Microbial factors associated with the natural suppression of take-all in wheat in New Zealand

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by Soon Fang Chng

Take-all, caused by the soilborne fungus, *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is an important root disease of wheat that can be reduced by take-all decline (TAD) in successive wheat crops, due to general and/or specific suppression. A study of 112 New Zealand wheat soils in 2003 had shown that *Ggt* DNA concentrations (analysed using real-time PCR) increased with successive years of wheat crops (1-3 y) and generally reflected take-all severity in subsequent crops. However, some wheat soils with high *Ggt* DNA concentrations had low take-all, suggesting presence of TAD. This study investigated 26 such soils for presence of TAD and possible suppressive mechanisms, and characterised the microorganisms from wheat roots and rhizosphere using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE).

A preliminary pot trial of 29 soils (including three from ryegrass fields) amended with 12.5% w/w *Ggt* inoculum, screened their suppressiveness against take-all in a growth chamber. Results indicated that the inoculum level was too high to detect the differences between soils and that the environmental conditions used were unsuitable. Comparison between the *Ggt* DNA

concentrations of the same soils collected in 2003 and in 2004 (collected for the pot trial), showed that most soils cropped with 2, 3 and 4 y of successive wheat had reduced *Ggt* DNA concentrations (by 195-2911 pg g^{-1} soil), and their disease incidences revealed 11 of the 29 test soils with potential take-all suppressiveness.

Further pot trials improved the protocols, such that they were able to differentiate the magnitudes of suppressiveness among the soils. The first of the subsequent trials, using 4% w/w *Ggt* inoculum level, controlled conditions at 16°C, 80% RH with alternate 12 h light/dark conditions, and watering the plants twice weekly to field capacity (FC), screened 13 soils for their suppressiveness against take-all. The 13 soils consisted of 11 from the preliminary trial, one wheat soil that had been cropped with 9 y of wheat (considered likely to be suppressive), and a conducive ryegrass soil. The results revealed that 10 of these soils were suppressive to take-all. However, in only four of them were the effects related to high levels of microbial/biological involvement in the suppression, which were assessed in an experiment that first sterilised the soils. In a repeat trial using five of the soils H1, H3, M2, P7 (previously cropped with 3, 3, 4 and 9 y successive wheat, respectively) and H15 (previously cropped with 5 y of ryegrass), three of them (H1, H3 and M2) had reduced *Ggt* DNA concentrations (>1000 pg g^{-1} soil reductions), and were confirmed to be suppressive to take-all. A pot trial, in which 1% of each soil was transferred into a γ-irradiated base soil amended with 0.1% *Ggt* inoculum, indicated that soils H1

and H3 (3 y wheat) were specific in their suppressiveness, and M2 (4 y wheat) was general in its suppressiveness.

The microbial communities within the rhizosphere and roots of plants grown in the soils, which demonstrated conduciveness, specific or general suppressiveness to take-all, were characterised using PCR-DGGE, and identities of the distinguishing microorganisms (which differentiated the soils) identified by sequence analysis. Results showed similar clusters of microorganisms associated with conducive and suppressive soils, both for specific and general suppression. Further excision, re-amplification, cloning and sequencing of the distinguishing bands showed that some actinomycetes (*Streptomyces bingchengensis*, *Terrabacter* sp. and *Nocardioides* sp.), ascomycetes (*Fusarium lateritium* and *Microdochium bolleyi*) and an unidentified fungus, were associated with the suppressive soils (specific and general). Others, such as the proteobacteria (*Pseudomonas putida* and *P. fluorescens*), an actinomycete (*Nocardioides oleivorans*), ascomycete (*Gibberella zeae*), and basidiomycete (*Penicillium allii*), were unique in the specific suppressiveness. This indicated commonality of some microorganisms in the take-all suppressive soils, with a selected distinguishing group responsible for specific suppressiveness. General suppressiveness was considered to be due to no specific microorganisms, as seen in soil M2.

An attempt to induce TAD by growing successive wheat crops in pots of *Ggt*-infested soils was unsuccessful with no TAD effects shown, possibly due to variable *Ggt* DNA concentrations in

the soils and addition of nutrients during the experiment. Increasing numbers of *Pseudomonas fluorescens* CFU in the rhizosphere of plants, during successive wheat crops was independent of the *Ggt* DNA concentrations and disease incidence, suggesting that increases in *P. fluorescens* numbers were associated with wheat monoculture.

This study has demonstrated that TAD in New Zealand was due to both specific and general suppressiveness, and has identified the distinguishing microorganisms associated with the suppression. Since most of these distinguishing microorganisms are known to show antagonistic activities against *Ggt* or other soilborne pathogens, they are likely to act as antagonists of *Ggt* in the field. Future work should focus on validating their effects either individually, or interactively, on *Ggt* in plate and pot assays and under field conditions.

Keywords: *Gaeumannomyces graminis* var. *tritici*, take-all, successive wheat, DNA, inoculum, suppressive soils, take-all decline, denaturing gradient gel electrophoresis, DGGE, microbial populations, *Pseudomonas fluorescens*.

Table of Contents

Title Page ... i

Abstract ... ii

Table of Contents ... vi

List of Tables ... xi

List of Figures ... xiii

List of abbreviations and symbols ... xviii

List of companies in text ... xx

Acknowledgements ... xxi

Chapter 1 Introduction ... 1

1.1 Wheat production in New Zealand ... 1
1.2 Significance of take-all in wheat ... 1
1.3 Biology of *Gaeumannomyces graminis* var. *tritici* and related fungi ... 2
1.4 Isolation of *Ggt* from infected roots ... 3
1.5 Disease symptoms and damage ... 3
1.6 Disease cycle ... 5
1.7 Factors affecting *Ggt* as soil inoculum ... 6
1.7.1 Competitive saprophytic ability ... 6
1.7.2 Nitrogen ... 7
1.7.3 Temperature and moisture ... 9
1.8 Management of take-all ... 11
1.8.1 Chemical controls ... 11
1.8.1.1 Soil fumigation ... 11
1.8.1.2 Soil fungicides ... 12
1.8.1.3 Seed treatment ... 13
1.8.1.4 Foliar treatment ... 14
1.8.2 Cultural practices ... 14
1.8.2.1 Crop rotation ... 15
1.8.2.2 Direct drilling ... 17
1.8.2.3 Soil fertilisation and host-plant nutrition ... 18
1.8.3 Disease prediction by quantitative soil analysis of *Gaeumannomyces graminis* var. *tritici* ... 19

1.8.4 Biological control 20

1.8.4.1 Take-all suppressive soils 20

1.9 Mechanisms of take-all decline 24

1.9.1.1 Interactions of microbial antagonists with *Ggt* 26

1.10 Characterising microbial components associated with soil suppressiveness 28

1.11 Overall aim and hypothesis of PhD research 30

1.12 References 31

Chapter 2 *Gaeumannomyces graminis* var. *tritici* concentrations in soils from successive wheat cropping and resulting disease incidence 38

2.1 Abstract 38

2.2 Introduction 39

2.3 Materials and methods 40

2.3.1 Soil sampling and processing 40

2.3.2 Pot experiment 42

2.3.3 Disease assessment 46

2.3.4 Statistical analyses 46

2.4 Results 47

2.4.1 *Ggt* DNA concentrations in field soils with different periods of successive wheat cropping.. 47

2.4.2 Disease incidence from the pot experiment 49

2.4.3 Soil *Ggt* DNA concentrations and disease incidence from the pot experiment 50

2.4.4 Fresh and dry weights from pot experiment 53

2.5 Discussion 55

2.6 References 61

Chapter 3 Inoculation of soils with different levels of *Gaeumannomyces graminis* var. *tritici* to detect differing take-all suppressiveness 65

3.1 Abstract 65

3.2 Introduction 65

3.3 Materials and methods 66

3.3.1 Soil sampling and processing 66

3.3.2 *Ggt* inoculum production 67

3.3.3 Pot experiment 68

3.3.4 Disease assessment and other measurements 68

3.3.5 Statistical analyses 70

3.4 Results 70

3.4.1 Disease incidence 70

3.4.2 Plant dry and fresh weights 72

3.4.3 Water uptake 74

3.5 Discussion 75
3.6 References 80

Chapter 4 Manipulating controlled environmental conditions to maximise efficacy in screening of take-all suppressive soils 82
4.1 Abstract 82
4.2 Introduction 82
4.3 Materials and methods 83
4.3.1 Soil samples 83
4.3.2 Pot bioassay experiment 84
4.3.3 Disease assessment and other measurements 87
4.3.4 Statistical analyses 87
4.4 Results 87
4.4.1 Effects of environmental conditions on disease incidence 87
4.4.2 Effects of environmental conditions on plant dry weights 89
4.4.3 Water uptake 94
4.5 Discussion 96
4.6 References 101

Chapter 5 Take-all decline in New Zealand wheat soils and its potential mechanisms 104
5.1 Abstract 104
5.2 Introduction 105
5.3 Materials and methods 107
5.3.1 Soil samples 107
5.3.2 Experiment 1: Screening of soils for suppressiveness 107
5.3.2.1 Pot bioassay 107
5.3.2.2 Disease assessment 108
5.3.2.3 Statistical analyses and other calculations 111
5.3.3 Experiment 2: Reproducibility of the screening experiment 112
5.3.4 Experiment 3: Transferability of suppressive properties between soils 113
5.3.4.1 Inoculum preparation 113
5.3.4.2 Pot bioassay 113
5.4 Results 114
5.4.1 Occurrence of take-all suppressive soil 114
5.4.1.1 Experiment 1: Screening of soils for suppressiveness 114
5.4.1.2 Experiment 2: Reproducibility of the screening experiment 118

5.4.2 Experiment 3: Transferability of suppressive properties between soils ... 121
5.4.3 Comparative analyses of the suppressiveness of soils between the three pot experiments ... 122
5.5 Discussion ... 123
5.6 References ... 132

Chapter 6 Characterisation of the microbial populations in the rhizosphere and the roots of wheat plants grown in soils with different suppressiveness to take-all using denaturing gradient gel electrophoresis ... 136

6.1 Abstract ... 136
6.2 Introduction ... 137
6.3 Materials and methods ... 140
6.3.1 Part 1: Confirmation of *Ggt* isolates used in the pot assays ... 140
6.3.1.1 *Ggt* isolates ... 140
6.3.1.2 DNA extraction from isolates ... 140
6.3.1.3 The amplification and sequencing of the ITS-region ... 141
6.3.2 Part 2: Characterisation of the microbial populations associated with take-all suppressiveness ... 142
6.3.2.1 DNA extraction from the root/rhizosphere samples ... 142
6.3.2.2 PCR amplification of group targeted rDNA gene fragments for DGGE analysis ... 144
6.3.2.3 Denaturing gradient gel electrophoresis (DGGE) ... 146
6.3.2.4 Band scoring and statistical analyses ... 146
6.3.2.5 Recovery, re-amplification, cloning and sequencing of the distinguishing DGGE bands 147
6.4 Results ... 148
6.4.1 Identity confirmation of the *Ggt* isolates ... 148
6.4.2 Microbial communities in the rhizosphere and roots of wheat plants grown in soils of different suppressiveness ... 149
6.4.3 Similarities among the five soils in the banding patterns derived from DGGE analyses ... 150
6.4.4 Identification of bands that differentiated the soils ... 151
6.4.4.1 Principal component analyses ... 151
6.4.4.2 Sequence analysis of the distinguishing DGGE bands ... 157
6.5 Discussion ... 158
6.6 Acknowledgement ... 166
6.7 References ... 167

Chapter 7 Attempted induction of soil suppressiveness to take-all in a pot experiment 175

7.1 Abstract 175

7.2 Introduction 176

7.3 Materials and methods 177

7.3.1 Soil sampling 177

7.3.2 Pot Experiment-Induction of TAD 180

7.3.3 Disease assessment 181

7.3.4 Numbers of *Pseudomonas fluorescens* CFU in the rhizosphere and roots of wheat plants ... 181

7.3.5 Data analysis 182

7.4 Results 183

7.4.1 Disease incidence 183

7.4.2 *Ggt* DNA concentrations 184

7.4.3 Numbers of *Pseudomonas fluorescens* colonies in the roots of wheat plants 184

7.4.4 Soil pH 185

7.4.5 Nutrient status in the soil 187

7.4.6 Correlations between the parameters 191

7.5 Discussion 193

7.6 References 199

Chapter 8 Concluding discussion 203

8.1 Prediction of take-all risk in subsequent wheat crop by quantification of *Ggt* DNA concentrations in soil 203

8.2 Efficacy of the pot assays used for screening of take-all suppressive soils 205

8.3 New Zealand TAD soils and their possible mechanisms 205

8.4 Microorganisms associated with the suppressiveness of TAD 207

8.5 Fluorescent *Pseudomonas* spp. as potential biological control agents of *Ggt* 209

8.6 Soil nutrition and TAD induction 210

8.7 The modified hypothesis 211

8.8 Recommendations for future research 211

8.9 Concluding remarks 214

8.10 References 215

Appendices 218

Appendix 1 Growth stages of cereals 218

Appendix 2 Soil types of tested soils and descriptions of the methods used for basic soil and mineral N analyses 219

Appendix 3 General methods 221

Appendix 4 Media, gels and solutions 225

Appendix 5 Additional results and ANOVA tables of statistical analyses 227

List of Tables

Table 1.1 Examples of risk of take-all in susceptible cereals after different crops (Hornby & Beale undated). 16

Table 1.2 Characteristics of the seven types of take-all suppression suggested by Hornby (1983). 22

Table 1.3 Causes and the main characteristics of two types of suppressiveness to take-all in soils (Hornby *et al.* 1998). 25

Table 1.4 Advantages and disadvantages of molecular-based microbial community fingerprinting methods (Garbeva *et al.* 2004) 29

Table 2.1 Site information and cropping histories of fields (2003/04) where soils were sampled. The 15 soils sampled from the St Andrews region are shaded. 43

Table 2.2 Some physico-chemical properties of soils sampled. 44

Table 2.3 Sutiability of Nitrogen levels for New Zealand arable crops, as recommended by R. J. Hill Laboratories Ltd (2002). 45

Table 3.1 Field location and cropping history, as well as some properties of the three soils used in the pot experiment. 69

Table 3.2 Physico-chemical properties of the three soils used in the pot experiment. 69

Table 4.1 Field location and cropping history, as well as some properties of the three soils used in the pot experiment. 86

Table 4.2 Physico-chemical properties of the two soils used in the pot experiment. 86

Table 5.1 Field locations, cropping histories and some properties of the soils used in the pot experiments. 109

Table 5.2 Physico-chemical properties of the soils used in the pot experiments. 110

Table 6.1 The origins of the twelve *Ggt* isolates used in this study. The five isolates shaded in grey were used as a mixed inoculum in Chapters 2-5. 140

Table 6.2 Characteristics of the five soils, which were determined in the Chapter 5 studies, including their wheat cropping histories, increases in disease severity (TAR) after the addition of *Ggt*, and potential mechanisms of suppressiveness, used in the current DGGE study. 143

Table 6.3 Primers used to target and amplify the rDNA gene regions of bacteria, fungi, actinomycetes and ascomycetes. 145

Table 6.4 Vertical denaturing gradient of the 8% polyacrylamide gels used in DGGE to separate the double stranded rDNA of the targeted microbial groups. 146

Table 6.5 Genbank ITS sequence comparisons for the 12 *Ggt* isolates showing their nearest relatives in Genbank. 148

Table 6.6 Number of bands present in at least three replicates of each soil for the four microbial groups. Data in parentheses are the total number of bands separated by DGGE for that particular soil. 149

Table 6.7 a-c Results of the principal component analysis, showing the first three principal components and the bands responsible for the separation of the five soils. The number of replicates, in which the distinguishing bands occurred, is also included. Highlighted bands were excised, re-amplified, cloned and sequenced. 154

Table 6.8 Identities of microorganisms, indicated by matching Genbank database sequences to the sequences derived from the distinguishing bands generated on the DGGE profiles specific for the bacterial, fungal, actinomycete and ascomycete rDNA. 158

Table 7.1 Field locations, cropping histories and some properties of the soils used in the Experiment 1.... 178

Table 7.2 Physico-chemical properties of the soils used in Experiment 1.. 179

Table 7.3 A matrix showing the correlations between the parameters measured (disease incidence, *Ggt* DNA concentrations, or numbers of *P. fluorescens* CFU, pH and nutrient status). A negative correlation coefficient indicates that the two variables are inversely related. The highlighted coefficients are the ones that showed strong relationships (r > 0.7). 192

List of Figures

Figure 1.1 World distribution of take-all in 1972 (Hornby *et al.* 1998). 2

Figure 1.2 (a) Colony characteristics of 8 d old *Ggt* on PDA; (b) autumn wheat crop at GS 65 showing whitehead symptoms; (c) runner hyphae of *Ggt* on wheat roots and (d) blackening of wheat stem bases and root systems........ 4

Figure 1.3 Pre-sowing *Ggt* DNA concentrations of soils from 112 wheat fields, in relation to the take-all indices of the subsequent wheat crops grown in the same fields (Cromey, unpubl. data). Take-all risk categories for subsequent crops at the time of assessment are indicated by the graph segments as low, medium and high with *Ggt* DNA concentrations of 5-130, 131-325 and >325 pg g^{-1} soil, respectively........ 20

Figure 1.4 Diagrammatic illustration of take-all decline in continuous cereal crops and its effect on yield (Deacon 1997). 23

Figure 2.1 Set-up of pot assay in growth chamber. 46

Figure 2.2 The relationship between years of successive wheat cropping and the mean post-harvest *Ggt* DNA concentrations in soils in the 2003/04 season. Error bars are the least significant differences (LSD) at the 5% level (df = 115 for both analyses)........ 47

Figure 2.3 Pre-sowing and post-harvest *Ggt* DNA concentrations for field soils with 1-4 y of successive wheat cropping in the 2003/04 season (a - d). The red dotted lines represent the three take-all risk categories used at the time of soil selection, with the vertical and horizontal lines applied to the pre-sowing and post-harvest data, respectively. The area between the first two lines is low risk (5-130 pg g^{-1} of soil), between second and third lines is medium risk (131-325 pg DNA g^{-1} soil), and beyond the third line is high risk (>325 pg g^{-1} soil)........ 48

Figure 2.4 The logit-transformed mean percent infected roots of plants grown in (a) all the test soils and (b) in the 15 St. Andrews soils. Data in parentheses are the back-transformed mean percent infected roots. Error bars are the least significant differences (LSD) at the 5% level (df = 114)........ 49

Figure 2.5 Variations between soils with the same wheat cropping histories on the logit-transformed mean percent infected roots of plants grown in (a) all the test soils and (b) in the 15 St Andrews soils. Error bars are the least significant differences at the 5% level (df = 114)........ 50

Figure 2.6 Relationship between the mean *Ggt* DNA concentrations and the logit mean percent infected roots in St Andrews soils........ 51

Figure 2.7 Change in *Ggt* inoculum concentrations from pre-sowing to post crop harvest in the 2003/04 season for field soils with 1 to 4 y of successive wheat cropping (a – d), relative to mean percent infected roots (back-transformed) of wheat plants grown in the same soils in a pot experiment.. 52

Figure 2.8 Mean (a) fresh and (b) dry weights of plants grown in the 29 test soils naturally infested with *Ggt*. Error bars are the least significant differences (LSD) at the 5% level (df = 114)........ 53

Figure 2.9 Mean weights of plants grown in different soils of the same wheat cropping histories (a) root dry; (b) root fresh; (c) shoot dry; and (d) shoot fresh weights. Error bars are the least significant differences (LSD) at the 5% level (df = 114). 54

Figure 2.10 Mean (a) fresh and (b) dry weights of plants grown in the 15 St Andrews soils. Error bars are the least significant differences (LSD) at the 5% level (df = 114). 55

Figure 3.1 Mean incidence of infected wheat roots in soils with different levels of added *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59). 71

Figure 3.2 Mean incidence of infected wheat roots in the three soils treated with different levels of *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59). 71

Figure 3.3 Variations in incidence of infected roots on wheat plants grown in the three soils treated with different levels of *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59). 72

Figure 3.4 Mean weights of plants grown in soils amended with different levels of *Ggt* inoculum (a) root and shoot dry and (b) root and shoot fresh weights. Error bars are the least significant differences at the 5% level (d.f. = 59), and apply only to the individual plant variables. 73

Figure 3.5 Root and shoot (a) dry and (b) fresh weights of plants grown in the three soils amended with different levels of *Ggt* inoculum. Error bars are the least significant differences (LSD) at the 5% level (d.f. = 59), and apply only to the individual plant variables. 73

Figure 3.6 Mean total water uptake for plants grown in soils amended with different levels of *Ggt* inoculum. Error bar is the least significant difference at the 5% level (d.f. = 59). 74

Figure 3.7 Mean total water uptake for plants grown in the three soils amended with different levels of *Ggt* inoculum. Error bar is the least significant difference at the 5% level (d.f. = 59). 75

