAs showed reasonable amounts in all root samples at this time.

References

- Bekkara F, Jay M, Viricel M R, Rome S (1998) Distribution of phenolic compounds within seed and seedlings of two *Vicia faba* cvs differing in their seed tannin content and study of their seed and root phenolic exudations. *Plant and Soil* 203:27-36
- Koo B-J, Adriano D C, Bolan N S, Barton C D (2005) Root exudates and microorganisms. In: Hillel D, Hatfield J L, Powlson D S, et al. (eds) *Encyclopedia of Soils in the Environment*, Elsevier Academic Press, London. pp 421-428
- Kuzyakov Y, Siniakina S (2001) A novel method for separating root-derived organic compounds from root respiration in non-sterilized soils. *Journal of Plant Nutrition and Soil Science* 164:511-517
- Melnitchouck A, Leinweber P, Eckhardt K U, Beese R (2005) Qualitative differences between day- and night-time rhizodeposition in maize (*Zea mays* L.) as investigated by pyrolysis-field ionization mass spectrometry. *Soil Biology and Biochemistry* 37:155-162
- Neumann G, Römheld V (2007) The release of root exudates as affected by the plant's physiological status. In: Pinton R, Varanino Z, Nannipieri P (eds) *The Rhizosphere:*

- Biochemistry and Organic Substances at the Soil-Plant Interface, CRC Press, New York. pp 23-72
- Reichman S M, Parker D R (2007) Probing the effects of light and temperature on diurnal rhythms of phytosiderophore release in wheat. *New Phytologist* 174:101-108
- Rovira A D (1959) Root excretions in relation to the rhizosphere effect. IV. Influence of plant species, age of plant, light, temperature and calcium nutrition on exudation. *Plant and Soil* 11:53-64
- Smith W H (1972) Influence of artificial defoliation on exudates of sugar maple. *Soil Biology and Biochemistry* 4:111-113
- Walker T S, Bais H P, Halligan K M, Stermitz F R, Vivanco J M (2003) Metabolic profiling of root exudates of *Arabidopsis thaliana*. *J. Agric. Food Chem.* 51:2548-2554
- Watt M, Evans J R (1999) Linking development and determinacy with organic acid efflux from proteoid roots of white lupin grown with low phosphorus and ambient or elevated atmospheric CO₂ concentration. *Plant Physiology* 120:705-716

Appendix 4.4 Causes and Possible Solutions of the Errors in Calculation of Root Area using software

Objective

In situ sampling developed in this project using AEM in the rhizotrons showed many advantages over the commonly used methods (Chapter 3, Section 3.3.3.2). However, the exudate unit used in Chapter 3 was the amount of OAs collected over the whole membrane, which was not the case in many situations. From the recorded images, less than 25% of AEM area was directly in contact with root samples in the majority of cases. Therefore, software was designed to calculate the actual root areas in contact with AEM strips. Due to the bevel design of the endoscope camera lens and geometrical distortions during image recording (roundness of the access portal and flatness of the recorded image), the area calculated by the software may be different from the actual area. Hence, this experiment was carried out to test the error of this calculation software in the worst scenarios with the aim of recording photos with roots in most suitable positions, if possible, to reduce such errors.

Methods

Two photos, one with AEM-Whatman 3MM strip on top of roots, the other without AEM-Whatman 3MM strip, were taken in the same position with the endoscope camera during the sampling process (Chapter 4, Section 4.2.4.2). Two corresponding images with or without AEM were loaded up to the designed software and overlapped to identify the roots and surrounding soils covered by AEM-Whatman 3MM strip in a size of 1 x 2 cm. Based on the color difference between root and soil, the roots within a defined color range could be identified by the software. In some cases, roots showed a similar color as surrounding soil. Then, a boundary between roots and soil could be firstly defined in the image manually which allows the identification of roots based on color only in the defined area. The proportion of the identified area out of the total AEM strip area (2 cm²) was then calculated by the software.

Due to the bevel design of the endoscope camera lens (Figure 4-2b), the image can cause distortion during the recording step. An experiment was set up to test the error caused by this design. A graph paper with standard 1 x 1 mm square was indicated by arrows with a 1 x 2 cm square (same size as AEM strip) and inside of this square, a small area (2 x 7 mm) was marked by pen to mimic a root (Figure A4.3-1). The graph paper was

placed on a flat surface in a vertical angle to the surface of camera lens, and then parallel to the surface of camera lens, and finally horizontal to the camera lens (Figure A4.3-1). The images were recorded by an endoscope camera for analysis by the software.