Figure 4.1 Set up of pot experiment in C16. 85

Figure 4.2 Mean incidence of infected wheat roots in (a) different growth chambers and (b) soils, subjected to (c) the two watering regimes and (d) with sieving. Error bars are the least significant differences (LSD) at the 5% level [df = 9 (a) and 45 (b-d)]. 88

Figure 4.3 Mean incidence of infected wheat roots on plants grown in different growth chambers (C16, C19 and MC16) and soils (P7, R1 and R1a) with different watering regimes (Wt = by weight and F = saturation). Error bar is the least significant difference (LSD) at the 5% level (df = 54). 89

Figure 4.4 Mean dry root and shoot weights of plants grown (a) in different growth chambers, (b) in different soils, (c) with different watering regimes and (d) in soils with sieving treatments. Error bars are the least significant differences (LSD) at the 5% level (df = 9 (a) and 45 (b-d) and apply only to the individual plant variables. 90

Figure 4.5 Mean root dry weight of plants grown in (a) soils maintained in different growth chambers, (b) watering regimes carried out in different growth chambers and (c) soils with different watering

regimes. Error bars are the least significant differences (LSD) at the 5% level (df = 46, 31 and 45, respectively). 91

Figure 4.6 Comparative sizes of root systems of plants grown in the three soils subjected to the two watering regimes and maintained in the three growth chambers. 92

Figure 4.7 Mean shoot dry weights of plants grown in different soils (P7, R1 and R1a) subjected to different watering regimes (Wt = by weight and F = saturation) maintained in growth chambers. Error bar is the least significant difference at the 5% level (df = 50). 93

Figure 4.8 Mean water uptake by Wt plants in the three (a) different growth chambers and (b) soils. Error bars are the least significant differences (LSD) at the 5% level (df = 9 and 18, respectively). 94

Figure 4.9 Mean biweekly water uptake over time by Wt plants. Error bar is the least significant difference (LSD) at the 5% level (df = 216). 95

Figure 4.10 Mean water uptake over time by plants watered by Wt during the pot bioassay in the three growth chambers (C16, C19 and MC16) and soils (P7, R1 and R1a). Error bar is the least significant difference (LSD) at the 5% level (df = 216) 95

Figure 5.1 The mean TAR (take-all ratings) of plants grown in different soils. Error bar is the least significant difference (LSD) at the 5% level (df = 114). 115

Figure 5.2 The mean TAR (take-all ratings) of plants grown in soils subjected to the three soil sterilisation and *Ggt* inoculation treatments. Error bar is the least significant difference (LSD) at the 5% level (df = 114). 115

Figure 5.3 TAR (take-all ratings) of plants grown in soils with or without sterilisation prior to adding *Ggt* inoculum. Error bar represents the least significant difference (LSD) at the 5% level (df = 114). 116

Figure 5.4 The mean TAR (take-all ratings) of plants grown in non-sterilised soils with or without added *Ggt* inoculum. Error bar represents the least significant difference (LSD) at the 5% level (df = 114). 117

Figure 5.5 Increases in the mean TAR (take-all ratings) of plants, which were grown in non-sterilised versus sterilised soils, both being inoculated with *Ggt* before planting (x-axis), relative to the increases in the mean TAR of plants grown in non-sterilised soils with and without added *Ggt* inoculum (y-axis). The vertical dotted line represents the least significant difference (LSD) at the 5% level compared to zero or non-significant increase in the mean TAR (df = 114) for the x-axis, while the horizontal dotted line is the LSD at the 5% level compared to zero or non-significant increase in the mean TAR (df = 114) for the y-axis. 118

Figure 5.6 Mean TAR (take-all ratings) of plants grown in different soils. Error bar is the least significant difference (LSD) at the 5% level (df = 42). 119

Figure 5.7 Mean TAR (take-all ratings) of plants grown in soils subjected to the three sterilisation and *Ggt* inoculation treatments. Error bar is the least significant difference (LSD) at the 5% level (df = 42). 119

Figure 5.8 Mean increases in the TAR (take-all ratings) of plants, which were grown in non-sterilised versus sterilised soils, both being inoculated with *Ggt* before planting (x-axis), relative to the differences in plants grown in non-sterilised soils with and without added *Ggt* inoculum (y-axis). The vertical dotted line represents the least significant difference (LSD) at the 5% level compared to zero or non-significant increase in TAR (df = 42) for the x-axis, while the horizontal dotted line is the LSD at the 5% level compared to zero or non-significant increase in TAR (df = 42) for the y-axis. 120

Figure 5.9 Mean TAR (take-all ratings) of plants grown in the γ-irradiated base soil, to which was added 1% of the five different test soils and ± *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (df = 27). 121

Figure 5.10 Increases in the mean TAR (take-all ratings) of plants due to addition of *Ggt* inoculum to the γ-irradiated base soil, to which was added 1% of the five different test soils. The dotted line is the least significant difference (LSD) at the 5% level compared to '0' or non-significant increase in TAR after the addition of *Ggt* inoculum (df = 27). 122

Figure 5.11 Increases in the mean TAR (take-all ratings) of plants due to addition of *Ggt* inoculum to the five soils in the three experiments. Error bars are the least significant differences (LSD) at the 5% levels compared to '0' or non-significant increase in TAR after the addition of *Ggt* inoculum (df = 114, 42 and 27, respectively). 123

Figure 6.1 The mean similarity matrix and dendogram showing the relationships among the five soils from cluster analysis of the 152 bands separated on the four DGGE gels specific for bacteria, fungi, actinomycetes and ascomycetes. Similarity is expressed as a value of the Jaccard correlation coefficient with a value of '0' indicating the soils had no bands in common (i.e. completely different), whereas a value of '1' indicated the soils had the same bands present (i.e. completely identical). The letters S, C and G represent specific suppressive, conducive, general suppressive soils, respectively. The numbers next to the soil codes are the replicate numbers. 150

Figure 6.2 A scatter plot matrix of the first three principal components showing the separation of the five soils from the analyses on the bands separated on the four DGGE gels specific for bacteria, fungi, actinomycetes and ascomycetes, respectively. The plots represent three side views (faces a, b and c) of a single cube formed by plotting the data using the three axes (components) in three dimensions. The letters, C, S and G in parentheses, represent conducive, specific and general suppressive soils, respectively. The component coefficient refers to the length of the vector in relation to the principal component of interest. 152

Figure 6.3 DGGE analyses with the primers specific for the general (a) bacterial and (b) fungal populations in the rhizosphere/roots of wheat plants grown in the five soils. M represents the marker lane, which comprised the four *Ggt* isolates. The numbers 1-4 in both gels represent the replicate number of the soil. Other numbers with arrows indicate the bands excised. 155

Figure 6.4 DGGE analyses with the primers specific for (a) actinomycetes and (b) ascomycetes in the rhizosphere/roots of wheat plants grown in the five soils. The numbers 1-4 in both gels represent the replicate number of the soil. Other numbers with arrows indicate the bands excised. 156

Figure 7.1 Set up of the pot experiment in the growth chamber. 181

Figure 7.2 Fluorescent colonies of *Pseudomans fluorescens* at 10^{-4} dilution under UV light. 182

Figure 7.3 Mean incidence of take-all infected roots on wheat plants grown in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111). 183

Figure 7.4 Mean *Ggt* DNA concentrations in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111). 184

Figure 7.5 Mean numbers of *Pseudomonas fluorescens* CFU in the roots of wheat plants grown in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111). 185

Figure 7.6 Mean pH of (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111). 186

Figure 7.7 Mean pH of the four soils sown with successive seasons of wheat crops. Arrow indicates the time when the second nutrient solution was added into the soils. Error bar is the least significant difference (LSD) at the 5% level (df = 111). 187

Figure 7.8 Mean Magnesium concentrations in the four soils tested over seven successive wheat seasons. Error bar is the least significant difference at the 5% level (df = 111). 188

Figure 7.9 Mean calcium concentrations of (a) soils grown with successive seasons of wheat crops and (b) in the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences at the 5% level (df = 111). 188

Figure 7.10 Mean concentrations of (a) available nitrogen, (b) Olsen phosphorus, (c) sodium, (d) potassium, (e) magnesium and (f) calcium in the four soils sown with successive seasons of wheat crops. Arrows indicate the time when the second nutrient solution was added into the soils. Error bars are the least significant differences at the 5% level (df = 111). 190

List of abbreviations and symbols

a.i.	active ingredient
bp	base pair
ANOVA	analysis of variance
CFU	colony forming unit
dia	diameter
DNA	deoxyribonucleic acid
F	Watering by continuously sitting pots in containers of water
FC	field capacity
g	gram
Gga	*Gaeumannomyces graminis* var. *avenacea*
Ggg	*Gaeumannomyces graminis* var. *graminis*
Ggt	*Gaeumannomyces graminis* var. *tritici*
GS	growth stage
h	hour (s)
ha	hectare
ITS	internal transcribed spacer
K	potassium
kg	kilogram
m	metre
MC	moisture content
mg	milligram
Mg	magnesium
min	minute (s)
mL	millilitre
mth	month (s)
N	nitrogen
ng	nanogram
P	phosphorous
PCR	polymerase chain reaction
PDA	potato dextrose agar
pg	picogram
S	sulphur
TAD	take-all decline
TAR	take-all rating (s)
wk	week (s)

Wt	watering by weight twice per week to field capacity
y	year (s)

List of companies in text

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New Zealand Institute for Crop & Food Research Ltd, Lincoln, New Zealand
Plant Pest Information Network (PPIN) database, Ministry of Agriculture and Forestry, Lincoln, New Zealand
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Chapter 1
Introduction

1.1 Wheat production in New Zealand

In New Zealand, wheat is primarily grown for domestic consumption and is milled for flour. Some wheat grain and the by-products of flour milling, bran and pollard, are used for stock feed. An estimated 40, 000 hectares (ha) was grown with wheat, producing 328, 000 tonnes of grain in the year ended June 2007 (Ministry of Agriculture and Forestry 2007). Nearly 70% of New Zealand wheat crops are grown in Canterbury, where the growing and harvesting conditions are most favourable (Ministry of Agriculture and Forestry 2005). The wheat industry was worth NZ$113.3 million per annum during the year ended June 2007, at a contract price of NZ$315 tonne^{-1}, contributing significantly to the New Zealand arable sector, which was worth NZ$379 million (Ministry of Agriculture and Forestry 2004, 2007).

1.2 Significance of take-all in wheat

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) Arx and Oliver var. *tritici* Walker (*Ggt*) is considered one of the most damaging root diseases of wheat (*Triticum aestivum* L.) worldwide (Wiese 1998). It was first found in South Australia in the 1850s (Hornby *et al.* 1998) and now has a cosmopolitan distribution in temperate regions of the world (Figure 1.1), extending to the tropics at high elevations (Mathre 1992). Recently, take-all has been found in arid wheat producing areas of the world, where irrigation has been used, including Montana, Texas, North Dakota, Idaho, Washington, and Oregon in the USA, and in Israel (Mathre 2000). It was first reported on wheat in New Zealand in 1913 (Cockayne 1913; Pennycook 1989).

In Australia, take-all was estimated to have caused an average of 18% crop loss in 1986/87 (Oerke *et al.* 1994). Information on the impact of take-all on wheat grown in New Zealand is limited as research focusing on this disease only began in recent years. In a survey for the 1995-96 growing season, take-all was found in 10% of the 157 wheat fields assessed (Braithwaite *et al.* 1998). An extensive survey by Cromey *et al.* (2006) in New Zealand wheat and barley fields over three growing seasons during 1999-2002 (38, 91 and 113 fields for respective seasons) found that in total, 30% of the fields had greater than 20% incidence of take-all. This would reduce crop yield in individual fields by at least 15% and result in yield loss of around 5% across all the fields surveyed. Assuming wheat is valued at $300 tonne^{-1}, and the total annual New

Zealand production is 328, 000 tonnes (Ministry of Agriculture and Forestry 2007), a conservative estimation of NZ$5 million could be lost each year due to this disease.

[This figure has been removed for copyright compliance.]

Figure 1.1 World distribution of take-all in 1972 (Hornby *et al.* 1998)**.**

1.3 Biology of *Gaeumannomyces graminis* var. *tritici* and related fungi

Ggt belongs to the phylum *Ascomycota*; sub-division *Ascomycotina*; class *Ascomycetes*; order *Diaporthales*; family *Magnapothaceae* (Hornby *et al.* 1998), and tends to have a *Phialophora*-like anamorph (Walker 1981; Hornby *et al.* 1998), which allows it to be identified in culture. The three other varieties closely related to *Ggt* are var. *avenae* (*Gga*), var. *graminis* (*Ggg*), and var. *maydisal* (*Ggm*). All these varieties are similar in their morphological characteristics, but can be differentiated by the size of their ascospores, pathogenicity and extent of infection on the roots of hosts. *Ggt* attacks wheat, barley and some susceptible grasses but not oats (Hornby *et al.* 1998; Wiese 1998), whereas *Gga* infects oats, wheat, barley and other grasses. Oats are resistant to *Ggt* mainly because of the four antifungal avenacins, A-1, A-2, B-1 and B-2, present in their roots (Crombie & Crombie 1986). *Ggg* is pathogenic to turf grasses but is weakly pathogenic to wheat (Wiese 1998). *Ggm*, a maize pathogen in China, is the most recently proposed *Gaeumannomyces* variety (Hornby *et al.* 1998). *Ggt*, *Gga* and *Ggm* produce simple hyphopodia on the surfaces of host tissues while *Ggg* produces both simple and lobed hyphopodia. *Gga* generally has longer ascospores (65 μm to 176 μm) than *Ggt* (27 μm to 124 μm) (Mathre 1992), whereas those of

Ggg are similar in length to those of *Ggt* (27 µm to 124 µm) (Hornby *et al.* 1998). *Ggm* has ascospores 55.5 µm to 85.5 µm in length with one end being pointed (Hornby *et al.* 1998).

1.4 Isolation of *Ggt* from infected roots

The fungus can be readily isolated from infected crop debris and plant parts such as stem bases, sub-coronal internodes, crown roots or seminal roots (Hornby *et al.* 1998). Isolation from soil can be achieved by growing susceptible hosts as baits and by subsequently isolating the fungus from the lesions on infected roots (Cunningham 1981; Hornby *et al.* 1998). Potato dextrose agar (PDA) amended with antibiotics, and semi-selective media SM-*GGT*3 (Juhnke *et al.* 1984), R-PDA (Duffy & Weller 1994) and SM-*GGT*4 (Elliott 1991), can be used to aid the isolation and identification of the fungus. Both SM-*GGT*3 and SM-*GGT*4 contain PDA amended with L-DOPA, which turns black in close proximity to growing hyphae of *Ggt*, plus antibiotics and antifungal agents to restrict growth of competing organisms. R-PDA consists of diluted PDA amended with rifampicin and tolclofos-methyl; *Ggt* is able to alter the colour of rifampicin in R-PDA from orange to purple in as little as 24 h.

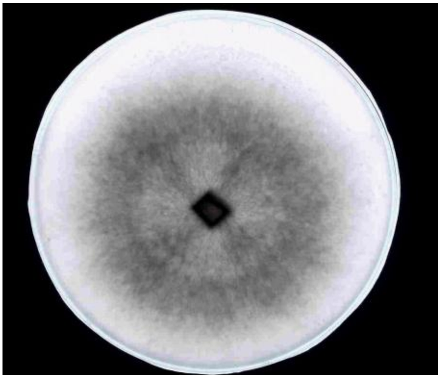
Colonies of *Ggt* grown for 2-4 d on PDA at 20°C display a pronounced whorled appearance in culture, caused by the curling back of hyphal tips (Cunningham 1981; Hornby *et al.* 1998). Hyphae are generally pale in colour, and the majority darken with age. The intensity of pigmentation is a hereditary trait, but is affected very much by the substrates present in the medium (Cunningham 1981). Figure 1.2a shows the colony characteristics of *Ggt* on PDA.

1.5 Disease symptoms and damage

Early infection during the seedling stage (infection of seminal roots) can affect yield by limiting tiller formation and causing premature death of tillers (Wiese 1998). Wheat infected with take-all generally shows above ground symptoms such as yellowing of the lower leaves on tillers and stunting of growth. The small heads may also mature early or appear bleached (Figure 1.2b), when they are usually sterile due to restriction of water flow to the tops (Cook 2003). The shrivelled grains caused by early maturation of tillers is also highly susceptible to infection by fungi such as *Cladosporium herbarum* or *Alternaria* spp. (Colhoun 1971). Severe infection by *Ggt* may also weaken the culm bases (Wiese 1998), allowing the plants to be easily pulled from the soil or broken off near the soil line (Wiese 1998; Cook 2003). Below ground symptoms, including dark lesions caused by the dark runner hyphae of *Ggt* (Figure 1.2c) (Colhoun 1971;

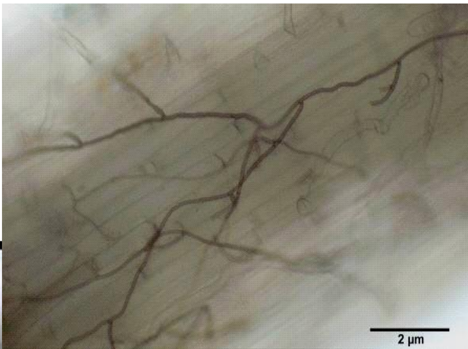
Wiese 1998), are restricted to infected roots if soil moisture is limited during early crop development. With sufficient soil moisture throughout the whole growing season, the blackening will extend into the crown and up the culm base, where it is visible beneath the lowest leaf sheath as a distinct, superficial, dark, shiny mycelium plate surrounding the culm (Figure 1.2d) (Wiese 1998).

(a)



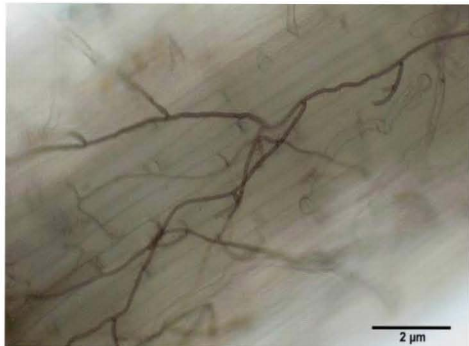
(b)

(c)



(d)

Figure 1.2 (a) Colony characteristics of 8 d old *Ggt* on PDA; (b) autumn wheat crop at GS 65 showing whitehead symptoms; (c) runner hyphae of *Ggt* on wheat roots and (d) blackening of wheat stem bases and root systems.



1.6 Disease cycle

Take-all infection can occur at any growth stage of the wheat plant (Wiese 1998). Primary infection of autumn-sown wheat by *Ggt* takes place from growth stages (GS) 15 to 29 (seedling to tillering) (see Appendix 1 A1. 1 for cereal growth stages) as the roots grow through the soil near, or come into contact with, infested debris from the previous growing seasons (Wiese 1998; Hornby & Beale undated). The dark runner hyphae of *Ggt* (Figure 1.2c) residing in the crop debris will first colonise the roots superficially, and then penetrate directly by hyaline hyphae beneath the hyphopodia into the root cortices and across the endodermis into the stele, where they obtain nutrients, carbon and energy (Cook 2003). Infection in this way can occur throughout the whole growing season, but temperatures between 10 and 20°C are optimal (Wiese 1998), meaning that primary infection usually occurs in autumn and there will be very little further root infection and symptom development in the winter.

Lesion development and secondary infection by root-to-root contact of the wheat, often begins in spring around GS30 (stem elongation), when temperatures start to rise (Hornby *et al.* 1998; Hornby & Beale undated). From late spring to summer when the crop is at GS45 (booting stage) to before anthesis (GS69), plants may start showing symptoms such as uneven growth, stunting, and stem blackening. At completion of anthesis (GS69), white heads may become apparent (Hornby *et al.* 1998).

Spread of *Ggt* via ascospores is rather insignificant and relatively unimportant epidemiologically (Hornby *et al.* 1998; Wiese 1998). Ascospores from perithecia on the stem bases or stubble, may be discharged into the air by active ejection from the ascus and dispersed by splashing rain, and to some extent by wind (Hornby 1981). At least 0.25 mm of rain is required early in the season to cause release of spores that infect the crop. Infection can be through the proximal parts of the seminal roots of seeds sown on the soil surface (not deep down into the soil), or through root hairs by tropical growth of the hyphae towards the roots (Hornby 1981; Skou 1981). Ascospores discharged during the summer are not able to survive and germinate under dry conditions, nor can they enter the soil for the hyphae to infect the roots, therefore rarely cause infection. However, susceptible grass weeds infected by the ascospores in the early season may act as primary or secondary infection sources throughout the growing season (Skou 1981; Hornby *et al.* 1998; Wiese 1998).