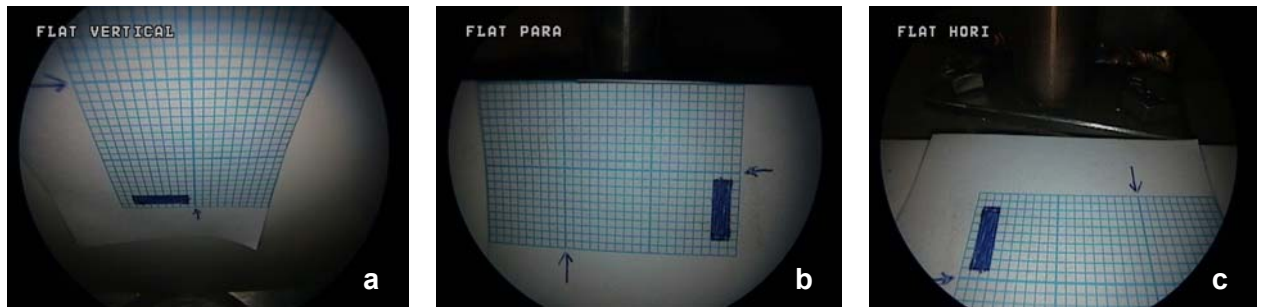


Figure A4.3-1 Testing the distortion caused by the bevel design of camera lens by placing the graph paper in a flat surface which was a). vertical or b). parallel and c). horizontal to the surface of camera lens.

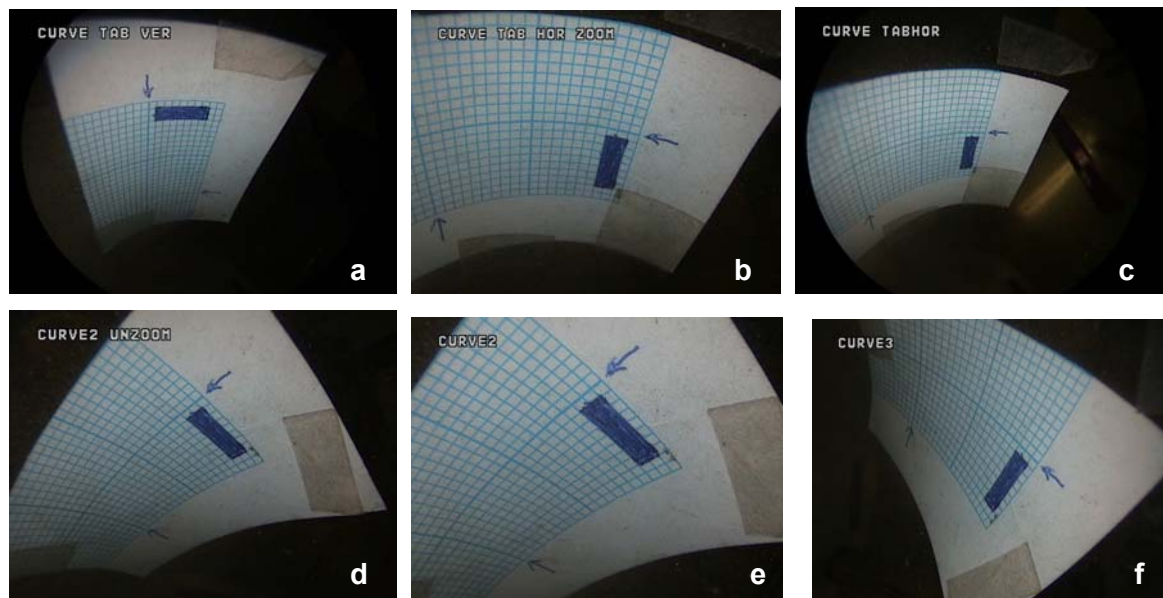


Figure A4.3-2 Testing the geometric distortion of image while placing the graph paper on the portal.

Due to the roundness of the access portal and the flatness of recorded image, the geometrical distortion cannot be avoided during the image recording and this software could not correct this distortion. An estimate of the error caused by this distortion in the access portals in rhizotrons was made. The graph paper was placed on the surface of a clear plastic tube which had the same diameters as access portals in the rhizotrons with vertical, horizontal and also random position to the camera lens (Figure A4.3-2). All the images were analysed by the developed software.

Results

Error caused by the bevel design of camera lens

Depending on the position of the “root”, the error caused by the bevel design of the endoscope camera lens ranged from -37% to 16%. This error could easily change depending on the position and the size of the roots in the image. From Figure A4.3-1 and Table A4.3-1, it is noticeable that the ‘root’ located far away from the camera lens resulted in a large negative error and the ‘root’ located near the camera lens could cause a positive error. Therefore, if possible, the roots covered with AEM when sampling should be positioned in the middle of the image to reduce the error caused by the camera lens.

Table A4.3-1 Estimation of error caused by the camera lens.

Position	Calculated area (cm ²)	Error (%)
flat vertical	0.089014	-37.3137
flat parallel	0.164861	16.09905
flat horizontal	0.158961	11.94468

Error caused by geometric distortion

In the tested situations (Figure A4.3-2), the error of calculated “root” area ranged from 5 to 89% (Table A4.3-2). As discussed above, this error can change depending on the position and the size of roots. In addition, the bevel camera lens contributed part of the error, although the ‘root’ was placed in the middle of the image while recording. The geometric distortion error became larger when the long marked site (7 mm) was vertical to the depth of the portal (Figure A4.3-2a) compared to the situation in Figure A4.3-2b, and reflected in the image with larger area be distorted. In Figure A4.3-2a, there was little geometric distortion for the short marked site (2 mm) as it is along the depth of the access portal. The error for the ‘root’ in Figure A4.3-2b was much smaller (4%) in comparison with the situation in Figure A4.3-2a (89%), as the long marked site was along the depth of access portal (Table A4.3-2). When placing the graph paper randomly in the access portal (most likely in the real situation), the error was among the two extreme scenarios with the same marked area. As the software calculates the proportion of root area in the total strip, whether or not the zoom is used will not result in any difference. However, the position of the roots in the image may affect the result slightly.

Table A4.3-2 Estimation of error caused by the geometric distortion.

Position	Calculated area (cm ²)	Error (%)
curve tab vertical	0.268444	89.04479
curve tab horizontal	0.147691	4.00752
curve tab horizontal zoomed	0.149106	5.003939
curve 2	0.228274	60.75628
curve 2 not zoomed	0.226174	59.27752
curve 3	0.223134	57.13689

Conclusion

The calculation of root area surface which is directly in contact with AEM strips from two images (with or without AEM strips) recorded at the same spot during the sampling could be achieved using the developed software. Although the error of this calculation caused by the bevel design of camera lens and geometric distortion could not be corrected using this software, efforts had been made to reduce these errors during the image recording process. If possible, roots growing along the depth of access portals were selected to reduce the error caused by geometric distortion at a maximum level, although it is heavily dependent on how roots appeared in the access portal. When recording image, the roots should be located in the middle part of the image to reduce the error caused by the bevel design of camera lens. All the information obtained here was applied when collecting exudates *in situ* in Chapter 4, Section 4.2.4.2.

Appendix 4.5 Shannon Diversity Indices in Soil Microbial Communities Associated with Pine Trees

Table A4.4-1 Shannon diversity indices in microbial communities in rhizosphere and non-rhizosphere soil samples collected at the second sampling ($n=8$ for rhizosphere and non-rhizosphere soils), and the P -values of ANOVA analysis across the treatments. Values are presented as the mean \pm 1 standard error for samples in each treatment.

2 nd sampling	Taxonomic group	GM 10cm	WT 10cm	GM 20cm	WT 20cm	P -value
Rhizosphere soil	Bacterial 16S	3.67 \pm 0.023	3.65 \pm 0.033	3.68 \pm 0.035	3.64 \pm 0.032	0.818
	α -Proteobacteria	2.91 \pm 0.109	2.85 \pm 0.093	2.84 \pm 0.091	2.85 \pm 0.093	0.76
	β -Proteobacteria	2.46 \pm 0.061	2.56 \pm 0.083	2.41 \pm 0.042	2.37 \pm 0.066	0.201
	Pseudomonads	2.96 \pm 0.074	2.93 \pm 0.061	2.88 \pm 0.051	2.84 \pm 0.048	0.371
	Fungal 18S	2.71 \pm 0.055	2.69 \pm 0.059	2.67 \pm 0.066	2.81 \pm 0.064	0.454
Non-rhizosphere soil	Bacterial 16S	3.63 \pm 0.030	3.40 \pm 0.149	3.63 \pm 0.028	3.62 \pm 0.023	0.099
	α -Proteobacteria	2.83 \pm 0.102	2.68 \pm 0.095	2.91 \pm 0.063	2.83 \pm 0.047	0.062
	β -Proteobacteria	2.56 \pm 0.066	2.53 \pm 0.091	2.57 \pm 0.099	2.54 \pm 0.098	0.971
	Pseudomonads	2.83 \pm 0.087	2.66 \pm 0.142	2.93 \pm 0.053	2.84 \pm 0.086	0.094
	Fungal 18S	2.80 \pm 0.057	2.70 \pm 0.082	2.86 \pm 0.082	2.92 \pm 0.040	0.21

Table A4.4-2 Shannon diversity indices in microbial communities in rhizosphere, non-rhizosphere and bulk soil samples collected at the third sampling ($n=4$ for bulk soils), and the P -values of ANOVA analysis across the treatments. Values are presented as the mean \pm 1 standard error for samples in each treatment.