1.7 Factors affecting *Ggt* as soil inoculum

1.7.1 Competitive saprophytic ability

Ggt is a root inhabiting fungus with little competitive saprophytic ability (CSA) and growth in soil in the absence of host-plant roots (Skou 1981). Between crops, it survives by living saprophytically in infested host residue from previous crops, and as a pathogen of susceptible grass weed species, acting as inoculum sources for the subsequent crops (Garrett 1981; Shipton 1981). Over 402 grass species have been listed as hosts of *Ggt* (Nilsson 1969), including species of *Bromus*, *Hordeum*, and *Triticum*, and species of *Dactylis*, *Festuca*, *Lolium*, *Elytrigia* and *Poa* , but not oats (Nilsson & Smith 1981). The amount of *Ggt* inoculum available to infect subsequent crops depends on the ability of the fungus to survive in the field and compete with other microflora for substrates in the dead host tissues (Garrett 1970).

Ggt lacks the ability to persist in the rhizosphere of non-hosts (Skou 1981), hence, even when the *Ggt* infested stubble was in close proximity to other dead debris (such as ploughed-in wheat straw) in the field, the extent of its saprophytic colonisation by growing through the soil, was found to be very minimal (Shipton 1981). Poor or decreased CSA of *Ggt* with increasing levels of competition from other microorganisms, was confirmed by Butler (1953) and Garrett (1970) in pot experiments, through the addition of *Ggt* inoculum and non-sterilised soils to sterilised soils. Garrett (1976) later showed that the penetration rate of the fungus into mature cell walls of the straws, was dependent on the rate of enzymic degradation of the walls around the apices of penetrating hyphae. Other attributes contributing to the poor CSA of *Ggt*, include slow germination and growth, poor production of celluloytic and lignolytic enzymes to degrade the cell walls of the roots for hyphal penetration and many other interacting factors, which in turn, dictate the potential for its pathogenic activity (Shipton 1981).

All the above information indicates that, in the absence of susceptible hosts, the quantity of *Ggt* inoculum present in the infested crop debris is on a path of continual reduction and degradation, as it is a poor competitor in the soil, and without CSA, further saprophytic colonisation is minimal.

1.7.2 Nitrogen

Once *Ggt* becomes established on a suitable substrate, maintenance of inoculum (resting mycelium) becomes vital for its survival (Garrett 1970). The supply of soluble nitrogen (N) to *Ggt* within the straw, or diffusing in from the soil, is one of the most important factors regulating its survival (Huber 1981). This was demonstrated by Garrett (1938), who found that *Ggt* died out early from almost undecomposed straw in induced N-deficient soils. When the soil was amended with dried blood (containing 13% N), the death of the fungus was delayed even when the straws became almost fully decomposed. In contrast, decline of the fungus was accelerated by the addition of rye-grass meal, which delayed decomposition of the straw by taking up the available N. He later concluded that ample N would promote a greater and more sustained development of the dark mycelium of *Ggt* within the infected straw as the fungus assimilated more of the undecomposed carbohydrate reserves of the straw (Garrett 1940). However, if the soil is well aerated, it might favour microbial activity and hasten the disappearance of *Ggt* by promoting more rapid consumption of the available food material by both the pathogen itself and by other associated microorganisms (Garrett 1940).

Similar results were reported also by Butler (1953), who found that *Ggt* remained viable in 89% of the test straws for 20 wk in N-enriched soil, but its viability fell rapidly after 12 wk in N-deficient soil. However, the direct effects of N-amendment (in contrast to its effect as mediated by other microorganisms) on the viability of *Ggt* were not considered, as non-sterilised soils were used in their studies.

Chambers and Flentje (1969) used gamma-irradiated N-enriched soils and managed to separate the direct effect of N on *Ggt* from the effect mediated by other soil microbes. They reported 86% of straws buried in un-amended (no N added), irradiated soil for 24 wk still contained viable *Ggt*, but only 67% of straws still contained viable *Ggt* when soil microorganisms were included by using the unsterilised soil. Conversely, 99% of straws still contained viable *Ggt* after 24 wk when N was added to the unsterilised soil, which contained soil microorganisms. They suggested that the survival of *Ggt* was dependent on the availability of N rather than on the competition and antagonism of other soil organisms.

Garrett (1963; 1966; 1967; 1970; 1976; 1976) examined the effect of decomposition rate of the cellulose of straws on the saprophytic survival of *Ggt*. He concluded that the

relationship between the cellulolysis rate of straw tissue by the fungus and the general metabolic rate of the fungus are the two main factors determining the saprophytic survival of the fungus in the straw. General metabolic rate determines the rate at which carbohydrates are consumed by the processes of respiration and growth, while cellulolysis rate determines the rate at which soluble carbohydrates are made available for the metabolism of the fungus (Garrett 1970). Only when the cellulolysis rate is adequate to maintain the general metabolic rate, will a fungus be able to continue to survive in the straw tissues. Hence, a fast-growing fungus with a high metabolic rate will require a higher rate of cellulolysis to supply its needs than will a slow-growing fungus with a low metabolic rate.

A cellulolysis adequacy index (C.A.I.), which is a ratio obtained by dividing cellulolysis rate by the linear growth rate of the fungus (as a general metabolic rate) was proposed by Garrett (1963) to express these relationships. If the CAI is less than unity, it indicates a need for extra N for maximum survival, whereas an index of more than unity indicates that increased supply of N will reduce longevity. *Ggt* has a CAI of between 0.3 and 0.5 and therefore, will respond positively to N-amendment (Garrett 1967). However, he also found that the *Ggt* inoculum potential (ability to cause maximum root infection) developed more quickly at the higher N levels, at which cellulolysis was most vigorous, but rapidly declined at the highest N level. This means that whether in the presence of microbial competitors or not, the optimum level of N for *Ggt* is that just sufficient to maintain the cellulolysis at a rate adequate for its survival. In contrast, Scott (1969a) reported that *Ggt*'s survival was determined mostly by the degree of success in saprophytic competition with other microorganisms in high N soils.

For decomposition to take place at a high rate, the level of N must be quite high (Deacon 1997). This is expressed as the carbon and nitrogen (C:N) ratio of a material assuming that both C and N substrates are in utilisable forms. For instance, wheat straw has a C:N ratio at about 80:1, whereas fungal hyphae have a C:N ratio at about 10:1 depending on their age and other factors. During fungal growth, one third of C will be converted into cellular material, the other two thirds being respired as carbon dioxide (CO_2). Similar things happen when a fungus is growing on plant material that is breaking down. For instance, for a material with C:N ratio of 100:1, when 10 of its C units are combined with one N unit in the fungal mycelium, this will release 20 C units as CO_2 and there will still be 70 C units left in the residual substrate. Decomposition will stop at this point because the fungus is

starved from the lack of N for growth and enzyme production. With the above information, the conclusion given by Chambers and Flentje (1969) on the acceleration of the loss of the *Ggt* viability in colonised straws buried in irradiated N-deficient soil with other introduced microorganisms, could be explained. Introduced microorganisms could have competed with *Ggt* to use the available N to convert C for growth and enzyme production. In addition, nitrogen depletion could also drive fungal succession to take place (Deacon 1997). The early colonisers, which used up the available nitrogen, might not be efficient in recycling their mycelial nitrogen reserves, thereby resulting in their starvation. Other fungi would replace them, using their lytic products as a nitrogen source (Garrett 1940).

In summary, *Ggt* survives well in infested wheat residues with increasing soluble N, but only up to a point where excess N will hasten decomposition of the residues and promote succession of other microorgnisms.

1.7.3 Temperature and moisture

Temperature and moisture are important in determining the survival of *Ggt* inoculum and hence take-all (Cook 1981). Work summarised by Deacon (1997) showed that when wheat plants were grown in sterilised soil inoculated with *Ggt*, the pathogen was able to cause progressively more disease on the roots (>50% - 90% roots infection) as the soil temperature was raised from 13 to 23°C, or even 27°C, which is the optimum temperature for its growth on agar. However, in natural, non-sterile soil, the amount of disease declined as the temperature was raised above 18°C (Deacon 1997). The inference drawn from the study was that higher temperatures favour other microorganisms even more than they favour the *Ggt*. These other microbes may be able to inhibit and antagonise the *Ggt* or to compete for saprophytic degradation of the wheat residues, causing death of *Ggt* (Garrett 1934; Deacon 1997), thus other interacting factors must be taken into account in natural conditions. This information also indicates that temperatures between 12 to 18°C are sufficient for infection to take place.

Studies have indicated that take-all is prevalent in moist soil in the field, largely due to the high water potential requirement for the pathogen to grow and infect the host in the top 25 cm of soil, where it is first to dry when rain or irrigation ceases (Cook 1981). *Ggt* does not grow at soil matric potentials drier than -3.5 to -4.0 MPa and its growth rate is reduced by half at -1.5 to -2.0 MPa, which is quickly achieved in the top layer of soil during

prolonged periods with no rain or irrigation. Hence, water must be supplied to the soil regularly for severe take-all to develop.

A few researchers have studied the effects of temperature and moisture on the survival of *Ggt* during soil storage, but they have had varied results. The fungus itself is reported to be capable of tolerating extreme temperatures during soil storage. When introduced into sterilised soil, its viability was not affected by repeated temperature alternations between 21 and -29°C, and it tolerated 45°C for 30 min (Shipton 1981). MacNish (1973) conducted a bioassay using soil cores stored at various environmental conditions. He found that the viability of *Ggt* was not affected when soils were stored for at least nine wk in dry and cool conditions (-25 MPa to -98 MPa at 15°C) and moist, cool conditions (-0.4 MPa to -0.7 MPa at 15°C). Disease incidence, however, decreased by 50% for soils stored under very dry and hot soil regimes (-98 MPa at 35°C) for 9 wk, and was totally eliminated from the soils stored under wet and hot conditions (-0.01 MPa to -0.2 MPa at 35°C) for 4 wk.

Wong (1984) incubated soils inoculated with *Ggt*-infected wheat straws to four temperature-moisture regimes for 3 months and later assessed the lesions on the roots of wheat seedlings planted in the soils as baits. He found that *Ggt* survived best in cool dry soil (15°C at < -10 MPa) (100% survival), followed by warm dry soil (30°C at <-10 MPa) (63 to 97% survival). The fungus was eliminated from warm, moist soil (30°C at -0.3 MPa), but in cool moist soil (15°C at -0.3 MPa), its survival was 18 to 40%. Exactly how these percentage survival rates of *Ggt* were derived was however not explained by the author.

As the elimination of *Ggt* was based on ‘zero’ disease incidence observed on the roots of the tested wheat seedlings for both researchers, and re-isolation of the fungus from the soils was not attempted, it is not clear whether the fungus was truly eliminated as it could have lost virulence or pathogenicity under undesirable storage conditions. The various temperature-moisture regimes used in their studies suggested however, that *Ggt* was able to survive even under the very dry conditions, which were up to 65 times (at -98 MPa) beyond permanent wilting point (approximately -1.5 MPa), and that cool temperatures were preferred to warm temperatures. While the soils tested were stored at constant conditions for a period, the survival and infectivity of *Ggt* under wetting and drying cycles were not explored.

1.8 Management of take-all

1.8.1 Chemical controls

Effective chemical control of many root diseases has almost always depended on the use of soil fumigants or fungicides, which may be applied to the soil as drenches and/or incorporated mechanically, as sprays and in seed treatments (Hornby *et al.* 1998; Cook 2003). To provide effective control for crops known to be at risk from severe take-all, fungicide applications could be incorporated into an integrated disease management programme (Bateman 1989).

1.8.1.1 Soil fumigation

Soil applied fumigants will decrease *Ggt* to insignificant levels, but they are costly and therefore not commercially practical for an arable crop such as wheat (Hornby *et al.* 1998; Cook 2003). Fumigation is useful as an experimental tool, in particular, where larger factorial experiments are involved. For instance, an average yield increase of about 1 t ha^{-1} for spring and winter wheat representing five locations over the years 1993 to 1996 after fumigation, has been reported in the USA (Cook *et al.* 2002). It has been suggested that the increased crop yield may be due to the flush of N, released by the killed microbial biomass in the soil following fumigation, but this cannot account for the amount of increased crop yield (Cook 1992). It was subsequently shown that the increased growth and yield response of crops by fumigation is due to improved root health and better nutrient exploration by the roots because of the death of soil pathogens (including *Ggt* and nematodes) (Cook 2003). However, there may also be a rapid build-up of *Ggt* following the year of treatment because of a decreased abundance and diversity of non-target antagonistic microflora in the soil (Hornby *et al.* 1998). Consequently, severe infection often results if another cereal crop is grown without re-application of the chemical.

The large volumes and high concentrations needed, and hence the risk of contaminating groundwater, also preclude fumigation as a practical treatment for controlling take-all (Hornby *et al.* 1998). In addition, their high cost, broad spectrum of activity and environmentally unfriendly effects (such as the effect of methyl bromide on the ozone layer) means that it is still impossible to use fumigants for a large scale crops such as commercially grown wheat.

1.8.1.2 Soil fungicides

Considerable work has been carried out in the UK. with fungicides applied to the soil as drenches and/or incorporated mechanically (Bateman 1980; Bateman & Nicholls 1982; Bateman 1984; Bateman *et al.* 1994). Bateman *et al.* (1994) suggested that for a soil-applied fungicide to be effective in controlling take-all, it has to meet the following requirements: (i) good intrinsic fungitoxicity, (ii) some mobility in soil water, and (iii) season-long persistence. In their evaluation of the efficacy of six fungicides in controlling naturally-occurring take-all on winter wheat at two sites from 1986 to 1987, they reported nuarimol and triadimenol (sterol biosyntheis-inhibiting fungicides) to be the most effective. Triadimenol and nuarimol, were able to control take-all and improve yields when applied to the soil at 1-2 kg ha^{-1}, but these rates are considered to be uneconomical for a low-value crop such as wheat.

Workers in Victoria, Australia tested the efficacy of different systemic fungicides and found that both benzimidazole and triazole, when formulated into clay granules or pellets and applied at 1.5 kg ha^{-1} in the seed furrow at the time of seeding, gave control in naturally infested fields (Ballinger & Kollmorgen 1986a, 1986b). However, in a similar trial, Cook (2003) found that greater rates were needed (>1.5 kg ha^{-1}) to give useful control of up to 100% fewer deadheads and more than 200% greater yield, thus, making this approach uneconomical.

The cost of treatment and the fact that control is only partial and variable, are the main reasons why soil-applied fungicides are not being used commercially for control of take-all. Moreover, fungicides applied to the soil may contaminate groundwater, hence it is important that they are applied at the smallest possible effective rates (Bateman 1989). Besides, soil fungicides may also decrease the abundance and diversity of non-target antagonists of *Ggt*, some of which are implicated in useful, natural biological control of take-all, thereby delaying the onset of take-all decline (TAD) (Wong 1981). For instance, in continuous winter wheat experiments at Rothamsted, a benomyl trial had slightly increased take-all in the second year after treatment; which did not occur with nuarimol, probably because it was more persistent (Bateman 1984). In the third year after treatment, neither of these fungicides appeared to have delayed the onset of TAD, which is the result of natural biological control and often occurs in monocultures after the peak of take-all has been reached (Bateman 1989). TAD will be described in Section 1.8.4.

1.8.1.3 Seed treatment

Research on chemical control over the past 20 years has mostly focused on fungicide treatment of seeds as only small quantities of fungicides are required to provide early season control (Cook 2003). Again, sterol biosynthesis-inhibiting fungicides, particularly triadimenol, have shown potential (Cook 2003). Triadimenol was first found to be useful in delaying take-all development on irrigated spring wheat in Montana by Mathre *et al.* (1986). They reported that triadimenol, when applied at 0.22 ml a.i. kg^{-1} of seeds, was able to delay above ground symptoms on wheat by up to 53 d and to reduce numbers of root lesions at harvest by 50%. They explained that this chemical was able to delay infection by *Ggt* long enough to provide yield increases in winter wheat.

In France, silthiofam (Latitude®) has been reported as the most promising chemical to be used for controlling take-all. Schoeny and Lucas (1999) did a comprehensive study on the epidemiology of take-all to evaluate the efficacy of this fungicide at 0.25 and 0.5 g a.i. kg^{-1} of seeds. They found that throughout the cropping season take-all incidence in plants treated with the higher rate of fungicide was delayed by 3 to 5 wk, and disease severity decreased by 67 to 81%. However, if the epidemic started late in the growing season only moderate control was obtained, and fungicides applied at both rates were able to increase yield by 10 and 15%, respectively.

A trial conducted in New Zealand in the 2002/2003 season, to compare various fungicide treatments, however, exhibited some controversial results. The per kg rates used to treat autumn-sown seeds were 0.1 and 0.2 g a.i. triadimenol (Baytan Universal®), 0.07 and 0.14 g a.i. silthiofam (Latitude®), 1.5 and 3.0 g a.i. fluquinconazole and 0.3 and 0.6 g a.i. prochloraz (Jockey®). Results showed that at both application rates, triadimenol and silthiofam were able to reduce disease severity on the roots, early in the season, by 32 and 25%, respectively, but did not affect disease severity or symptom development (whiteheads) later in the season. These two fungicides however did not significantly increase yield. The combined fluquinconazole and prochloraz (Jockey®), on the other hand, decreased whiteheads by an average of 84% at both rates and disease severity by 57 and 75%, respectively. Conversely, Jockey also reduced seedling emergence by 47.6% and did not increase yield (Cromey, unpublished data).

The usefulness of triadimenol in controlling take-all has been inconsistent mainly because, like most systemic fungicides, triadimenol moves very inefficiently, or not at all, to the

roots where the early protection is needed (Cook 1994). Seeds treated with this chemical were reported to have only 0.2 to 0.7% of the chemical in the seedling roots, compared to 7.7% in the shoot, and 12 to 18% in the seed up to 28 d after sowing. About 56 to 74% of the chemical entered the soil where potentially it could provide direct inhibition of the pathogen (Cook 1994).

1.8.1.4 Foliar treatment

Foliar application of the many systemic phloem-translocated fungicides used to control foliar diseases, was found to be ineffective in controlling take-all, unless applied at high frequencies and concentrations (Bateman 1989). For instance, azoxystrobin (Amistar®), a broad spectrum fungicide with systemic translaminar activity, had to be applied at 250 g ha^{-1} (500 g a.i. kg^{-1} soil) on a wheat crop, in autumn, spring and summer (at GS21, 31 and 45, respectively), to reduce take-all severity (from TAR = 200 to 165) (Jenkyn *et al.* 2000). The fungicide efficacy in this trial was probably due to the high number of sprays applied, the ability of the chemical to move through the soil to the roots to control *Ggt* and the improved capacity of the plants to resist attack by other pathogens (Jenkyn *et al.* 2000).

Development of fungicides capable of being translocated in the phloem of the plants has raised the possibility of controlling root diseases (Bateman 1989; Hornby *et al.* 1998). However, most of the phloem-translocated fungicides currently available are active against oomycete pathogens, and none is active against *Ggt* (Hornby *et al.* 1998). Furthermore, the relatively low application rates and the inaccessibility of the fungicide to the inoculum, mean that conventional spray application of fungicides is unlikely to be successful in controlling take-all.

1.8.2 Cultural practices

Extensive efforts have been made trying to find resistance genes in wheat, barley and their closest relatives to *Ggt*, but to date, useful genes for resistance are still absent. According to Hornby *et al.* (1998), the most effective control strategy for decreasing the effects of take-all was by manipulating the agronomic practices, which have subsequently been shown to reduce the severity of take-all in fields. However, none of these practices provides more than a modest level of control (Cook 2003).

1.8.2.1 Crop rotation

With the specialised nature of *Ggt* and its dependency on infested host remains for its survival within a food base, it is not surprising that crop rotation is the only cultural control method that works consistently and is economic for controlling take-all (Cook 2003). The time required for the break crops before returning to a susceptible cereal varies from area to area, depending on the climate and soil characteristics. In warm rainy areas (much of Europe and the southeast of USA), 1 y out of wheat after a wheat harvest will allow sufficient time for the decomposition of infested host remains, thereby reducing *Ggt* inoculum potential to below an economic threshold (Cook 2003). On the other hand, soils in the rain-fed (non-irrigated) intermountain Pacific Northwest are too dry for microbial activity during the warm summer and autumn months and too cold during the wet winter and spring months. Therefore, it will take at least 2 y out of wheat to lower the inoculum potential of *Ggt* below an economic threshold in this region (Cook 2003).

Hornby *et al.* (1998) recommended short rotations that prevent the field from ever developing severe take-all, or continuous cropping with a single cereal species which exploits the TAD phenomenon, over the intermediate rotations that are so often practised. For instance, if a break crop is introduced every fifth year, at least two of the four cereal crops in the rotation are likely to suffer from moderate to severe take-all. However, the need for profitability, lack of suitable alternative crops and unsuitability of some wheat-growing areas for alternative crops, means that much wheat is grown as part of short runs (1-3 y) of cereals, in which the take-all problem persists and TAD (will be discussed in Section 1.8.4) is unlikely to develop (Hornby & Beale undated). Table 1.1 shows some examples of the risk of take-all in susceptible cereals after different crops. Barley, rye and triticale are less susceptible to take-all than wheat, hence will be safer to be grown in high-risk soils or situations. Oats being resistant to *Ggt*, make an acceptable break crop, except where *Gga* is known to be a problem (Hornby *et al.* 1998).

Table 1.1 Examples of risk of take-all in susceptible cereals after different crops (Hornby & Beale undated).

Risk of take-all	Previous crop or land use
High	Wheat, barley, triticale
Medium	Rye, grassland, set-aside
Low	Oilseed rape, linseed, maize, sugar beet, potatoes, peas, beans, oats

It is important to plan the crop rotation carefully as crops such as oilseed rape, if followed by beans and then wheat, can promote increase of take-all in the subsequent second wheat crop. This is because oilseed rape leaves more residual N in the soil, which could favour the saprophytic survival of *Ggt* (Hornby & Beale undated). In addition, this crop also allows more cereal volunteers to carry *Ggt* over to the next crop, because of minimal cultivation before sowing giving rise to more inoculum developing in the next wheat crop after beans (Hornby & Beale undated). Rape also has high glucosinolate content, which may cause natural bio-fumigation and decrease the microflora antagonistic to *Ggt*, thus increasing take-all in subsequent wheat crops. Pastures such as cocksfoot, clovers and ryegrass (used for animal grazing) that are free from grass weeds (such as *Elytrigia repens* and *Bromus* spp.), also make acceptable breaks, and although they may carry *Ggt* they also carry the antagonist, *Phialophora graminicola*, which is able to suppress the pathogen's development in a subsequent cereal crop (Hornby *et al.* 1998).

The trend towards increasing the proportion of non-cereal crop in arable rotations has been limited by pathological considerations as well. For example, over-cropping with pulses can lead to problems with a foot rot complex (mainly caused by *Fusarium* and *Phoma* spp.). Rape, spring beans and linseed are all susceptible to *Sclerotinia sclerotiorum*, and winter beans, if cropped too frequently, can suffer severe attacks by *Sclerotinia trifoliorum*. Short sequences of cereals can, thus, be valuable in reducing disease risks for other crops in the rotation, but of course such sequences carry their own disease problem (Hornby *et al.* 1998).

1.8.2.2 Direct drilling

Direct drilling or "no-till", by which seeds are drilled directly into the soil without prior cultivation or disturbance of soil and stubbles, is becoming a more common method in wheat production in many countries (Cook 2003). Direct-drill systems can affect take-all in several ways, and these effects are summarised by Yarham (1981) as below:

- Non-disturbance of the soil favours the development of perennial grass weeds, which if susceptible to *Ggt*, can act as carriers for the inoculum.
- Larger fragments of wheat crop debris left near the soil surface may result in a concentration of inoculum in the top few centimetres of soil into which the seed is drilled, thereby increasing chances of infection.
- *Ggt* inoculum levels can decline more markedly in unploughed soils grown with consecutive wheat crops between late winter and mid-summer than in ploughed soils, due to increased decomposition of debris and increased biological activity by competitors and antagonists of *Ggt* in the soil closer to the surface (Hornby 1975).
- The increase in bulk density (i.e. decrease in pore size) and the increased biological activity and aerobic respiration in the upper layers of the soil, result in increased carbon dioxide (CO_2) levels (Deacon 1997). Combinations of depleted oxygen and high CO_2 in the water film in the soil, and high bulk density, are sufficient to restrict rooting and reduce *Ggt*'s survival and take-all development.
- The marked stratification of soil will cause phosphorus (P) to accumulate in soil surface layers, and the better-nourished plants will then be less susceptible to take-all.
- Mineralisation of soil nitrogen will also be decreased, thereby, influencing disease development through its effect on the nutrition of both host and parasite.

In New Zealand, a 3 y field trial which included two tillage systems (disc cultivation and direct drilling), was established as part of the study investigating the effects of various crop management regimes (tillage, residue management and types of winter crops) on take-all (Cromey *et al.* 2004). The results showed significantly lower disease incidence (13% infected tillers) on crops established from direct drilling, than those established after discing (57% infected tillers. This conclusion was based on assessing the tillers from about 100 plants (Cromey, personal communication), and indicated that direct drill could help

reduce the risk of take-all. In the trial, other management regimes, including the removal of residue from a previous crop and growing wheat after 2 y of ryegrass, were also demonstrated to reduce the risk of take-all in the field. With so many interacting factors influencing disease development, it is not surprising that reports on the effect of direct drilling on take-all have been variable.

1.8.2.3 Soil fertilisation and host-plant nutrition

Plants with missing and damaged roots because of take-all are less efficient in the uptake of nitrogen, phosphorus, potassium and other major and trace nutrients (Cook 2003). The addition of these nutrients, especially nitrogen (N) during tillering, stem elongation, or both stages of plant development, will lessen the limiting effects of take-all, helping the crop to outgrow the disease (Cook 2003). In general, increased N decreases the disease, but high soluble N level in the soil actually favours the survival of the pathogen in the absence of hosts up to a point where excess levels of N will hasten decomposition of the residues by a succession of other microorganisms, that can compete with *Ggt* for available N (discussed in Section 1.7.2). However, managing take-all by adding more nutrients can also lead to leaching of unused nutrients, especially soluble N causing more groundwater pollution (Huber 1981; Cook 2003).

When plants already deficient in the relatively immobile nutrients, such as phosphorus and other trace nutrients (e.g. copper, zinc and manganese), are infected by *Ggt*, their damaged roots will have reduced absorptive capacity for nutrients and water (Huber 1981). Deficiencies in the immobile nutrients will then become more acute, thereby increasing the susceptibility of the host and causing rapid development of the disease (Cook 1982, 2003). The availability of trace nutrients to the crop is markedly affected by pH (Huber 1981). Alkaline soils are generally more conducive to take-all and tend to lower the availability of many trace elements including iron, zinc, manganese, copper and boron but not molybdenum, while many of these trace elements are more available in acid soils (Huber 1981). The degree of nutrient deficiency caused by the loss of absorptive capacity however, can vary considerably from plant to plant and from area to area within the field. Attempts to overcome these localised and sporadic deficiencies with increased applications over the entire field may be unnecessary in regions where the crop is healthy (Cook 2003).

1.8.3 Disease prediction by quantitative soil analysis of *Gaeumannomyces graminis* var. *tritici*

Recent advances in molecular techniques, such as real-time PCR (polymerase chain reaction), have allowed workers in South Australia (CSIRO[1] Entomology and SARDI[2]) to develop a quantitative test for *Ggt* DNA concentrations in the soil using Taq-Man® real-time PCR, (Herdina & Roget 2000; Ophel-Keller & McKay 2001; Ophel-Keller *et al.* 2008). The *Ggt* DNA concentrations can be used to predict take-all severity/risk prior to sowing subsequent wheat crops. Risk categories for take-all were developed by relating disease levels obtained from bioassays to the subsequent development of take-all in the field (Hornby 1981; Herdina & Roget 2000) (Ophel-Keller *et al.* 2008). The DNA concentrations were then calibrated by standardising the bioassay results on a 0-100 scale to maintain continuity (Ophel-Keller *et al.* 2008). The risk categories developed in Australia were: below detection limit (<5 pg g^{-1} soil), low (5-130 pg g^{-1} soil), medium (131-325 pg g^{-1} soil) and high (>325 pg g-1 soil) (Ophel-Keller *et al.* 2008). These risk categories are referred to throughout this thesis. Since completion of this research, SARDI has modified the risk categories to suit New Zealand conditions. These are: below detection limit (<5 pg g^{-1} soil), low (5-96 pg g^{-1} soil), medium (96-256 pg g^{-1} soil) and high (>256 pg g^{-1} soil). Direct measurement of *Ggt* DNA concentrations in the soil enables growers to decide whether fields can be safely replanted with wheat or barley, or whether break crops are needed. However, studies carried out in Australia, which compared their *Ggt* DNA concentrations in soils from the fields with subsequent disease ratings obtained from wheat plants grown in these soils in pot assays, have not always shown good correlations (Herdina & Roget 1999, 2000).

In New Zealand, the *Ggt* DNA concentrations in the soils of 112 paddocks with varying wheat cropping histories (previously cropped with 0-3 y of wheat) were assessed by the RDTS[3], SARDI in April 2003, prior to sowing autumn wheat (Cromey *et al.* 2004). Results showed increased mean *Ggt* DNA concentrations from 106 to 1419 pg g^{-1} of soil reflecting the years of prior wheat crops (0-3 y) (2002/03 season). The 'pre-sowing' *Ggt* DNA concentrations of these soils correlated moderately with the take-all severity (expressed as a disease index) in subsequent crops (2003/04 season) ($r = 0.5$), which at the time, would have been cropped with 1-4 y of wheat (Figure 1.3) (Cromey, unpul. data). It appears that

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[1] Commonwealth Scientific and Industrial Research Organisation
[2] South Australian Research & Development Institute
[3] Root Disease Testing Service

direct measurement of *Ggt* inoculum in the soil with this method predicts the take-all risk in the subsequent wheat crops moderately well. This service for measuring soil *Ggt* DNA concentrations, has been available commercially to New Zealand growers since 2008. These results on the correlation of the pre-sowing *Ggt* DNA concentrations, with the actual take-all severity of a subsequent crop, were not yet available when this literature review was prepared.

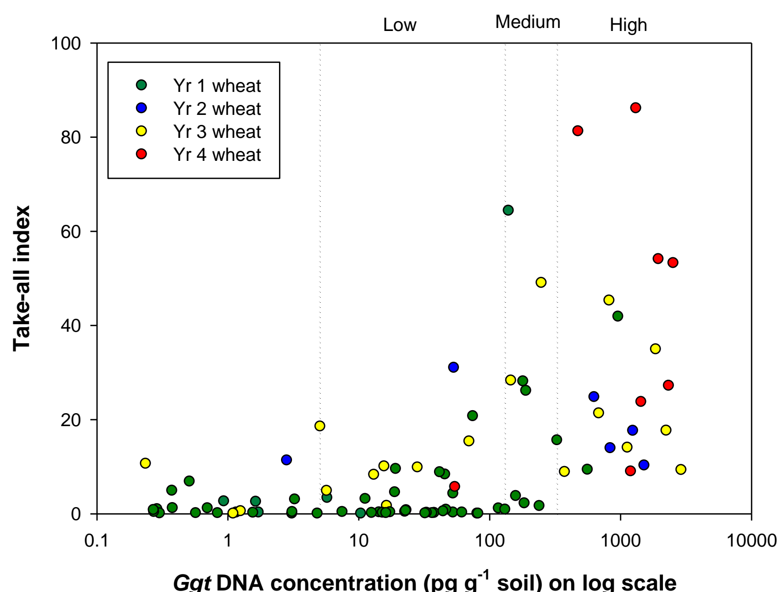

Figure 1.3 Pre-sowing *Ggt* DNA concentrations of soils from 112 wheat fields, in relation to the take-all indices of the subsequent wheat crops grown in the same fields (Cromey, unpubl. data). Take-all risk categories for subsequent crops at the time of assessment are indicated by the graph segments as low, medium and high with *Ggt* DNA concentrations of 5-130, 131-325 and >325 pg g^{-1} soil, respectively.

1.8.4 Biological control

Increasing public concern over the use of agrichemicals in the environment, which causes groundwater and environmental pollution, and the lack of effective fungicides to control take-all, have resulted in escalating pressure to search for alternative control measures and biological control has received increasing attention.

1.8.4.1 Take-all suppressive soils

Soil suppressiveness is a widespread and well-known phenomenon, which is usually complex and not easily understood. Baker and Cook (1974) classified the inhospitality

characteristics of certain soils to plant pathogens into these categories: (1) the pathogens cannot establish in the soil, (2) they establish in the soil but fail to cause disease symptoms on the plants, or (3) they establish in the soil and initially cause disease symptoms on the plants, but disease severity declines over time with successive planting of the same crop in the soil. These categories however, do not imply that only biological principles are responsible for regulating the suppressiveness, since abiotic factors such as environmental conditions (temperature and moisture), soil, fertilisers and pH can play a part in regulating the survival and activities of pathogens.

Hornby (1983) summarised the types of suppressive conditions, including TAD, that decrease take-all severity (Table 1.2). He suggested that the differences between the seven types of suppression are not distinctive. It is possible that types I and II are closely related, IV and VI may be modifications of general antagonism by non-hosts. Type VII has been added to accommodate disease suppression that occurs after exposure of the host to avirulent and hypovirulent fungi (i.e. cross-protection). Type VI suppression is non-transferable due to general antagonism, while types I, IV and possibly VII are transferable (Hornby 1983). According to Hornby (1983), any of the take-all suppression types may take place but the most commonly occurring suppression type is TAD. The author mentioned that low soil pH (3.5-5.0 and sometimes pH5.0-6.0) might promote *Ggt*'s production of diffusible inhibitors that can prevent its growth, and thus the disease, and therefore development of TAD. However, he did not discuss the roles played by the abiotic factors, such as nutrients, soil characteristics, soil pH, water and climate on the mechanisms involved in the other six types of take-all suppression.

When wheat crops are grown successively in a field, the level of take-all disease can build up progressively, reaching a peak in the third or fourth year. Provided that sufficient N is available, disease levels can then decline spontaneously in the following years, so that wheat can be grown continuously with acceptable yields (Figure 1.4). This phenomenon, which has been reported in many countries, including Australia, Canada, USA, Switzerland, UK, France, Yugoslavia, Denmark and Holland, is known as TAD (Rovira & Wildermuth 1981; Deacon 1997; Mazzola 2002; Weller *et al.* 2002).

Table 1.2 Characteristics of the seven types of take-all suppression suggested by Hornby (1983).

Suppression type	**Suppression characteristic**	**Development requirements**			**Development period**	**Characteristics of suppressive soil (SS) type**
		H	**P**	**D**[a]		
I	Take-all decline (TAD)	+	+	S+[b]	Few seasons	Induced (by monoculture)
II	Long-term	+	+	+	Over 200 y of permanent cultivation and cropped with wheat	Long-standing
III	Long-term, without severe disease	+	+	-	Many years of wheat	Long-standing
IV	Non-host (crop-dependent)	-	-	-	One to several seasons	Induced generally by rotations, by grass or grass/legume leys
V	Added pathogen	-	+	-	Short	Induced by natural antagonists in the soil
VI	General antagonism	-	-	-	'Immediate'	Not usually considered within SS range
VII	Cross-protection	+	-[c]	-	'Immediate'	Introduced[d]

[a]H, host; P, pathogen; D, disease.
[b]Disease levels required (+), required to be severe (S+) or not required (-).
[c]Avirulent pathogen may be added to the soil.
[d]Where organisms are added to the soil rather than on the seed.

[This figure has been remove for copyright compliance.]

Figure 1.4 Diagrammatic illustration of take-all decline in continuous cereal crops and its effect on yield (Deacon 1997).

TAD is the most thoroughly studied of the various types of take-all suppression. For this phenomenon to occur, it requires monoculture of a susceptible host such as wheat for several years and presence of *Ggt* with at least one severe outbreak of take-all (Weller *et al.* 2002). The number of wheat crops required before the onset of TAD can vary considerably depending on the location of the field, soil type and environmental conditions (Weller *et al.* 2002). Although TAD suppressiveness can be reduced or eliminated by breaking monoculture with a non-host crop, a field with a long history of TAD can regain suppressiveness once a wheat or barley crop is again grown (Weller *et al.* 2002). In countries such as the USA, farmers who practise intensive or continuous cereal cropping for economical reasons are incorporating TAD in their farming systems (Cook 2003).

Most New Zealand growers tend to grow wheat crops within short runs of cereals (such as barley, triticale and oats) to avoid severe take-all outbreaks. During the 2-3 y of cereals, the take-all problem frequently increases but the cereal-growing period is probably too short to allow development of TAD. To date, it is not known whether TAD or any other form of take-all suppressive soils occur in New Zealand as research in this areas has not yet been conducted. However, Cromey *et al.* (2004) reported increasing mean *Ggt* inoculum concentrations in New Zealand soils with each consecutive wheat crop from 0 to 3 y, which suggests that the take-all risk in New Zealand wheat crops increases at least into the fourth year of continuous wheat.

1.9 Mechanisms of take-all decline

The mechanisms involved in TAD are thought to include the combined effects of specific suppression of the pathogen and general suppression prior to its development (Gerlagh 1968; Graham & Mitchell 1999).

General suppression develops when the soil conditions are suitable for many antagonistic effects by the microbiota in the rhizosphere. It includes competition for resources (such as carbon and energy in the food base), antagonism in lesions by secondary colonists, and stimulation of host defence mechanisms, which together act to lower the inoculum potential of *Ggt*, hence no one microorganism is responsible for it (Cook 2003). The suppression factor is not destroyed by heating soil to 60°C, and is not transferable with introduction of small soil volumes to non-suppressive soils (Rovira & Wildermuth 1981).

The occurrence of specific suppression is due, at least in part, to the activities of individual species or select groups of microorganisms during some stage in the life cycle of the pathogen. It is a result of the previous cropping system, which for *Ggt* is cereal monoculture, and occurs in the rhizosphere and in lesions rather than in the bulk soil. This suppression factor is transferable and can be destroyed at 60°C (Rovira & Wildermuth 1981; Cook 2003). Characteristics of specific and general suppressions are shown in Table 1.3.

The terms ‘pathogen-suppressive’ and ‘disease-suppressive’ have been used to describe suppressive soils but these terms seem to have been used interchangeably and the differences between them are not clear. Hornby (1983) described pathogen-suppression as being the suppression which occurs when the pathogen is growing saprophytically in the debris or surviving in the soil (resting phase), with disease-suppression being the suppression of the pathogen’s parasitic growth in the host. However, there is strong support for presence of antagonistic mechanisms in suppression, indicating that the term ‘pathogen suppression’ may be more appropriate (Hornby 1983). The potential for disease control by natural antagonism exists in many soils, but its expression depends on a fine balance between conditions favourable to the pathogen versus conditions favourable to antagonists (Cook 1982).

Table 1.3 Causes and the main characteristics of two types of suppressiveness to take-all in soils (Hornby *et al.* 1998).

Suppression type	Causes	Characteristics
Specific (transferable) suppression	Continuous wheat with take-all	Eliminated by moist heat (60°C for 30 min)
	Additions of *Ggt* mycelia to soil	Eliminated by chemical fumigants
	Addition of other fungi (antagonists/competitors) to soil	Operates below 20°C and masked by non-transferable suppression above 25°C
		Operates primarily in the rhizosphere
General (non-transferable) suppression	Increased microbial activity with increased organic amendments and fertility build-up	Not eliminated by moist heat
	NH_4^+-N uptake by roots	Reduced but not eliminated by fumigation
	Non-host plants	Operates at all soil temperatures but increases above 5°C
	High soil temperatures	Operates primarily in bulk soil

Mazzola (2002) on the other hand, considered that all soils capable of suppressing soil-borne plant diseases were disease-suppressive soils. Although it has been suggested that the term 'disease-suppressive' should be limited to situations involving a clearly identified biological component, abiotic elements of the soil have been shown to have a role in disease suppression as well. Abiotic characteristics, including chemical and physical attributes of the soil (such as pH, organic matter and clay content), can suppress plant diseases directly or indirectly through their impact on soil microbial activity (Mazzola 2002).

Simon *et al.* (1987) investigated the association between pathogen-suppressive effects and disease suppression, and found that soils cropped with consecutive wheat (7-8 y) were able to suppress both saprophytic growth and the parasitic activity of *Ggt*. They suggested that pathogen-suppression and disease-suppression did not occur independently, and that disease-suppression was a result of pathogen-suppression. Whether the suppressiveness of the soils investigated was of the TAD phenomenon was not mentioned. It seems that disease suppression, pathogen suppression, specific suppression and general suppression share some similar principles and suppressive characteristics, but because most suppressive

soils are so poorly understood, it is difficult to assign them to either of the disease or pathogen suppressive categories.

To date, it is still uncertain whether an individual or a select group of microorganisms in the soil causes TAD. In addition, most studies on TAD (Wildermuth 1980; Andrade *et al.* 1994) were carried out on a few confirmed TAD sites, which did not allow for wider investigations into the variations between field locations and other factors on a larger scale. As TAD is a common suppression phenomenon and has been reported in many countries, it is likely that it could occur in New Zealand. Once the mechanisms for TAD suppression, including the effect of soil characteristics, are clearly understood, it should be possible to reproduce the phenomenon in many locations, including New Zealand. Moreover, it is necessary to verify the relationship between *Ggt* inoculum levels and disease incidence in TAD soils in New Zealand, as well as the other interacting factors (outlined in the previous sections) that could also play a part in the microbial activities which are responsible for the onset of TAD.

1.9.1.1 Interactions of microbial antagonists with *Ggt*

Past studies have indicated that biological factors are responsible for the mechanisms of soil suppressiveness, mainly because sterilisation of soils removes the suppressive activity (Baker & Cook 1974; Van Os & Van Ginkel 2001; Mazzola 2004). Hence, most investigations into the biological control of *Ggt* have focused on the interaction among the pathogen, host, and bio-control agents introduced into the rhizosphere (Weller *et al.* 2002). They include the following:

1. Introduction of cold tolerant strains of *Gaeumannomyces graminis* var. *graminis* and a *Phialophora* sp. with lobed hyphopodia via seed-furrow to cross-protect the hosts from being infected by *Ggt* (Wong *et al.* 1996).