3 rd sampling	Taxonomic group	GM D1	WT D1	GM D2	WT D2	GM D3	WT D3	P -value
Rhizosphere soil	Bacterial	3.53 \pm 0.044	3.53 \pm 0.057	3.46 \pm 0.029	3.50 \pm 0.037	3.51 \pm 0.025	3.52 \pm 0.010	0.726
	α -proteobacteria	2.81 \pm 0.066	2.97 \pm 0.119	2.86 \pm 0.080	2.96 \pm 0.130	2.73 \pm 0.119	2.98 \pm 0.119	0.437
	β -proteobacteria	2.47 \pm 0.075	2.50 \pm 0.037	2.58 \pm 0.043	2.52 \pm 0.052	2.54 \pm 0.066	2.67 \pm 0.063	0.249
	Pseudomonas	2.89 \pm 0.041	2.90 \pm 0.063	3.00 \pm 0.065	2.84 \pm 0.202	2.93 \pm 0.034	2.98 \pm 0.120	0.512
	Fungal	2.39 \pm 0.106	2.46 \pm 0.168	2.57 \pm 0.122	2.60 \pm 0.073	2.53 \pm 0.092	2.57 \pm 0.026	0.874
Non-rhizosphere soil	Bacterial	3.52 \pm 0.027	3.54 \pm 0.021	3.48 \pm 0.020	3.51 \pm 0.097	3.56 \pm 0.033	3.55 \pm 0.016	0.798
	α -proteobacteria	2.74 \pm 0.068	3.06 \pm 0.099	2.86 \pm 0.052	2.72 \pm 0.072	2.81 \pm 0.061	2.83 \pm 0.144	0.178
	β -proteobacteria	2.63 \pm 0.091	2.63 \pm 0.114	2.77 \pm 0.110	2.76 \pm 0.031	2.42 \pm 0.055	2.74 \pm 0.093	0.094
	Pseudomonas	2.96 \pm 0.023	2.98 \pm 0.070	2.97 \pm 0.051	3.10 \pm 0.025	2.95 \pm 0.043	2.90 \pm 0.060	0.184
	Fungal	2.68 \pm 0.084	2.56 \pm 0.100	2.51 \pm 0.066	2.67 \pm 0.024	2.62 \pm 0.068	2.68 \pm 0.073	0.106
Bulk soil	Bacterial	3.57 \pm 0.036	3.55 \pm 0.027	3.58 \pm 0.009	3.60 \pm 0.025	NA [#]	NA	0.758
	α -proteobacteria	2.99 \pm 0.021	2.75 \pm 0.140	2.86 \pm 0.077	2.82 \pm 0.140	NA	NA	0.161
	β -proteobacteria	2.62 \pm 0.179	2.51 \pm 0.115	2.55 \pm 0.118	2.38 \pm 0.093	NA	NA	0.28
	Pseudomonas	3.02 \pm 0.024	2.93 \pm 0.079	3.05 \pm 0.064	2.84 \pm 0.066	NA	NA	0.339
	Fungal	2.71 \pm 0.072	2.63 \pm 0.094	2.77 \pm 0.018	2.53 \pm 0.072	NA	NA	0.069

*D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm. [#]NA = not applicable

Appendix 4.6 ANOVA Analysis of Individual OAs between Water-soluble Exudate Solutions from Root-rhizosphere and Non-rhizosphere soils

Table A4.5-1 Probability values analysed by ANOVA in the concentrations of individual OA present between the water-soluble exudate solutions from root-rhizosphere soils and non-rhizosphere soils, with each sample under the null hypothesis that all the samples have the same mean. The probability values with significant difference ($P < 0.05$) are shown in bold. “-” indicates that no P -value was applicable for some anions as these anions were not detected in any of the non-rhizosphere exudates extracted by water.

Organic anion	1 st sampling	2 nd sampling	3 rd sampling
formate	<0.001	0.082	-
acetate	<0.001	0.102	-
quininate	<0.001	<0.001	<0.001
shikimate	<0.001	<0.001	<0.001
malate	<0.001	<0.001	<0.001
succinate	-	0.002	0.003
lactate	0.028	<0.001	<0.001
malonate	-	<0.001	<0.001
citrate	-	<0.001	<0.001
fumarate	<0.001	<0.001	<0.001
maleate	<0.001	-	0.233
tartarate	-	<0.001	0.025

Appendix 4.7 Soil pH and Moisture in Non-rhizosphere Soil from the 1st and 3rd Samplings in Chapter 4

Table A4.6-1 Measurements of soil moisture and pH in non-rhizosphere and bulk soil samples collected at the a). first sampling and b). third sampling and *P*-values analysed by ANOVA across the treatments. Values are presented as the mean \pm 1 standard error ($n=8$ for the non-rhizosphere soils, $n=4$ for the bulk soils). The probability values with significant difference ($P<0.05$) are shown in bold. Different letters indicate significantly different ($P<0.05$) between treatments.

a. 1 st sampling		GM 10 cm	GM 20 cm	WT 10 cm	WT 20 cm	<i>P</i> -value	
Soil moisture (%)		20.24 \pm 0.375	20.41 \pm 0.298	20.33 \pm 0.614	20.32 \pm 0.321	0.835	
Soil pH		5.18 \pm 0.017 ^b	5.02 \pm 0.054 ^a	5.23 \pm 0.047 ^b	5.07 \pm 0.012 ^a	<0.001	

b. 3 rd sampling		Soil	GM D1*	WT D1	GM D2	WT D2	GM D3	WT D3	<i>P</i> -value
Soil moisture	non-rhizosphere soil		12.07 \pm 0.543	9.67 \pm 1.532	9.97 \pm 0.731	8.32 \pm 0.514	8.58 \pm 0.293	7.90 \pm 0.183	0.132
	bulk soil		11.92 \pm 1.592	10.64 \pm 1.311	10.61 \pm 1.341	9.38 \pm 1.208	NA [#]	NA	
Soil pH	non-rhizosphere soil		4.97 \pm 0.089 ^{abc}	4.99 \pm 0.047 ^{abc}	4.90 \pm 0.052 ^a	4.94 \pm 0.031 ^{ab}	5.15 \pm 0.038 ^d	5.08 \pm 0.022 ^{cd}	0.03
	bulk soil		4.94 \pm 0.056 ^{ab}	5.05 \pm 0.040 ^{bcd}	5.02 \pm 0.037 ^{abc}	4.99 \pm 0.030 ^{abc}	NA	NA	

*D1 = 0-14 cm; D2 = 14-28 cm D3 = 28-45 cm. [#]NA = not applicable

Appendix 4.8 Representative DGGE Gel of Basidiomycete Communities in the Rhizosphere and Non-rhizosphere Soil Samples Associated with GM and WT Radiata Pines at the 3rd Sampling

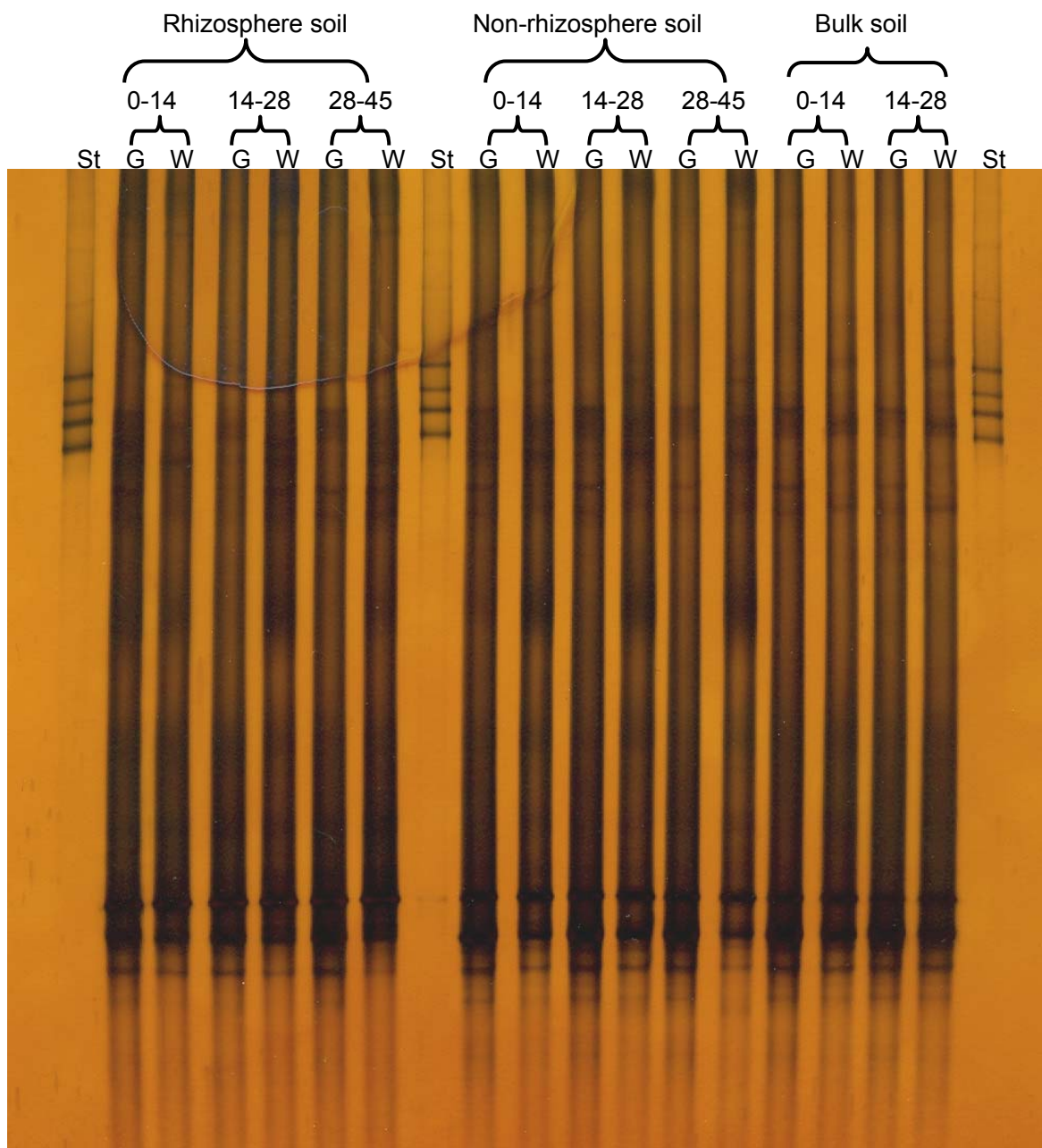


Figure A4.7-1 DGGE profiles of Basidiomycete community in the rhizosphere, non-rhizosphere and bulk soils associated with GM and WT radiata pines grown in one of the rhizotron containers from the 3rd sampling. St: fungal 18S DGGE marker consisted of bulked fungal 18S PCR products from strain *Umbelopsis ramanniana*, *Trichoderma reesei*, *Mortierella chlamydospora*, *Phoma cucurbitacearum*, *Clonostachys rosea*.

Appendix 4.9 Representative DGGE Gel of *gacA* Gene in *Pseudomonas* in the Rhizosphere and Non-rhizosphere Soil Samples Associated with GM and WT Radiata Pines at the 1st Sampling