2. Seed or soil inoculation with a sterile red fungus to limit take-all through competition for thiamine and induce host plant resistance (Shankar *et al.* 1994; Aberra *et al.* 1998). This fungus was identified as *Limonomyces roseipellis* in a rDNA variation study (Andjic *et al.* 2005).

3. Seed inoculation with a *Bacillus* sp. that produced a broad-spectrum antibiotic inhibitory to *Ggt* and other wheat root pathogens, *Rhizoctonia solani* AG8 and *Pythium* spp. (Kim *et al.* 1997).

4. Seed inoculation with a *Pseudomonas fluorescens* isolate that produced the antibiotic (phenazine-1-carboxylic acid or PCA) (Cook 2003). It delayed take-all infection by competing with *Ggt* to colonise the root tips, and exploiting the nutrients released from the damaged tissues in root lesions, so that less *Ggt* inoculum was left for the following wheat crop (Deacon 1997).

5. Incorporation of the non-pathogenic *Phialophora* spp. close to the seeds at planting, to provide a source of competition in the wheat rhizosphere and to induce host plant resistance (Mathre *et al.* 1998).

6. Seed inoculation with *Microdochium (Idriella) bolleyi* to allow colonisation of the naturally senescing cortical cells of cereal roots during the early stages of the crop, to outcompete *Ggt* for infection sites and nutrients (Kirk & Deacon 1987a, 1987b).

7. Antagonism of *Ggt* on cultures by *Trichoderma lignorum*, *Penicillium waksmanii*, *P. restrictum*, *P. oxalicum* and *P. multicolor*, which were isolated from wheat soils (Hornby *et al.* 1998).

Of the above, *Pseudomonas fluorescens* varieties which are naturally present in TAD soils, are believed to play the major role in suppressing take-all by producing the antibiotic 2,-4-diacetylphloroglucinol (DAPG). Extensive studies carried out by Raaijmakers and co-workers (1997; 1998; 1999; 2001) revealed that DAPG-producing *P. fluorescens* populations present in TAD soils at rhizosphere populations of 10^5 to 10^6 CFU g^{-1} of roots, were sufficient to fully suppress take-all. Suppression was lost when DAPG-producing strains were eliminated from TAD soils but was restored by adding a small amount of TAD soil mixed with DAPG-producing strains at less than 10^5 CFU g^{-1} of roots.

Although *P. fluorescens* has been found to be effective against the take-all pathogen, the expectation that one type of soil bacterium could effectively control a disease in field soils may be too optimistic. This is because the diversity of soil flora and fauna, as well as the physical characteristics of natural soil environments can affect the potential activities of the antagonists and pathogens. In order to understand soil suppressiveness better, the identity

and the biological activity of the diverse microbial populations/community that inhabit the rhizosphere in both take-all suppressive and conducive soils must also be considered, particularly if some of these species display different or complementary modes of action or abilities to colonise the roots. Future research needs to pay more attention to the mechanisms operating in a natural biological control phenomenon, such as TAD, which appears to be more reliable and effective than the vast majority of introduced biological control agents.

1.10 Characterising microbial components associated with soil suppressiveness

Numerous methods, such as fumigation/extraction, plate counts, direct microscopic counts, enzyme assays and substrate utilisation profiles, have been used to investigate the microbial communities in soils. However, these methods have their strengths and weaknesses. They may only determine the general microbial biomass (in the case of fumigation/extraction), or their activities (in the case of enzyme production), without concern for the identification or characterisation of individuals within the community (Mazzola 2004). Substrate utilisation profiles (e.g. carbon utilisation and lipid composition) can allow identification of individuals from different taxa (Garbeva *et al.* 2004), but cannot monitor the abundance of specific microorganisms (Mazzola 2004). Conventional cultivation techniques (e.g. plate and direct microscopic counts) do not reflect the actual microbial community as many microbes from the soil environments cannot be cultured (Hartman *et al.* 1997).

In contrast, methods involving applications of molecular techniques based on nucleic acid compositions can provide information on the relative abundance and the activities or functions of microbial populations over a range of taxonomic levels (Weller *et al.* 2002). Although DNA-based methods are in principle, reproducible and robust, they are susceptible to the potential biases that are inherent in both nucleic acid extractions and PCR amplifications (Garbeva *et al.* 2004). Some of the advantages and disadvantages of using molecular-based fingerprinting methods are outlined in Table 1.4.

Table 1.4 Advantages and disadvantages of molecular-based microbial community fingerprinting methods (Garbeva *et al.* 2004)

Advantages	Disadvantages
1. Dependence on efficient cell lysis only and not on the physiological status of cells.	1. Incomplete lysis of some species, notably gram-positive spore-formers.
2. Direct picture of the diversity of dominant microbial types, including the unculturables.	2. Possible biases in DNA extraction and PCR amplification due to inhibition by soil compounds.
3. Direct assessment of shifts in microbial community structure.	3. Possible presence of one particular sequence or band in different organisms.
4. Ease in handling, thus allowing simultaneous analysis of high sample numbers.	4. Heterogeneous bands that may originate from one bacterial strain due to heterogeneity in the rDNA genes producing double bands.
5. Reproducible results.	5. Phylogenetic information only is usually obtained, and the link to functional information is difficult.
6. Generation of sequences resulting in identification and specific probes to track the specific organism in the ecosystem	

Denaturing gradient gel electrophoresis (DGGE) is one of the most commonly used techniques for characterising/profiling the microbial communities in soil and rhizosphere (Garbeva *et al.* 2004). It involves DNA extraction, purification and specific amplification of the rDNA segments (most commonly) by using PCR with universal primers, which target the 16S region of bacteria and ITS, 18S or 28S regions of fungi. The double stranded PCR products have a GC clamped end to avoid complete disassociation, and are subjected to a DGGE gel containing a linearly increasing gradient of denaturing chemicals. The DNA fragments are separated in the gradient gels because as they reach higher levels of denaturing chemicals they begin to ‘melt’ at a point in the gel that is determined by the sequence of the fragment, which differs for different microbial (Muyzer *et al.* 1993; O'Callaghan *et al.* 2003). Once the double strands are split completely (but held together by the GC clamp), they are physically prevented from migrating any further on the gel. PCR mixes containing numerous species will produce a profile in which each band will represent a taxon (O'Callaghan *et al.* 2003). Subsequent cloning and sequence analysis of the excised bands from the DGGE profiles, can provide information on the identities of the microorganisms through database searches (Muyzer *et al.* 1993; O'Callaghan *et al.* 2006).

The DGGE method can be used to compare the microbial populations present in take-all suppressive and conducive soils in New Zealand and to identify the microorganisms that inhabit the roots and the rhizosphere soil. Knowledge of those which may be associated

with take-all suppressiveness, either individually or as a group, could allow development of better strategies for control of take-all. For instance, if an individual or a specific group of microorganisms is implicated as being associated with suppressive soils, more studies can be developed to determine their functions/roles in the suppressiveness. Once these functions are known, the microorganisms could be introduced into the soil. Alternatively, genetic markers can also be developed to enumerate the specific populations in the suppressive soils.

1.11 Overall aim and hypothesis of PhD research

The underlying hypothesis of this research was that TAD was a specific suppression caused by an individual or a selected group of microorganisms, which suppressed *Ggt* in the rhizosphere and roots of the host. The specific objectives involved in providing evidence for this hypothesis were:

1. To investigate the effects of successive wheat cropping on *Ggt* DNA concentrations and disease incidence and to identify soils in New Zealand that are suppressive to take-all.

2. To optimise the efficacy of pot assays used for screening take-all suppressive soils.

3. To determine the mechanisms involved in TAD suppressiveness in pot assays.

4. To characterise and compare the microbiota in the roots and rhizosphere of wheat plants grown in take-all suppressive and conducive soils using DGGE techniques.

5. To investigate the complex interactions between take-all development and the population dynamics of *Pseudomonas fluorescens* in soils which were naturally infested with *Ggt*, and the possible involvement of some soil physico-chemical properties over successive wheat cropping.

The individual chapters in this thesis consist of their own Abstract, Introduction, Materials and Methods, Discussion and Reference sections in a similar style to that prepared for submission for publication. This format has sometimes, inevitably, resulted in repetition of content in some chapters. However, attempts have been made to keep this repetition to the minimum where possible. All ANOVA tables derived from statistical analyses of the results from the experiments are presented in Appendix 5.

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Chapter 2
Gaeumannomyces graminis var. *tritici* concentrations in soils from successive wheat cropping and resulting disease incidence

2.1 Abstract

The 26 soils naturally infested with *Gaeumannomyces graminis* var. *tritici* (*Ggt*) were chosen after a survey on 112 New Zealand cereal fields in 2003/04 season had indicated their potential in suppressing take-all development. Some of the soils were from fields previously cropped with 3-4 y of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*) or triticale, which were considered likely to express take-all decline, and some were from fields with high natural *Ggt* inoculum concentrations before sowing but low take-all severity in the subsequent crops. Of the 26 soils, 12 were collected from the St Andrews region, South Island, New Zealand, and represented 1-4 y of successive wheat cropping (three of each). Three soils from ryegrass fields (2, 2 and 5 y), also from the St Andrews region, were included to represent 0 y of wheat cropping, giving 29 soils in total. The natural *Ggt* DNA concentrations in the soils were determined using real-time PCR and all soils were amended with 12.5% w/w *Ggt* inoculum. The relationship between disease incidence and successive years of wheat crops, and plant growth were investigated with a pot experiment.

Overall, the results showed that there was high variation between soils, which masked the potential trends. This was probably caused by too high an inoculum level and differences in soil origins. However, results from the 15 St Andrews soils showed that greater years of successive wheat cropping resulted in higher *Ggt* DNA concentrations ($P<0.001$) and disease incidence ($P<0.05$), but variable fresh and dry weights of roots and shoots ($P<0.001$). The mean *Ggt* DNA concentrations and disease incidences on the roots of potted plants were also highly correlated ($r = 0.76$). In addition, comparisons of the *Ggt* DNA concentrations in some soils before sowing and after the last harvest of wheat, with the disease incidence in the pot trial, showed that 11 out of the 29 soils had the potential for take-all suppressiveness.

Keywords: *Gaeumannomyces graminis* var. *tritici*, take-all, successive wheat, DNA, inoculum, suppressive soils, take-all decline, plant growth

2.2 Introduction

Control of take-all, in many areas of the world, is achieved by limiting the numbers of successive susceptible crops such as wheat (*Triticum aestivum* L.), or barley (*Hordeum vulgare* L.) to no more than two (Graham & Mitchell 1999). However, continuous cropping of wheat in the same field has been reported to result in take-all decline (TAD), in which the disease is naturally suppressed (Cook *et al.* 1986). TAD has been shown to be associated with the depletion of the nutrients required by the pathogen, due to natural decomposition of plant materials, and the presence of competing and antagonistic soil microorganisms, such as fluorescent *Pseudomonas* spp. (Graham & Mitchell 1999). In the development of the TAD phenomenon under successive years of wheat crops, the disease peaks at least once and declines to low but fluctuating levels (Cook & Naiki 1982; McSpadden Gardener & Weller 2001).

The association of disease severity or incidence with host crop successions, has been widely studied, with TAD reported to commence from the second to seventh year of wheat succession (Hornby & Henden 1986; Werker *et al.* 1991; Lebreton *et al.* 2004). Past studies that investigated the relationship between *Ggt* inoculum concentrations and years of successive wheat, were mostly by examining the extent of hyphal growth within the roots (Pope & Jackson 1973; Wildermuth & Rovira 1977), counting colonised residual wheat material or other propagules, lesion sizes (Cook *et al.* 1986) and pathogen recovery frequencies using conventional isolation techniques (Cook & Naiki 1982). Advances in the development of molecular tools in recent years have allowed investigations into the relationships between *Ggt* population structure (Lebreton *et al.* 2004), genotypic frequencies (Lebreton *et al.* 2007) and disease severity with successive wheat cropping.

A quantitative real-time PCR (polymerase chain reaction) has been developed to measure *Ggt* DNA concentrations in the soil to predict take-all risk prior to sowing subsequent wheat, and the service is provided to growers by CSIRO[1] Entomology and SARDI[2], Australia (Herdina & Roget 2000; Ophel-Keller & McKay 2001). However, the disease severity levels found in pot assays did not always show good correlations with the *Ggt* DNA concentrations in the corresponding soils (Herdina & Roget 1999, 2000). Similar poor correlations were obtained with the results in a New Zealand survey carried out

[1] Commonwealth Scientific and Industrial Research Organisation
[2] South Australian Research & Development Institute

during 2002-04 on 112 soils from fields with varying wheat cropping histories by Cromey *et al*. (2004). They found *Ggt* DNA concentrations of 106-1419 pg g^{-1} soil from fields with 0-3 y of previous successive wheat (in the 2002/03 season), suggesting that take-all risk in New Zealand wheat could increase up to the third or fourth year of continuous wheat. These 'pre-sowing' *Ggt* DNA concentrations correlated marginally with the take-all severity (expressed as a disease index) in subsequent crops (2003/04 season) (r = 0.5), which at the time, would have been cropped with 1-4 y of wheat (Cromey, unpul. data). However, a number of the soils with relatively high *Ggt* inoculum concentrations (>325 pg g^{-1} soil), had low take-all severity (take-all index <20) in subsequent crops. This raised the question of whether these fields might be experiencing some form of suppression. According to Hornby (1983), there are seven types of take-all suppression including TAD, and any soils that suppress plant disease, or the pathogenic fungi within them, are considered as suppressive soils (Hornby *et al.* 1998). To date, the occurrence of TAD or any other forms of take-all suppressive soils has not been reported in New Zealand.

This chapter reports on (1) changes in soil *Ggt* DNA concentrations from before sowing and after crop harvest in fields that had 1-4 y of successive wheat crops; (2) a pot experiment investigating wheat take-all incidence and plant growth; (3) the potential for take-all suppressiveness in these soils, determined by comparing soil *Ggt* DNA concentrations with disease incidence.

2.3 Materials and methods

2.3.1 Soil sampling and processing

In March 2004, soils were collected after crop harvest from 26 wheat fields and three ryegrass fields in the South Island, New Zealand. These fields were chosen from those in an earlier take-all survey carried out from 112 wheat fields for 2003/04 seasons by Cromey *et al.* (2004) to identify potential take-all suppressive soils. The fields selected represented a range of cropping histories, including fields previously cropped with 3-4 y of wheat, which could potentially express TAD, and fields with high *Ggt* inoculum concentrations (greater than 325pg *Ggt* DNA g^{-1} soil) but low take-all severity (disease index<20), at the time of assessment.

Among the 29 sites chosen, a subset of 15 were from the same region, St Andrews, and represented three sites each of 0-4 y of successive wheat crops. The three sites with 0 y of

wheat were cropped with *Lolium perenne* L. (perennial ryegrass) in the field (Table 2.1). Being from the same region with similar environmental conditions, the 15 soils were anticipated to be relatively similar in their physical properties, and thus would be ideal for investigating the effects of different successive wheat cropping histories on *Ggt* soil inoculum and disease levels and their effects on subsequent plant growth.

To provide the amount of soil required from each field, soil blocks of at least 10 cm × 10 cm × 10 cm were cut with a spade from ten sampling points spaced within a 1 ha zone of the field along a 'W' pattern (Van Elsas *et al.* 2002). This provided at least 10,000 cm^3 of soil from each site. Soils from each site were placed in two 30 L bins, partially dried at 25°C overnight, and then passed through a 4 mm sieve to homogenise the texture and remove any stones present. Crop debris caught in the sieve was returned to the soil as it was considered part of the natural *Ggt* inoculum. Soils from each site were pooled, thoroughly mixed together and stored until required at 4°C in 50 L bins covered loosely with plastic sheets. Prior to use, at least 300 g of each soil was sent to the Root Disease Testing Service (SARDI, Adelaide) for analysis of *Ggt* DNA concentrations.

The DNA analysis was performed by real-time PCR using TaqMan® MGB (tm) probes and Qiagen Quanti Tect probe Master Mix in 10 µL reactions on an ABI PRISM® 7900HT Sequence Detection System. Thermal cycling conditions were: an initial temperature of 95°C for 15 min to activate the Taq Polymerase followed by a melting step of 95°C for 15 s, and a combined annealing/extension step at 60°C for 1 min for 40 cycles. Standards were produced using DNA extracted from aseptically grown *Ggt* mycelium. The DNA was diluted though a 10-fold serial dilution from 200 pg μL^{-1} to 2 fg μL^{-1}. Due to commercial sensitivity, the procedures involved in the DNA extraction were not disclosed. The resulting *Ggt* DNA concentrations (in pg g^{-1} soil) quantified from the collected soils were treated as the 'post-harvest' data and were included in the analyses throughout the current study. Site information including locations determined by a global positioning system (GPS) and cropping histories are shown in Table 2.1.

Basic physico-chemical properties of the soils were determined by R. J. Hill Laboratories Ltd., Hamilton, New Zealand and Crop & Food Research Ltd., Lincoln, New Zealand (Table 2.2), and the soil types are listed in Appendix 2. The basic soil profile included pH, Olsen phosphorus, potassium, calcium, magnesium, sodium, cation exchange capacity and base saturation data. These tests are recommended on most agricultural or horticultural

soils (R. J. Hill Laboratories Ltd 2002). Anaerobically mineralised N (also known as available N) was included in the test because this nutrient has been found to be the most important fertiliser element in New Zealand cereal crops or arable soils. It is also a good indicator of biological activity and is closely related to microbial biomass (R. J. Hill Laboratories Ltd 2002). The main reason for conducting these tests was to rule out low/insufficient levels of nutrients as a limiting factor in plant growth. Investigation into the effects of nutrients on controlling take-all was not the focus in this study. All nutrients reported were in plant available forms and were compared to the desired/acceptable levels recommended for New Zealand crops. A brief description of the methods used in the basic soil and mineral N analyses are showed in Appendix 2. The test results for most soils indicated that the Olsen P and K concentrations were below the recommended range and that the N concentrations were at the lower end of the acceptable range (Table 2.3). The soils had cation exchange capacity (CEC) within the normal range, indicating that all soils were capable of storing nutrients (Morton *et al.* 2000). Microbial biomass C in the soils was also analysed to provide information on the active microbial population present in the soil. This test was done with hot-water extractable C method described by Ghani *et al.* (2003).

2.3.2 Pot experiment

The experiment, consisting of 29 soil samples, with or without added *Ggt* inoculum, was set out in a two-way randomised block design. The purpose of adding inoculum was to determine whether different soils had the capacity to inhibit the pathogenic activity of *Ggt* (Hornby 1983). Soils from the 15 St Andrews fields previously cropped with 0-4 y of wheat (three replicates of each) were treated as a subset. Each treatment was replicated four times to give a total of 232 pots.

Soil inoculation was carried out by hand mixing one part of pooled *Ggt* inoculum to seven parts of soil to give a final weight of 300 g of soil mixture per pot (size, 400 mL, height 10 cm). *Ggt* inoculum was prepared according to the method described by Hollins *et al.* (1986) except that autoclave bags (36 x 48 cm, able withstand temperatures up to 150°C; Raylab NZ Ltd., Auckland, New Zealand) instead of conical flasks were used for growing the inoculum. The pooled inoculum was made up of five *Ggt* isolates H11T3R1/3, A3SL4, BIOMILL1SC3, H9T3R1/1.2 and BIO3, which were obtained from *Elytrigia repens* rhizomes and wheat roots in 2002/03 (Crop & Food Research culture collection).

Table 2.1 Site information and cropping histories of fields (2003/04) where soils were sampled. The 15 soils sampled from the St Andrews region are shaded.