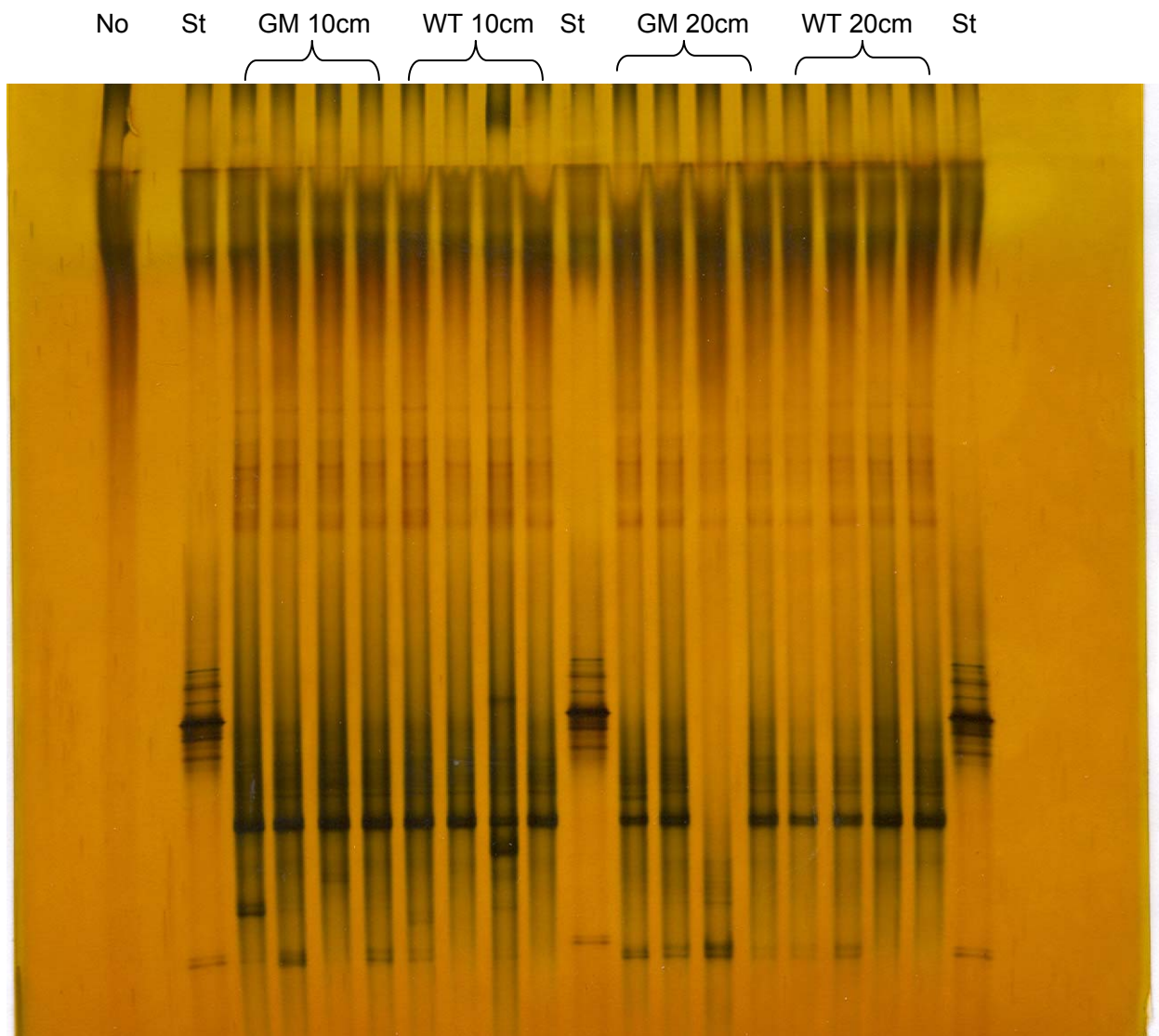


Figure A4.8-1 DGGE profiles of *gacA* gene from *Pseudomonas* communities in the rhizosphere and non-rhizosphere soils associated with GM and WT radiata pines grown in one of the rhizotron containers. No: negative control. St: bacterial 16S DGGE marker consisted of bulked bacterial 16S PCR products from strains *Pectobacterium carotovorum*, *Variovorax paradoxus* and *Arthrobacter* sp..

Appendix 5.1 HPLC Analysis of a Root Exudate Sample of Radiata Pine Seedling Collected by CaCl₂ Solution as Described in Appendix 3.1