Field code	[1]Pre-sowing *Ggt* DNA pg g^{-1} soil (2003)	[2]Risk	[3]Dec 03 Take-all index	[1]Post-harvest *Ggt* DNA pg g^{-1} soil (2004)	[4]Post-harvest *Ggt* DNA analysed from soils collected for the pot experiment 2004 (pg g^{-1} soil)	[5]Preceding crops 1999/00	2000/01	2001/02	2002/03	2003/04	2003/04 Years of wheat	Years of cereal	GPS Location
B3	840	High	13.88	750	834	W	P	W	W	W	3	3	S45°51.1', E168°34.0'
B6	1206	High	5.81	184	229	Dt	W	W	W	W	4	4	S45°51.0', E168°34.0'
B8	377	High	8.81	61	107	O	W	P	W	W	2	2	S45°53.4', E168°34.8'
C1	330	High	15.55	589	606	W	C	W	B	W	1	3	S43°55.4', E171°48.0'
C3	181	Medium	28.07	1026	1096	-	Po+W	W	B	W	1	4	S43°55.4', E171°48.2'
C6	3	BDL	11.28	111	136	-	B	W	W	W	3	4	S43°58.8', E171°48.2'
C12	2924	High	9.25	22	13	B	B	L	W	W	2	2	S43°45.2', E171°53.8'
G2	2251	High	17.59	1048	1723	W	W	Po	W	W+G	2	2	S43°58.5', E170°50.7'
H1	1250	High	17.56	148	171	B	G	W	W	W	3	3	S44°31.2', E171°06.9'
H2	687	High	21.26	686	195	C	Pa	Pa	W	W	2	2	S44°31.2', E171°06.8'
H3	1526	High	10.20	150	110	Pa	Pa	W	W	W	3	3	S44°31.1', E171°06.5'
H4	1	BDL	6.79	2	0	C	Pa	Pa	Pa	W	1	1	S44°31.0', E171°05.7'
H5	42	Low	8.77	244	491	G	W	W	G	W	1	1	S44°30.8', E171°05.6'
H6	250	Medium	48.99	1395	330	W	P	G	W	W	2	2	S44°30.5', E171°05.9'
H7	477	High	81.20	397	283	Pa	W	W	W	W	4	4	S44°30.5', E171°06.8'
H8	2540	High	53.18	1257	1745	Rg	W	W	W	W	4	4	S44°29.3', E171°04.6'
H9	54	Low	30.94	327	232	Pa	Pa	W	W	W	3	3	S44°29.2', E171°05.2'
H10	1321	High	86.06	1365	166	Pa	W	W	W	W	4	4	S44°28.6', E171°05.6'
H11	6	Low	4.83	105	12	W	W	G	W	W	2	2	S44°28.3', E171°05.7'
H12	19	Low	4.51	457	60	Pa	W	W	G	W	1	1	S44°28.2', E171°05.6'
H13	NA	NA	NA	NA	11	Rg	Rg	Rg	Rg	Rg	0	0	S44°29.7', E171°05.9'
H14	NA	NA	NA	NA	21	Rg	Rg	Rg	Rg	Rg	0	0	S44°30.7', E171°05.7'
H15	NA	NA	NA	NA	0	Rg	Rg	Rg	Rg	Rg	0	0	S44°30.5', E171°06.5'
I6	564	High	9.30	997	2052	BR	W	B	L	W	1	1	S44°09.3', E171°12.4'
L9	1136	High	13.97	256	1	G	Pa	Pa	W	W	2	2	S44°41.6', E171°04.7'
M2	2348	High	27.13	1683	719	G	W	W	W	W	4	4	S44°44.1', E171°06.5'
M3	1447	High	23.70	2992	2788	G	W	W	W	W	4	4	S44°44.1', E171°06.5'
M10	55	Low	5.64	1582	86	G	W	W	W	W	4	4	S44°44.0', E171°07.1'
M11	1961	High	54.03	2985	522	G	W	W	W	W	4	4	S44°44.3', E171°05.5'

[1]*Ggt* was analysed with soil collected from 25 positions (two soil cores at each position) along a 'W' pattern over the whole field using soil corers (20 mm dia., 10 cm depth).

[2]Take-all risk categories developed by SARDI for Australia at the time of quantitative DNA analysis of *Ggt* in 2003/04:

Low risk: 5-130 pg *Ggt* DNA g^{-1} of soil
Medium risk: 131-325 pg *Ggt* DNA g^{-1} soil
High risk: >325 pg *Ggt* DNA g^{-1} soil
BDL: DNA below detection limit

[3]Take-all disease index = (0a+10b+30c+60d+100e)/T
where a, b, c, d and e = number of plants in each of the infection categories below, and T= total number of plants.
Infection categories:
0 no infection
1 slight (1-10% of roots infected)
2 moderate (11-30% of roots infected)
3 high (31-60% of roots infected)
4 severe (61-100% of roots infected)

[4]*Ggt* was analysed with soil collected from 10 positions (2 × 10 cm^3 soil blocks at each position) along a 'W' pattern over the whole field using a spade.

[5]Preceding crops: wheat (W), pea (P), dogtail (Dt), oat (O), clover (C), grass (G), potato (PO), ryegrass (RG), pasture (PA), barley (B), borage radish (BR), linseed (L).

Table 2.2 Some physico-chemical properties of soils sampled.

Field code	Normal range	5.3-6.1	20-30	0.5-0.8	5.0-12.0	0.8-3	0-0.5	12-25	50-85	Min N ($\mu g\ g^{-1}$)	Microbial biomass C ($\mu g\ g^{-1}$)
	Soil texture	pH	Olsen P ($mg\ l^{-1}$)	[1]Potassium ($me\ 100\ g^{-1}$)	Calcium ($me\ 100\ g^{-1}$)	Magnesium ($me\ 100\ g^{-1}$)	Sodium ($me\ 100\ g^{-1}$)	[2]CEC ($me\ 100\ g^{-1}$)	Base Saturation (%)		
B3	Silt loam	5.6	14	0.30	7.30	0.31	0.09	16	51	24.52	366.36
B6	Silt loam	5.5	21	0.38	6.20	0.22	0.07	17	41	22.13	351.29
B8	Silt loam	5.8	21	0.53	6.60	0.33	0.07	16	46	15.51	345.02
C1	Silt loam	5.8	20	0.78	7.70	0.68	0.10	15	63	22.80	586.09
C3	Silt loam	6.3	16	0.90	10.70	0.83	0.11	16	76	26.32	549.09
C6	Silt loam	5.7	25	0.49	8.80	1.36	0.14	17	62	26.97	561.39
C12	Loamy silt	6.0	19	0.37	7.90	0.41	0.09	13	69	43.07	436.49
G2	Silt loam	6.0	29	0.38	8.70	0.38	0.14	17	58	38.82	660.53
H1	Silt loam	5.7	28	0.42	11.50	0.93	0.09	20	66	34.11	491.50
H2	Silt loam	6.0	14	0.34	11.80	1.07	0.10	18	74	25.09	496.01
H3	Silt loam	6.1	41	0.38	13.40	1.12	0.12	19	78	33.80	470.73
H4	Silt loam	6.0	20	0.29	9.50	0.95	0.11	16	66	26.07	451.76
H5	Silt loam	5.9	15	0.25	7.80	0.87	0.13	15	60	20.37	376.95
H6	Silt loam	5.9	19	0.21	9.50	1.01	0.11	16	69	26.51	406.83
H7	Silt loam	6.2	36	0.19	11.10	1.07	0.14	16	77	16.05	399.87
H8	Silt loam	6.1	18	0.27	10.80	1.22	0.13	17	73	41.07	577.11
H9	Silt loam	6.5	16	0.27	11.80	1.44	0.13	17	79	21.17	462.11
H10	Silt loam	6.1	16	0.43	8.60	1.09	0.09	15	67	29.46	510.14
H11	Silt loam	5.6	21	0.46	6.30	1.06	0.09	15	53	25.10	369.48
H12	Silt loam	6.4	14	0.39	11.20	1.14	0.11	16	80	21.66	490.13
H13	Silt loam	6.6	7	0.16	9.70	1.04	0.28	14	81	12.63	467.91
H14	Silt loam	6.2	14	0.14	8.90	1.05	0.21	14	74	25.38	457.89
H15	Silt loam	6.6	21	0.20	11.50	1.12	0.20	15	87	15.35	420.90
I6	Loamy silt	5.5	12	0.23	6.90	0.74	0.14	15	54	55.18	436.83
L9	Silt loam	6.2	13	0.22	8.80	1.07	0.12	15	66	16.67	509.70
M2	Silt loam	5.7	37	0.26	7.40	0.81	0.10	14	62	19.15	460.68
M3	Silt loam	5.8	43	0.26	7.40	0.75	0.10	14	62	21.24	382.33
M10	Silt loam	6.6	42	0.17	14.00	1.03	0.12	17	91	13.20	519.06
M11	Silt loam	6.9	43	0.26	12.60	0.90	0.24	14	100	17.94	476.41

[1] 'me' refers to milliequivalents

[2] Cation exchange capacity

Table 2.3 Sutiability of Nitrogen levels for New Zealand arable crops, as recommended by R. J. Hill Laboratories Ltd (2002).

Levels	Anaerobically mineralisable N (µg g^{-1} soil)	Available N (kg ha^{-1})
Very low	<35	<50
Low	35-50	50-150
Medium	50-80	150-250
High	80-240	250-350
Very high	>240	>350

Methods on preparation of *Ggt* inoculum and soil inoculation are described in Appendix 3, A3. 1. To ensure that all the *Ggt* isolates used were var. *tritici*, their pathogenicity on wheat and *Avena sativa* L. (oat), which *Ggt* does not infect (Deacon 1997), was determined earlier using the methods described by Chng *et al*. (2005) (Appendix 3, A3. 2). Each pot was planted with four pre-germinated healthy wheat seedlings (cultivar Regency, line 03/W/01P). Methods on pre-germination of seeds are presented in Appendix 3, A3. 3.

After planting, each pot received 20 mL of nutrient solution to give final concentrations for N, P, K and S of 150, 50, 100 and 20 µg g^{-1} soil, respectively (see Appendix 3, A3. 4 for nutrient calculations), before topping up with water to field capacity (FC) (-5 kPa) by weight (Appendix 3, A3. 5 and Appendix 5, A5. 1). The experiment was conducted in growth chambers (Conviron, Controlled Environments Ltd., Canada) maintained at 16°C with alternate 12 h light/dark photoperiods, photosynthetic photon flux density, (PPFD) of 375 µmols^{-1}m^{-2} at pot height (10 cm) and 80% relative humidity (RH) for 4 wk (Figure 2.1).

Due to the large size of the trial, two of the replicates (blocks) were run in each of the two growth chambers. The assumption was made that conditions in the growth chambers were similar for both times and that the replications would allow determination of any variations caused between growth chambers and time differences. All pots received water once weekly to FC by weight.

Figure 2.1 Set-up of pot assay in growth chamber.

2.3.3 Disease assessment

Roots were washed carefully in a bucket of tap water and assessed for infection. Infection was defined as at least one take-all lesion per root axis. Numbers of healthy and infected root axes on both seminal and nodal root systems were recorded and percent infected roots per pot were then calculated using the formula:

$$\text{Infected roots (\%) per pot} = \left(\frac{\sum (\text{Number of infected root axes per plant})}{\sum (\text{Number of root axes per plant})} \right) \times 100\%$$

Since there was a possibility that degree of infection could affect the root function and plant growth, fresh and dry weights of both roots and shoots were obtained before and after drying them at 70°C for 24 h.

2.3.4 Statistical analyses

Data on the post-harvest *Ggt* DNA concentrations for all the 29 test soils were analysed using one-way analysis of variance (ANOVA). The post-harvest *Ggt* DNA concentrations were those obtained from analysing the soils collected for the pot experiment (Table 2.1). *Ggt* DNA concentrations of the 15 St Andrews soils, which had been grown with 0-4 y of wheat, were analysed as a subset of the 29 soils using ANOVA. Data on percent roots infected were first transformed by adjusted logit to stabilise the variance and then

subjected to ANOVA. The fresh and dry weights of both the roots and shoots were also included in the analysis.

2.4 Results

2.4.1 *Ggt* DNA concentrations in field soils with different periods of successive wheat cropping

Results obtained from analysing the 29 soils showed that the mean *Ggt* DNA concentrations in the soils after crop harvest (2003/04) differed (P<0.001), with y 1 and 4 wheat soils having the highest natural *Ggt* DNA concentrations (718 and 817 pg g^{-1} soil, respectively) (Figure 2.2). Mean *Ggt* DNA concentrations in the 15 St Andrews soils varied significantly with years of successive wheat cropping (P<0.001) (Figure 2.2), with slightly increasing *Ggt* DNA concentrations during y 0-1 (0-200 pg g^{-1} soil), relatively constant concentrations during y 1-3, and then increasing concentrations after y 3.

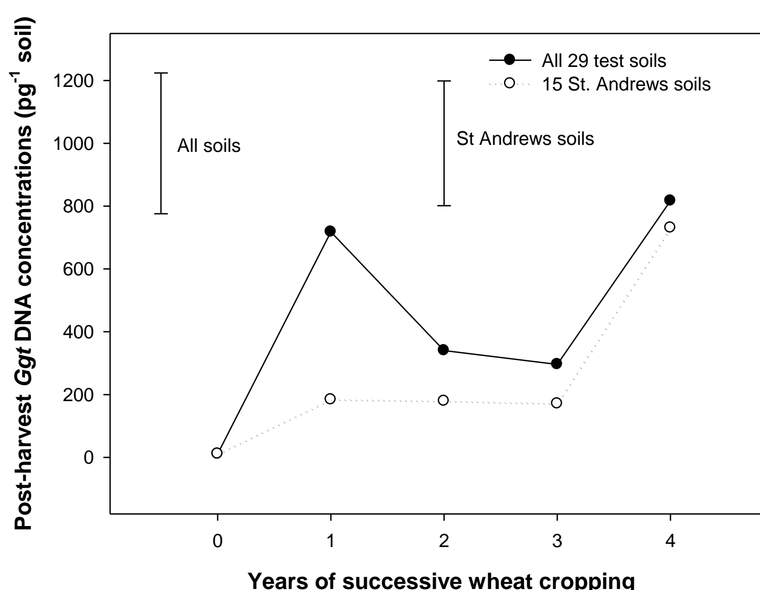

Figure 2.2 The relationship between years of successive wheat cropping and the mean post-harvest *Ggt* DNA concentrations in soils in the 2003/04 season. Error bars are the least significant differences (LSD) at the 5% level (df = 115 for both analyses).

Comparisons were also conducted between the pre-sowing and post-harvest *Ggt* DNA concentrations in individual soils (excluding the three ryegrass soils) (Figure 2.3). Results showed that a single wheat crop caused the *Ggt* DNA concentrations to increase or remain constant for most soils (Figure 2.3a), and to nearly always decrease after 2, 3 and 4 y of successive wheat crops (Figure 2.3b, c and d). The decrease in *Ggt* DNA concentrations

for a number of these soils (e.g. L9, C12 and H3), was sufficient to have their take-all risk levels reduced from high to medium or low. An exception was observed in soil M3, which had 4 y of successive wheat crops and experienced an increase in *Ggt* DNA concentrations (Figure 2.3d).

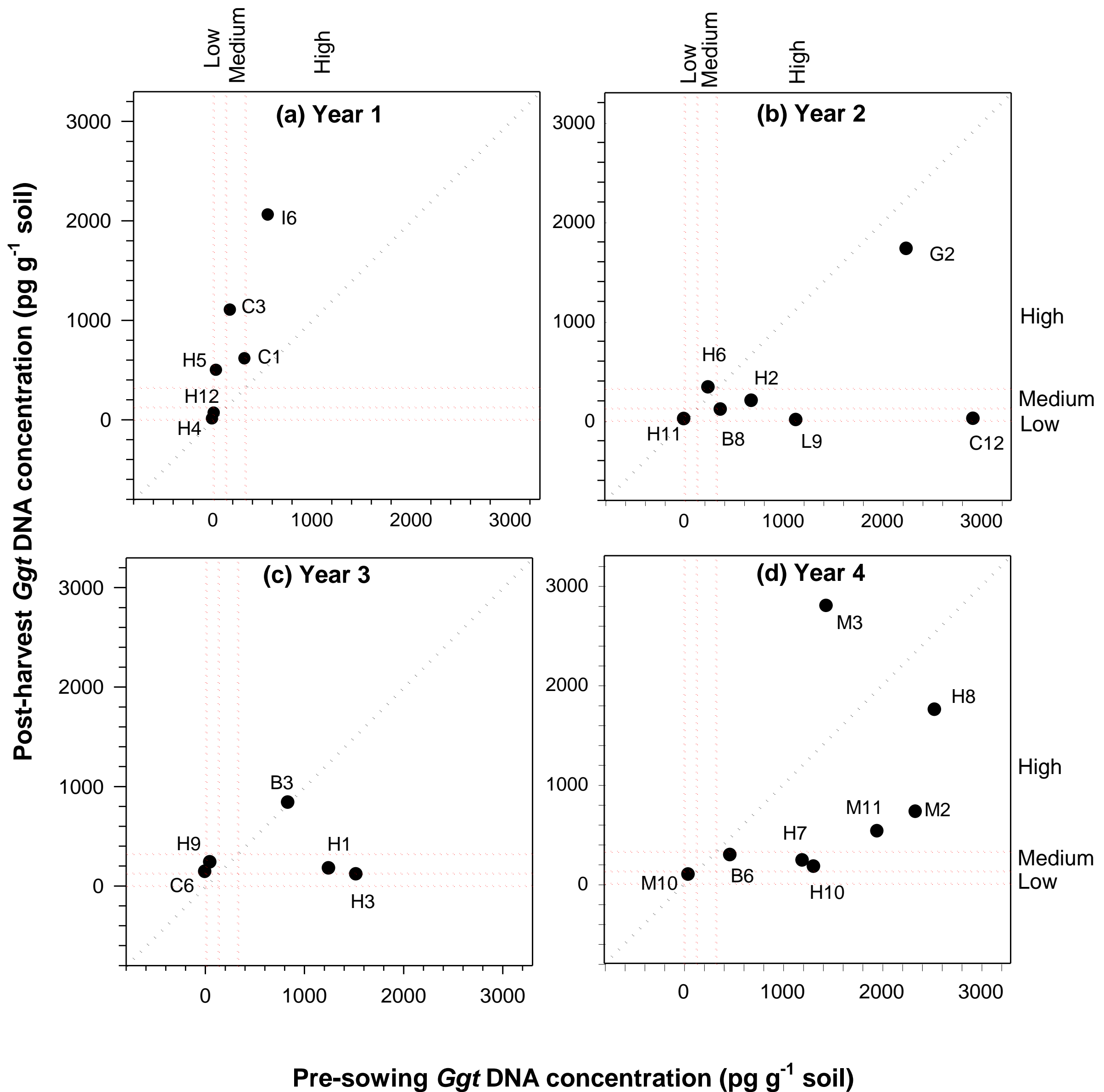

Figure 2.3 Pre-sowing and post-harvest *Ggt* DNA concentrations for field soils with 1-4 y of successive wheat cropping in the 2003/04 season (a - d). The red dotted lines represent the three take-all risk categories used at the time of soil selection, with the vertical and horizontal lines applied to the pre-sowing and post-harvest data, respectively. The area between the first two lines is low risk (5-130 pg g^{-1} of soil), between second and third lines is medium risk (131-325 pg DNA g^{-1} soil), and beyond the third line is high risk (>325 pg g^{-1} soil).

2.4.2 Disease incidence from the pot experiment

The purpose of adding inoculum was to determine whether different soils had the capacity to inhibit the pathogenic activity of the added *Ggt* (Hornby 1983). However, initial analysis of the full data set showed that roots of wheat plants grown in all soils with added *Ggt* inoculum were more severely infected than those grown in the same soils with no added *Ggt* inoculum ($P<0.05$) (73 and 9% infected roots, respectively), thus not differentiating the magnitudes of suppressiveness among the soils. Further analyses therefore focused only on plants grown in uninoculated soils, some of which were soils naturally infested with *Ggt*.

Results from the overall analysis of plants grown in uninoculated soils showed that mean disease incidence was significantly affected by successive wheat crops ($P<0.005$), with percent infected roots increasing in soils during 0-1 y and then remaining constant in soils during 1-4 y of successive wheat crops (Figure 2.4a). However, analysis from the 15 St Andrews soils (Figure 2.4b) showed a different trend from the overall analysis. There was a clear trend of increasing root infection with increasing successive wheat crops ($P<0.05$).

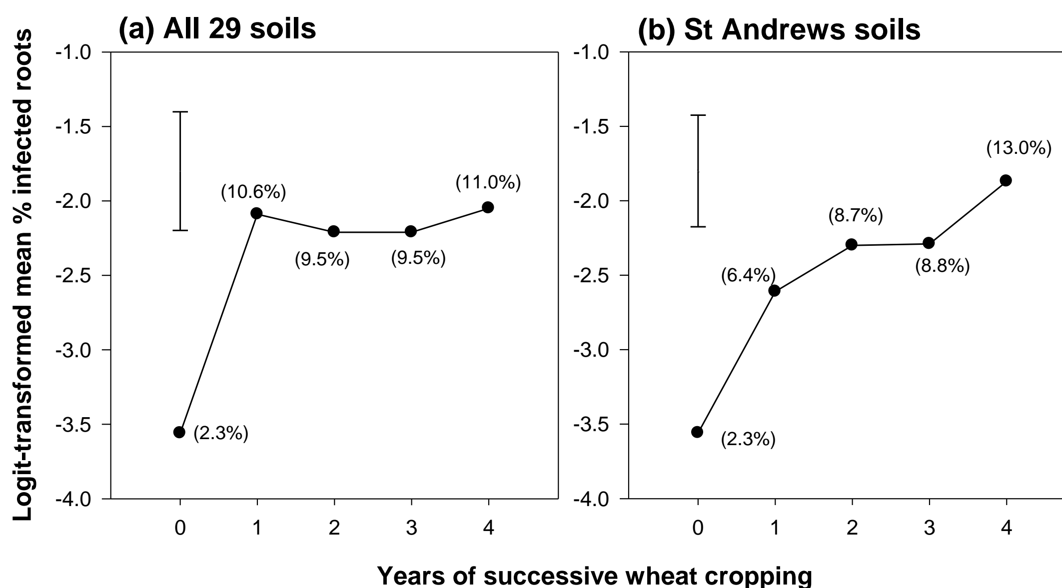

Figure 2.4 The logit-transformed mean percent infected roots of plants grown in (a) all the test soils and (b) in the 15 St. Andrews soils. Data in parentheses are the back-transformed mean percent infected roots. Error bars are the least significant differences (LSD) at the 5% level (df = 114).

There were also significant variations between different soils that had the same successive wheat cropping histories for both the overall analysis (29 soils) (P<0.05) (Figure 2.5a) and the analysis of the 15 St Andrews soils (P<0.05) (Figure 2.5b). Variation between the 15 St Andrews soils was, however, not as great as those in the 29 soils (overall analysis). The variation in St Andrews soils was mainly caused by the differences in percent infected roots between the three soils cropped with 0 y of wheat (i.e. H15, H14 and H13), and between H1 and H3, which were cropped with 3 y of wheat.

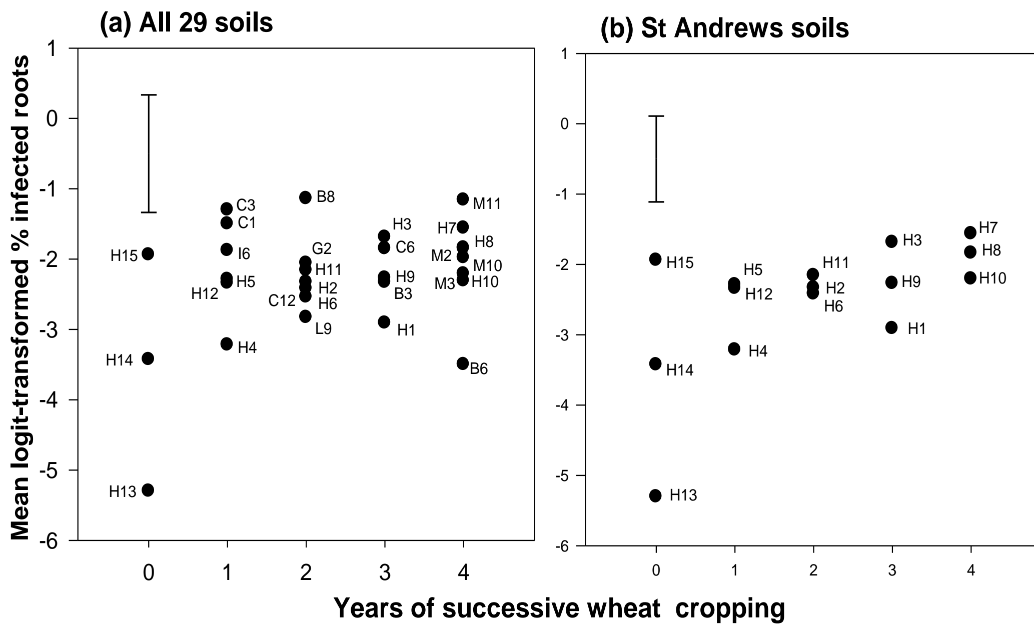

Figure 2.5 Variations between soils with the same wheat cropping histories on the logit-transformed mean percent infected roots of plants grown in (a) all the test soils and (b) in the 15 St Andrews soils. Error bars are the least significant differences at the 5% level (df = 114).

2.4.3 Soil *Ggt* DNA concentrations and disease incidence from the pot experiment

When comparing mean *Ggt* DNA concentrations (Figure 2.2) in the 29 soils used for the pot experiment with the back-transformed means of percent infected roots for the wheat plants grown in them (Figure 2.4a), there was no clear relationship between the pairs of values (r = 0.20). However, for the 15 St Andrews soils, the mean *Ggt* DNA concentrations were strongly correlated with the back-transformed means of percent infected roots (r = 0.76) (Figure 2.6). Comparisons using the raw data of percent infected roots for the 15 St Andrews soils, however, showed no relationship with *Ggt* DNA concentrations (r = 0.36).

The changes in soil *Ggt* DNA concentrations from pre-sowing to after crop harvest (2003/04 season), which might indicate the presence of a TAD soil, were plotted against the back-transformed mean percent infected roots in the pot experiment of the 26 soils (excluding the three ryegrass soils, Figure 2.7). Based on the information that diminishing pathogen in soil with declining, low or no disease during continued monoculture of wheat, are indications of TAD (Baker & Cook 1974), the results identified a number of soils with potential suppression. Eleven of the 29 soils, C12, H2, L9, H1, H3, B6, H7, H10, G2, M2 and H8, experienced large decreases in *Ggt* DNA concentrations that also coincided with the low percent infected roots (< 20% roots infected).

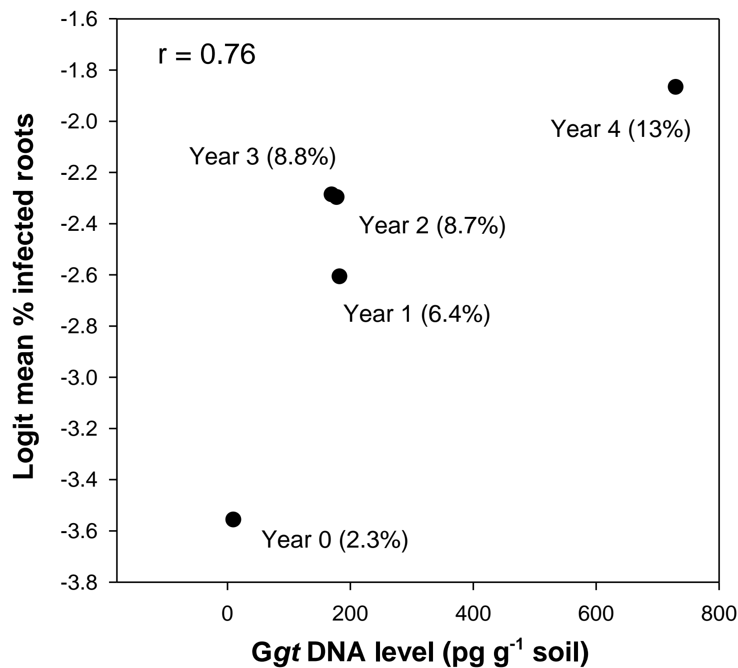

Figure 2.6 Relationship between the mean *Ggt* DNA concentrations and the logit mean percent infected roots in St Andrews soils.

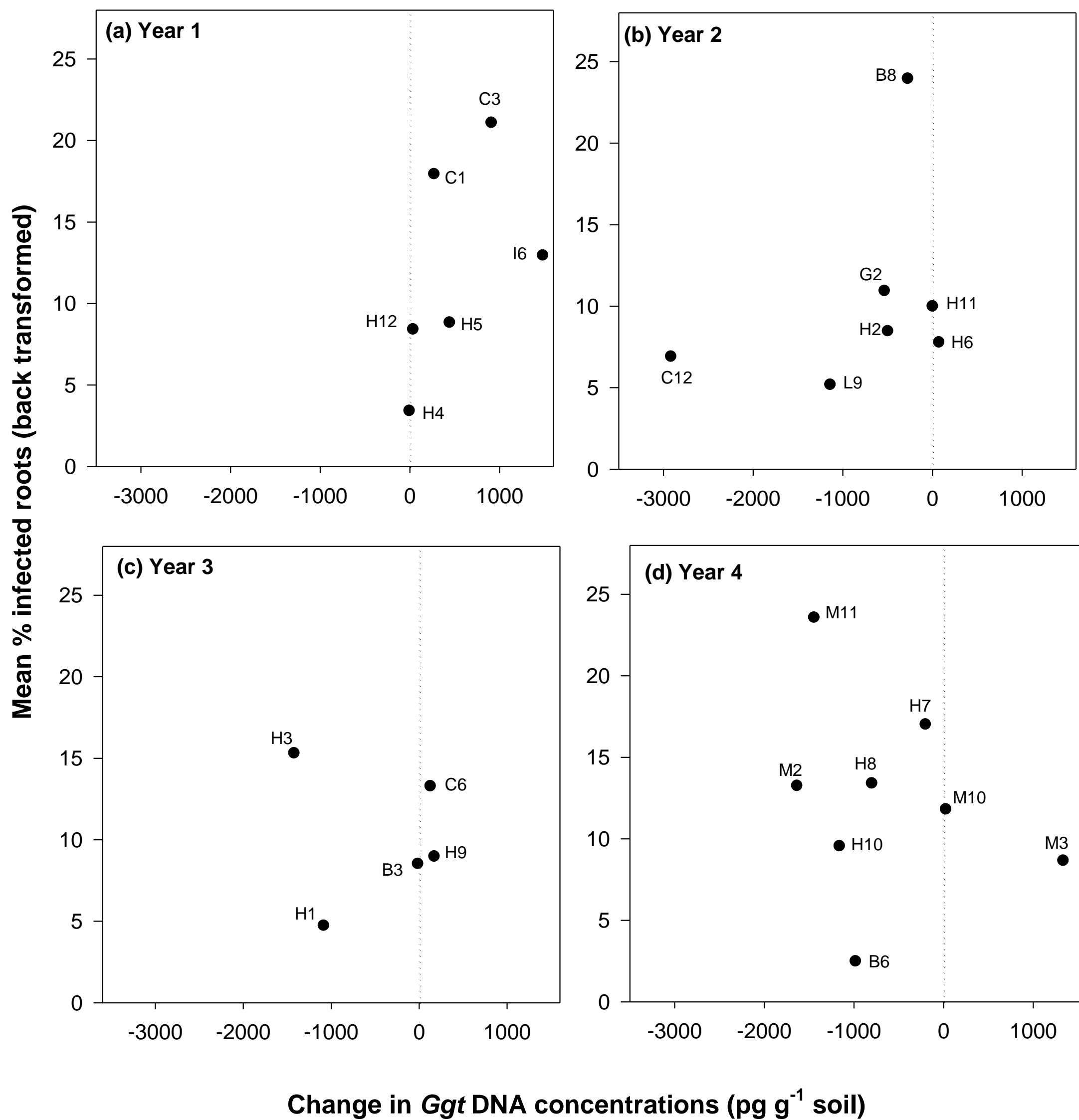

Figure 2.7 Change in *Ggt* inoculum concentrations from pre-sowing to post crop harvest in the 2003/04 season for field soils with 1 to 4 y of successive wheat cropping (a – d), relative to mean percent infected roots (back-transformed) of wheat plants grown in the same soils in a pot experiment.

2.4.4 Fresh and dry weights from pot experiment

From the overall analysis of plants grown in the 29 soils, shoot weights (both dry and fresh) were significantly affected by years of successive wheat cropping ($P<0.001$ for both dry and fresh weights). The lowest mean shoot weights were from plants grown in soils cropped with 2 y of successive wheat crops (fresh = 2.09 g and dry = 0.38 g) (Figures 2.8a-b). Root fresh and dry weights were however, not affected by years of successive wheat crops (Figures 2.8a-b). There were significant variations between different soils of the same wheat cropping histories in both the root ($P<0.005$) and shoot ($P<0.001$) weights (dry and fresh) (Figure 2.9).

Plants grown in the 15 St Andrews soils however, showed slightly different trends from the overall analysis. Both root and shoot dry and fresh weights of plants differed according to the years of successive wheat crops ($P<0.05$ for both root dry and fresh weights and $P<0.001$ for both shoot dry and fresh weights) (Figure 2.10a and b). Mean shoot fresh and dry weights (1.89 and 0.36 g, respectively) and root fresh and dry weights (3.23 and 0.41 g, respectively) were lowest on plants grown in soils with two successive years of wheat ($P<0.001$ for shoots and $P<0.05$ for roots). In general, roots had higher mean fresh and dry weights than shoots except for year 0, when the shoot mean dry weight (0.5g) was higher than that of roots (0.47g) (Figure 2.10b). There was no significant variation ($P\geq0.05$) between soils of the same wheat cropping histories for plant dry and fresh weights.

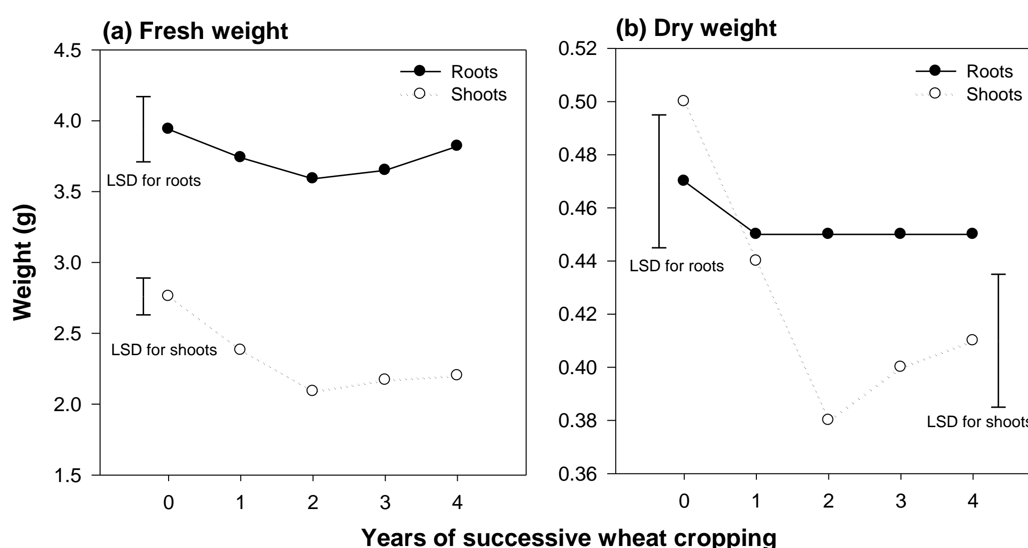

Figure 2.8 Mean (a) fresh and (b) dry weights of plants grown in the 29 test soils naturally infested with *Ggt*. Error bars are the least significant differences (LSD) at the 5% level (df = 114).

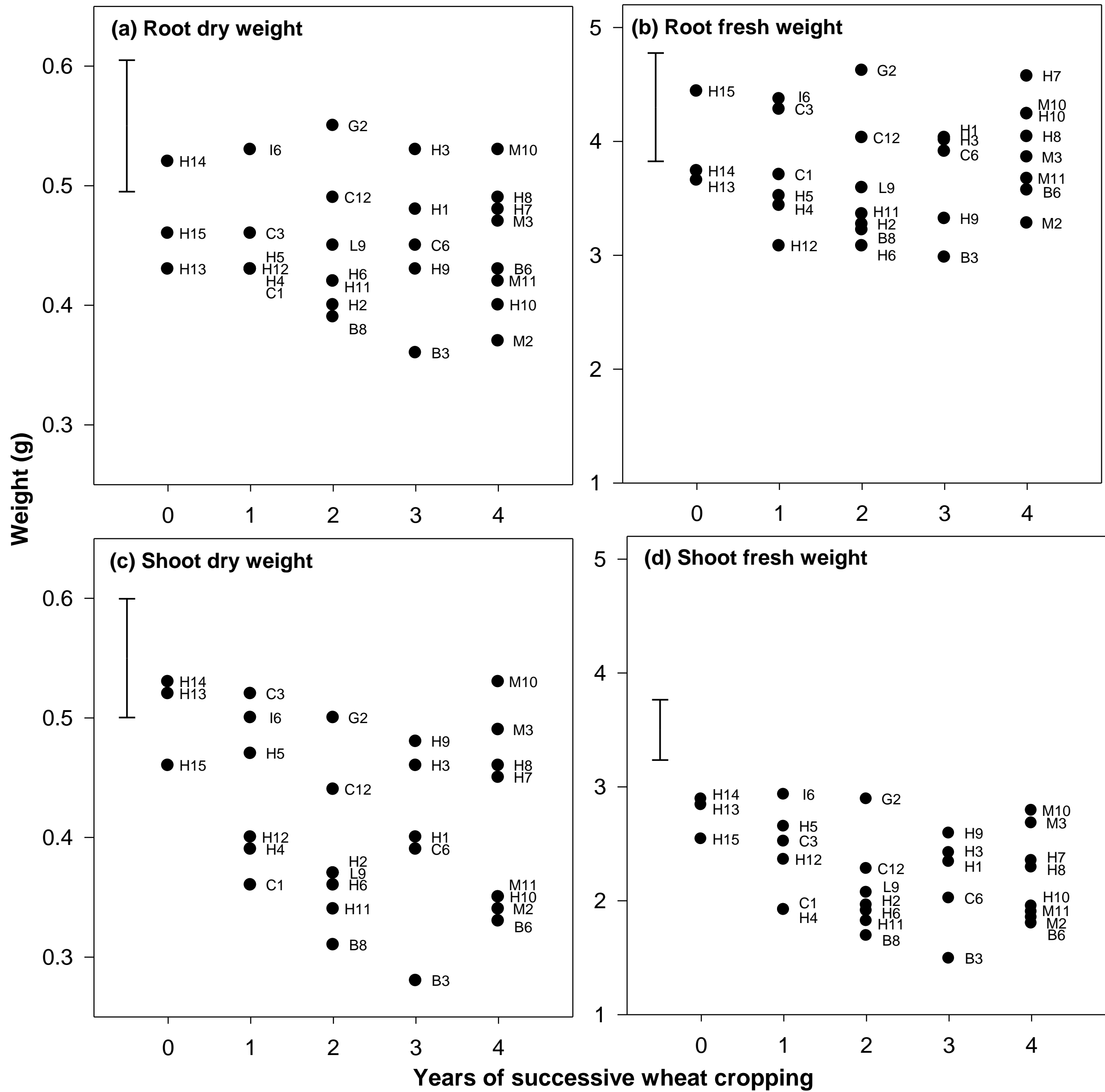

Figure 2.9 Mean weights of plants grown in different soils of the same wheat cropping histories (a) root dry; (b) root fresh; (c) shoot dry; and (d) shoot fresh weights. Error bars are the least significant differences (LSD) at the 5% level (df = 114).

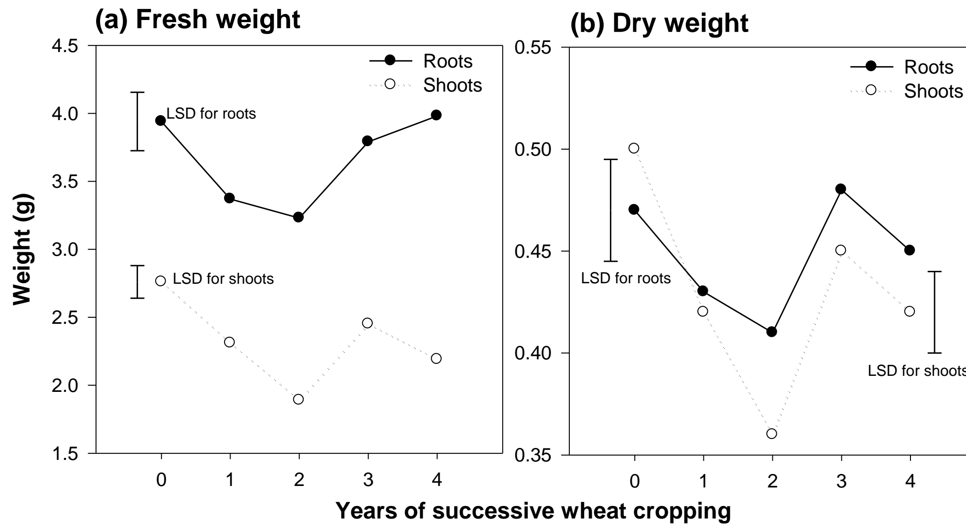

Figure 2.10 Mean (a) fresh and (b) dry weights of plants grown in the 15 St Andrews soils. Error bars are the least significant differences (LSD) at the 5% level (df = 114).

2.5 Discussion

The mean *Ggt* DNA concentrations for the 29 soils used in this study did not show a clear trend of increase with the increasing years of successive wheat crops (Figure 2.2). This was probably due to the large variations between soils from different regions. Many soil factors such as nutrition, physical properties, texture, structural stability and constitution, which may affect available water capacity and water release characteristics, can also affect plant growth and take-all severity (McLaren & Cameron 1996; Hornby *et al.* 1998). In this study, the test soils did vary in soil types, textures and water holding capacity at FC (Appendix 2, Appendix 5, A5. 1). However, their water release characteristics and so the amount of water available to the plants, were unknown. In addition, soils from different sites might have differential rates of decline in their *Ggt* inoculum concentrations depending on the environmental conditions and the time of soil sampling. For instance, Bithell *et al*. (in press: 2009) investigated the changes in *Ggt* DNA inoculum concentrations in 10 New Zealand second year wheat fields after crop harvest and reported monthly decreases of inoculum from 350 to 103 pg g^{-1} of soil from harvest. They attributed the decline of inoculums was related to the breakdown of viable *Ggt* infested propagules/host residues in the soil after crop harvest. Disease incidence in subsequent crops might also be affected by other factors such as variation of aggressive *Ggt* strains

between soils, and the gradual reduction and replacement of the aggressive strains by the less aggressive strains (Shipton 1977; Lebreton *et al.* 2004).