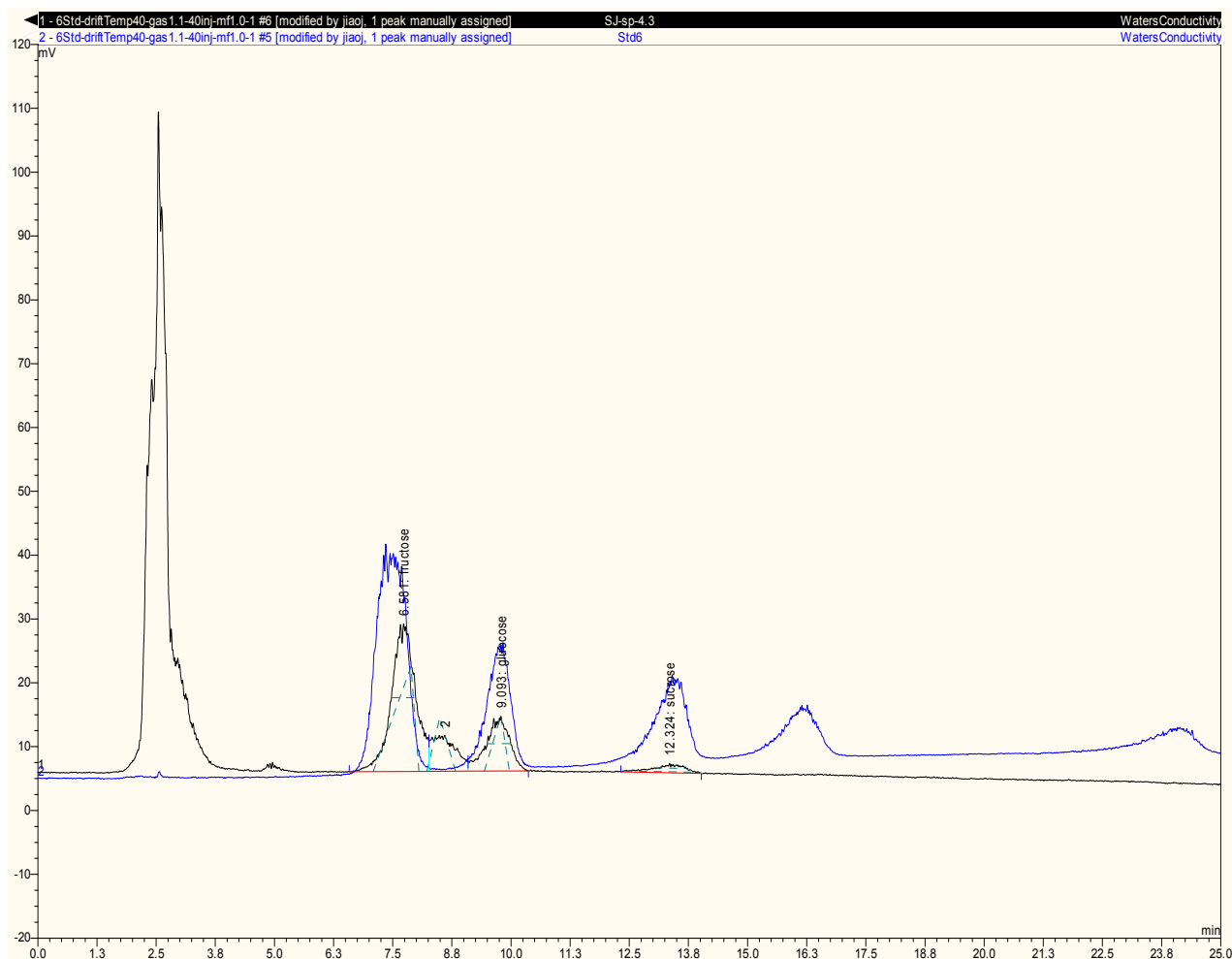


Figure A5.1-1 Chromatogram of a root exudate sample of radiata pine seedling collected by CaCl₂ solution (shown as black line) and standard sugar solutions containing fructose, glucose, sucrose, maltose, raffinose (shown as blue line). This exudate sample contained 72.54 mg l⁻¹ of fructose, 28.75 mg l⁻¹ of glucose and 10.36 mg l⁻¹ of sucrose.

Appendix 5.2 Archaeal 16S PCR

Archaeal 16S were amplified using the primer set 4Fa/1492R with cDNA templates of treatments Con, S, SQLM, SQ, SL and SM for phylochip analysis. The cDNA samples were converted by 1492R primer as described in Chapter 5, Section 5.2.4. The sequence for 4Fa is 5'- TCCGGTTGATCCTGCCRG -3' (Hershberger et al. 1996). The archaeal PCR reaction was carried out in 100 µl volumes containing 6 µl cDNA (1:10 diluted after RT-PCR) or RT-PCR negative control, 1 x OptiBuffer (Bioline, Australia), 0.2 mM dNTPs (Promega Corporation, WI, USA), 0.4 µM each primer, 2 mM MgCl₂, 0.4 mg ml⁻¹ BSA (Promega Corporation, WI, USA), 2% DMSO (PCR reagent, Sigma-Aldrich) and 6.4 U Bio-X-Act DNA polymerase (Bioline, Australia). PCR amplifications adapted from Brodie et al. (2007) were performed in a thermal cycler (I Cycler, Bio-Rad Laboratories, CA, USA) with an initial denaturing step at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 53°C for 30 s and 72°C for 1 min, before a final extension step at 72°C for 7 min. Amplified PCR products were checked by electrophoresis of 5 µl PCR products in 1% agarose gel with ethidium bromide staining (Figure A5.2).

Multiple bands were shown in most of PCR product samples on agarose gels with archaeal 16S PCR product (approximate 1500 bp) indicated by red arrows in Figure A5.2. The multiple PCR products in each sample were consistent with the products obtained by Brodie et al. (2007), DeAngelis et al. (2008) and K. DeAngelis (pers. comm.) using the same primer set and PCR conditions. With the exception of samples from treatment SM, all the other samples showed successful amplification of archaeal 16S gene sequence. No positive or very low amounts of archaeal 16S PCR products were obtained from either cDNA samples (Figure A5.2) or DNA samples (data not shown) of treatment SM. This result was consistent with repeated PCR amplifications, indicating the reduced archaeal populations in treatment SM compared to the control which showed strong amplifications of archaeal 16S (Figure A5.2).

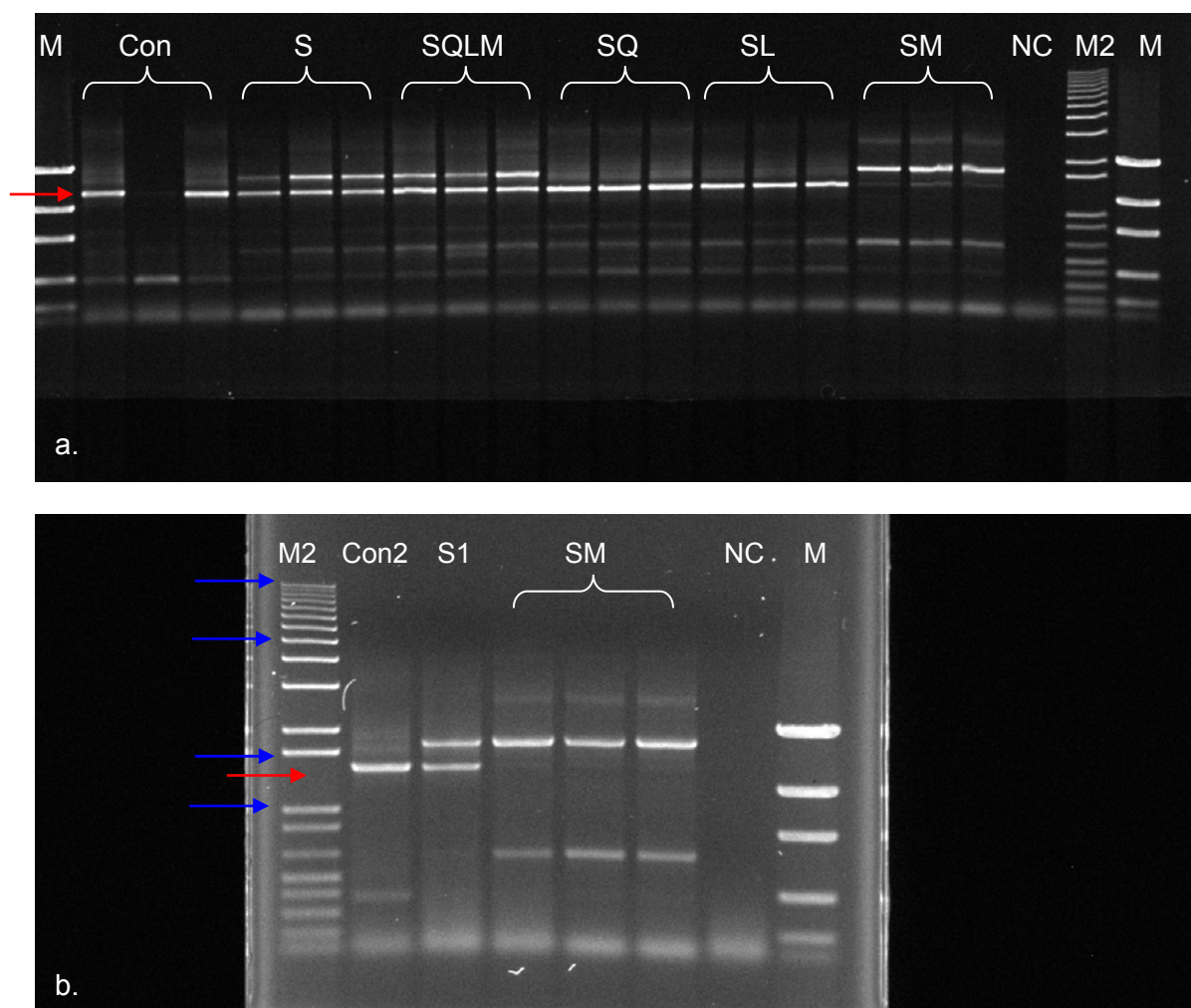


Figure A5.2 Agarose gels of archaeal 16S PCR products amplified from cDNA derived from soils amended daily with either DI water or various ARE solutions. a). PCR products of treatments Con, S, SQLM, SQ, SL and SM. b). PCR products of one sample from treatments Con, S and three samples from treatment SM (after another PCR reaction). The archaeal 16S PCR products are around 1500 bp and indicated by red arrows. M: Low DNA mass ladder (Invitrogen, USA) with band size (from top to bottom): 2000 bp, 1200 bp, 800 bp, 400 bp, 200 bp and 100 bp. M2: 1kb plus DNA ladder (Invitrogen) with marked (blue arrows) band size (from top to bottom): 12000 bp, 5000 bp, 1650 bp; 1000 bp. NC: PCR negative control using RT-PCR negative control as PCR template.

References

- Brodie E L, DeSantis T Z, Parker J P M, Zubietta I X, Piceno Y M, Andersen G L (2007) Urban aerosols harbor diverse and dynamic bacterial populations. *Proceedings of the National Academy of Sciences* 104:299-304
- DeAngelis K M, Brodie E L, DeSantis T Z, Andersen G L, Lindow S E, Firestone M K (2008) Selective progressive response of soil microbial community to wild oat roots. *The ISME Journal* 3:168-178
- Hershberger K L, Barns S M, Reysenbach A L, Dawson S C, Pace N R (1996) Wide diversity of Crenarchaeota. *Nature* 384:420-420
- K. DeAngelis, personal communication, April, 2009