In this study, additional assessment of the microbial biomass C concentrations (Table 2.2), which represented both the active and inactive microorganisms (Sparling 1997), were not significantly affected by the increasing successive wheat crops (Table 2.2), and were poorly correlated to the *Ggt* DNA concentrations in the 29 soils (r = 0.49). According to Deacon (1997), some of the different microorganisms present in the soils, could suppress the disease to some extent by antagonising or competing with *Ggt* to reduce/prevent infection of plant roots. However, direct measurement of the microbial biomass C levels in the soils, would not give an indication of the different groups of microorganisms, and the microbial activities (Nsabimana *et al.* 2004) present. This was because other parameters, such as CO_2 respiration and substrate utilisations, were not investigated. Therefore, the direct relationship between microbial biomass C and individual microbial populations cannot be determined. Future work will have to investigate the different populations of the microorganisms in soils cropped with different years of successive wheat crops.

For the 15 St Andrews soils, the mean *Ggt* DNA concentrations increased during y 0-1, remained constant during y -3 and increased after y 3 (Figure 2.2), results which are similar to those reported by Cromey *et al.* (2004), who used *Ggt* DNA concentrations instead of disease incidence or severity to explore the potential existence of TAD in New Zealand. As wheat is the most susceptible cereal crop to take-all (Scott 1981), it is expected that growing this crop successively, will result in carry-over of *Ggt* inoculum at a maximum level, into the next crop (Cromey *et al.* 2004). However, the phenomenon of TAD, which has been reported to occur after 4-6 successive wheat crops in the fields (McSpadden Gardener & Weller 2001; Mazzola 2002; Weller *et al.* 2002), was not observed from analysing the *Ggt* DNA data of the 15 St Andrews soils in this study. Similar to the 29 soils, additional analysis of the 15 St Andrews soils, also showed poor correlation between the *Ggt* DNA concentrations and the microbial biomass C (r = 0.49).

Although the 29 soils varied in their original *Ggt* DNA concentrations due to site variations, they were still useful in studying the effects of a single wheat crop in 2003/04 on *Ggt* DNA concentrations. For these 29 individual soils, the *Ggt* DNA concentrations increased for nearly all the soils in the first year of wheat cropping (Figure 2.3a-d). This was expected as most of the preceding crops grown in the fields (Table 2.1) were

susceptible to *Ggt*, thus providing sources of inoculum for the wheat crop (Nilsson & Smith 1981; Chng *et al.* 2005). Most soils in their second, third and fourth successive years of wheat cropping showed stable or decreasing *Ggt* DNA concentrations (Figure 2.3), suggesting that suppression of *Ggt* could have already begun to occur in these soils.

The initial aim of the current pot experiment was to investigate the potential differences in suppressiveness between the soils. However, given that the roots of all the plants grown in the inoculated soils were severely infected, it is likely that this inoculum dosage was too high to detect any potential differences in suppressiveness between the soils. Past research on screening for suppressive soil was mainly carried out by introducing uniform amounts of *Ggt* inoculum into samples of suspected suppressive soils, to determine whether the soils had the capacity to inhibit the pathogenic activity of *Ggt* (Hornby 1983). The putative suppressive soil was either mixed with a sterilised (steamed, autoclaved or fumigated) base soil, sand or potting media (Pope & Jackson 1973; Shipton *et al.* 1973; Pope & Hornby 1975; Andrade *et al.* 1994), or was tested on its own (Shipton *et al.* 1973; Pope & Hornby 1975; Andrade *et al.* 1994). Both of these methods were capable of determining the differing infectivity between the soils. However, if a test soil is suppressive, the addition of a small amount of it (1% w/w) into a non-suppressive soil (i.e. sterilised soil) will transfer its suppressive factor, resulting in the non-suppressive soil becoming suppressive (Wildermuth 1980). In the current study, the soils were used on their own mainly because of the impracticability of transferring 29 test soils into a sterilised base soil, which presumably would be required in a huge amount due to the pot replicates necessary for the experiment. The *Ggt* inoculum dosage used (equivalent to 12.5%) was recommended by Hollins *et al.* (1986) for investigating the relative resistance of various grasses to take-all, and was clearly too great for this wheat trial. A separate trial to determine a suitable inoculation dose for optimum disease expression, and detecting potential soil suppressiveness was later set up (Chapter 3).

In the pot experiment, the significant difference found in the mean percent infected roots of the 29 soils, which included ryegrass (y 0) soils and soils with successive wheat cropping histories (Figure 2.4a), might be due to the low *Ggt* DNA concentrations (nearly 0 pg g^{-1} soil) in the ryegrass soils. Since ryegrass is putatively less susceptible to *Ggt* (Nilsson & Smith 1981; Chng *et al.* 2005), these soils would have less inoculum in the pot trial. The poor correlations between the successive wheat crops and *Ggt* DNA concentrations, and

between the *Ggt* DNA concentrations and disease incidence, indicate that soil characteristics can complicate any causative relationships between the factors. There was also a possibility that the natural *Ggt* inoculums present in different soils might vary in their virulence and aggressiveness and the subsequent infectivity as well (Cunningham 1981).

For the 15 St Andrews soils, the mean percent infected roots also increased with successive wheat crops (Figure 2.4b). A similar trend was also reported at Rothamsted in the UK in research done from 1989 to 1994 on a wheat field naturally infested with *Ggt* with TAD happening after 4 y of successive wheat (Hornby *et al.* 1998). The variations in percent infected roots (Figure 2.5b) between different soils that had the same successive wheat cropping histories were, however, insignificant when compared to the same analysis for the 29 soils, indicating a lesser effect of soil physical properties in this case.

Despite the strong correlation between the mean *Ggt* DNA concentrations, and the mean percent infected roots (r = 0.76), it was not possible to confidently claim that the DNA-assay was able to accurately predict the disease levels from the pot experiment. This was mainly because of the poor correlation between the raw data of *Ggt* DNA concentrations and the raw data of percent infected roots (r = 0.36). These results agree with those of Herdina & Roget (1999; 2000), who reported poor predictions between the two parameters in their publications (R^2 = 0.43 and 0.63, respectively). They concluded that the problems could be attributed to insufficient soil samples collected from each field, and the difficulty in differentiating soils with low *Ggt* DNA concentrations from those with medium *Ggt* DNA concentrations. In the present study, all the plants were harvested at an early growth stage (GS15) (Tottman 1987) (after 28 d), and so it is anticipated that they were still epidemically at their primary state of infection, which may have contributed to the poor correlation between the *Ggt* DNA concentrations and the percent infected roots. Bailey & Gilligan (1999) investigated the dynamics of primary and secondary infection in take-all epidemics in a pot experiment, and predicted that plants grown at 15°C in a growth chamber, would begin secondary infection after 170 d. Their results also showed that the proportion of infected roots, both seminal and adventitious, would only reflect the levels of initial infective inoculum after about 380 d of growth, which was 30-50 d beyond the expected harvesting time.

To measure the capacity of soils to suppress the pathogen, most researchers compared the disease incidence or severity with that of a known disease suppressive soil, which had been in monoculture of wheat for 5 or more years, and had been observed to express the symptoms of TAD (Pope & Jackson 1973; Shipton *et al.* 1973; Wildermuth 1980; Cook *et al.* 1986; Simon *et al.* 1987). As TAD has not been reported in New Zealand, it was impossible to include a known TAD soil as a suppressive soil control in the present study. Hence, comparison was made between the differences in *Ggt* DNA concentrations from pre-sowing to after crop harvest in the field, with the mean percent infected roots of plants grown in the pots of individual soils (Figure 2.7). According to Baker & Cook (1974), soils showing diminished pathogen and declined disease incidence/severity during continued monoculture of wheat, could be undergoing TAD, therefore, 11 out of the 29 soils screened in this study could be potential take-all suppressive soils. Separate studies will have to be conducted to confirm their suppressiveness and the suppressive mechanisms (Chapters 5 and 6).

Plants grown in the 29 soils had decreasing shoot dry and fresh weights for the first 0-2 y and remained constant after the second year of successive wheat crops (Figure 2.8), indicating poorer plant growth in these soils. However, there were large variations in the root and shoot weights of plants from soils of different origins.

For the 15 St Andrews soils which had similar soil type, texture (Appendix 2) and physical properties, the fresh and dry weights (roots and shoots) better-reflected different successive wheat cropping histories. In these soils, the lowest above and below ground dry and fresh weights were found in plants grown in soils cropped with 2 y of successive wheat, with increases from y 3 soils and smaller decreases from y 4 soils (Figure 2.10). The reason behind this trend was unclear as there were no direct relationships between the shoot and root dry and fresh weights and the disease incidences ($r = 0.02$, 0.04, 0.2 and 0.20, respectively) or the *Ggt* DNA concentrations in the field soils before the pot trial ($r = 0.06$, 0.03, 0.01 and 0.02, respectively). Hornby (1981) also reported similar results with yield not being consistently related to soil inoculum levels or the incidence of disease. However, the moderate correlations between the root and shoot dry weights and between the root and shoot fresh weights, had indicated that the two growth components (roots and shoots) were greatly dependent on each other. In this trial, the soils were also very dry at the time of

harvest suggesting that watering once weekly might have been insufficient for growth of plants and might have not been ideal for optimising disease infection and expression.

Results in this chapter indicated that the 29 soils were more variable than the 15 St Andrews soils, and so less able to demonstrate effects of years of successive wheat cropping on disease progress, plant growth and natural *Ggt* DNA concentrations. This was probably due to the complex interactions between *Ggt* DNA concentrations, soil physical properties and the microbial activities in the different soils from different sites. Results from the St Andrews soils showed that both disease incidence and natural *Ggt* DNA concentrations increased with successive wheat crops. The TAD phenomenon was not observed in this analysis. However, when differences in *Ggt* DNA concentrations from field soils taken pre-sowing and after the last harvest were examined, most soils of 2, 3 and 4 y of successive wheat crops had more reduced *Ggt* DNA concentrations (195-2911 pg g^{-1} soil) than 1 y wheat soils, which had increased *Ggt* DNA concentrations (41-1488 pg g^{-1} soil). Even though there was a possibility that natural biological suppression on *Ggt* could be taking place from before and after crop growth, the likelihood of *Ggt* inoculum reductions in the soils due to seasonal changes, management practises before planting, and the breaking down of host residues for inoculums survival, cannot be dismissed. The individual soils from successive wheat cropping that had reduced *Ggt* DNA concentrations and low disease incidence (11 of the 29 test soils) were considered as potential take-all suppressive soils. More studies are required to confirm their suppressiveness and the suppressive mechanisms involved (Chapters 5 and 6).

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Chapter 3
Inoculation of soils with different levels of *Gaeumannomyces graminis* var. *tritici* to detect differing take-all suppressiveness

3.1 Abstract

The practice of introducing the pathogen, *Gaeumannomyces graminis* var. *tritici* (*Ggt*) into soils (as inoculum grown on oat grains, sand/maizemeal mixtures or in the form of infested soil residues), to screen for take-all suppressiveness has been widely used in field or laboratory trials but the amounts of *Ggt* inoculum reported have varied greatly. In our study, the effects of adding sand/maizemeal inoculum to soil at five different levels of *Ggt* (0, 0.2, 0.5, 1 and 4% w/w) were investigated in a pot assay using wheat plants. Three soils with different cropping histories (5 y ryegrass, 8 and 2 y wheat) had different concentrations of *Ggt* DNA (0, 200 and 1126 pg g^{-1} soil, respectively), and, therefore, represented soils likely to have different magnitudes of putative take-all suppressiveness (i.e. non-suppressive non-wheat, potentially suppressive wheat and non-suppressive wheat soils). After 4 wk growth at 19°C, plant assessments showed that introducing 4% w/w of *Ggt* inoculum effectively differentiated the suppressiveness among the soils ($P<0.01$). The take-all root incidences were 83, 69 and 81%, respectively. The 4% (w/w) inoculum rate did not reduce root growth of the wheat plants, and is therefore an appropriate level for investigating take-all suppressiveness in different soils.

Keywords: *Gaeumannomyces graminis* var. *tritici*, *Ggt*, take-all, inoculum, suppressive soils, DNA, plant growth

3.2 Introduction

The role of soils in suppressing wheat take-all, caused by the pathogenic fungus *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (*Ggt*), has been the subject of investigations by many researchers. This is particularly true with take-all decline (TAD), which is one of the most thoroughly studied of all suppressive soils (Hornby *et al.* 1998). TAD can develop during continuous wheat monoculture for 4 to 6 y (McSpadden Gardener & Weller 2001). It usually takes at least one severe outbreak of take-all, after which the disease may spontaneously reduce, resulting in increased yield (Weller *et al.* 2002).

Traditionally, assessment of take-all suppressiveness has been done by growing susceptible wheat plants in pots of the soil. Their subsequent symptoms indicate the soil's suppressive properties, without any need to amend the soil (Rovira & Wildermuth 1981). The control plants, which provided contrasts to the treatments, are usually grown in autoclaved or sterilised soil that had *Ggt* added to it. However, the problem with using un-amended soil is that one cannot determine whether the low disease levels are due to low inoculum potential (ability to cause an infection), or high suppressiveness (Rovira & Wildermuth 1981), a problem encountered in Chapter 2 as well.

The addition of *Ggt* inoculum to the soil may overcome this problem (Lester & Shipton 1967) provided that the added inoculum is made up of propagules with uniform size that are able to cause uniform and reproducible levels of disease on the roots (Simon *et al.* 1987). However, the amount of *Ggt* inoculum reported in the literature for screening take-all suppression properties has differed, with different types of culture media also being used. For instance, oat kernel inoculum has been used at 0.1% (Wildermuth 1982a, 1982b; Andrade *et al.* 1994a, 1994b), 0.15 and 0.45% (Weller *et al.* 1985), whilst ground oat was used at 0.1% (Wildermuth & Rovira 1977; Wildermuth 1980) and 0.5% (Hiddink *et al.* 2005), and a sand and wheat meal mixture at 20% (Lester & Shipton 1967). The need for a suitable *Ggt* inoculum concentration in the screening process for suppressive soil was also identified in Chapter 2. This research investigated the effects of soil amendment with various *Ggt* inoculum levels on plants that were grown in a range of soils previously selected for their differences in putative take-all suppressiveness, so that a suitable inoculums level could be determined and used in suppressive screening in the future.

3.3 Materials and methods

3.3.1 Soil sampling and processing

The three soils H15, P7 and R1 were collected from fields with different wheat cropping histories in St Andrews, Methven and Ashburton regions respectively of the South Island, New Zealand. Soil H15, which was included in a previous study (Chapter 2), was collected in March 2004 from a field cropped with 5 y of *Lolium perenne* L. (perennial ryegrass). This soil therefore represented a putative non-suppressive soil control that was not naturally infested with *Ggt*. Both P7 and R1 soils were collected in Nov 2004 from wheat fields known to have a history of take-all. Soil P7 was from a field cropped with 8 y of wheat, and so represented a soil with potential take-all suppressiveness that was naturally infested with *Ggt*. Soil R1 was

from a field cropped with 2 y of wheat, and so represented a putative non-suppressive soil that was naturally infested with *Ggt*.

Ten soil blocks of 10 cm × 10 cm × 10 cm were collected from each field using a spade to cut into the soil surface. The ten sampling points (providing at least 10,000 cm^3 of soil) were spaced within a 1 ha zone of the field along a 'W' pattern (Van Elsas *et al.* 2002). The soil from each field was placed in two 30 L bins, partially dried at 25°C overnight and then passed through a 4 mm sieve to homogenise the soil. Any stone caught in the sieve was discarded, while crop debris, which was considered as part of the inoculum, was returned to the soil. Sieved soils from each field were pooled and mixed thoroughly on a tarpaulin and then stored at 4°C in 30 L bins until use.

Samples were taken from each mixed soil for analysis; a 300 g sample was sent to SARDI for *Ggt* DNA analysis, and 150 g samples were sent to R.J. Hill Laboratories Ltd., Hamilton, New Zealand and to Crop & Food Research Ltd., Lincoln, New Zealand, for basic soil physico-chemical analyses. The methods on *Ggt* DNA quantification and other physico-chemical properties of the soils were outlined in Chapter 2. Tension tables were used to determine the moisture contents (MC) of all the soils at field capacity (FC) as described by Klute (1986) (Appendix 3, A3. 5). Table 3.1 shows the site information of the three soils including cropping histories, soil types, and textures, and *Ggt* DNA concentrations. Test results on the physico-chemical properties of the soils are presented in Table 3.2.

3.3.2 *Ggt* inoculum production

A mixed *Ggt* inoculum was made up of equal proportions of the five isolates H11T3 R1/3, A3SL4, H9T3 R1/1.2, Biomill1SC3 and BIO3, which were from the culture collections at the New Zealand Institute for Crop & Food Research Ltd. (Lincoln, New Zealand). Each culture was grown in a sand / maizemeal mixture, as described by Hollins *et al*. (1986), except that the growing medium was prepared in clear autoclavable bags (36 x 48 cm, capable of withstanding temperatures up to 150°C, Raylab NZ Ltd., Auckland, New Zealand) instead of conical flasks. Each bag had a 10 cm long, 5 cm diameter aluminium tube attached in the opening using rubber bands, and this was sealed by plugging with cotton wool and covering with foil. The medium was then thoroughly shaken and autoclaved at 121°C for 1 h.

Once the sterilised medium was cooled, each bag was inoculated with 40 potato dextrose agar (PDA) discs (8 mm diameter) cut from the actively growing edge of an 8 d old *Ggt* isolate culture. The inoculated medium was then shaken by hand before incubating at 23°C for 3 wk

and shaken weekly. Prior to use, the five different *Ggt* isolates grown in separate bags of sand/maizemeal media were pooled and mixed thoroughly in one of the bags.

3.3.3 Pot experiment

The pot experiment was set out in a two-way randomised block design consisting of three soils amended with *Ggt* inoculum at 0, 0.2, 0.5, 1 and 4% weight by weight (w/w) with four replicates of each treatment combination. Soil amendment was carried out by hand mixing the required amount of *Ggt* inoculum with the soil to give a final weight of 300 g of soil mixture per 400 mL pot.

Each pot was planted with four pre-germinated healthy wheat seedlings (cultivar Regency, line 03/W/01P) (Appendix 3, A3. 3) and received 20 mL of a nutrient solution to provide adequate nutrients in the soil (N = 150, P = 50, K = 100 and S = 20 µg g^{-1} soil) (Appendix 3, A3. 4). All the pots were then watered to FC (− 5 kPa) by weight (Appendix 3, A3. 5 and Appendix 5, A5. 1) and incubated in a growth chamber (Conviron, Controlled Environments Ltd., Canada) for 4 wk. The growth chamber was maintained at 19°C with alternate 12 h light/dark photoperiods, photosynthetic photon flux density (PPFD) of 375 µmol$s^{-1}m^{-2}$ at pot height (10 cm) and 80% relative humidity (RH). All the pots received water to their FC by weight twice per week.

3.3.4 Disease assessment and other measurements

Roots were washed carefully in a bucket of tap water and assessed for infection, being classed infected if they had at least one take-all lesion per root axis. It was assumed that the 4 wk duration of growth would allow primary infection, and so the extent of take-all lesions on the roots (severity) between plants from different soils would not be that great. Therefore, disease assessment was conducted by counting the numbers of healthy and infected root axes on both seminal and nodal root systems. Numbers of healthy and infected root axes on both seminal and nodal root systems were recorded and percent infected roots per pot were then calculated using the formula:

$$\text{Infected roots (\%) per pot} = \left(\frac{\sum (\text{Number of infected root axes per plant})}{\sum (\text{Number of root axes per plant})} \right) \times 100\%$$

Table 3.1 Field location and cropping history, as well as some properties of the three soils used in the pot experiment.

Field code	GPS Location	Soil type	Soil texture	[1]Preceding crops 1997/98	1998/99	1999/00	2000/01	2001/02	2002/03	2003/04	2004/05	Years of wheat	*Ggt* DNA pg g^{-1} soil	[2]Risk	Moisture content (% W/W) Original	At FC
H15	S44°30.5', E171°06.5'	Claremont silt loam	Silt loam	-	-	Rg	Rg	Rg	Rg	Rg	Rg	0	0	-	18.51	34.69
P7	S43°44.3', E171°38.6'	Mayfield silt loam	Silt loam	W	W	W	W	W	W	W	W	8	200	Medium	10.85	30.74
R1	S44°02.5', E171°43.3'	Templeton silt loam	Silt loam	-	-	-	P	W	Rg	W	W	2	1126	High	20.49	28.38
															$P<0.05$	$P<0.005$
															LSD = 7.46	LSD = 2.42

[1]Preceding crops: Ryegrass (RG)
Wheat (W)
Pea (P)

[2]Take-all risk categories developed by SARDI for Australia at the time of quantitative DNA analysis of *Ggt* in 2003/04:

Low risk: 5-130 pg *Ggt* DNA g^{-1} of soil
Medium risk: 131-325 pg *Ggt* DNA g^{-1} soil
High risk: >325 pg *Ggt* DNA g^{-1} soil

Table 3.2 Physico-chemical properties of the three soils used in the pot experiment.

Field code	Normal range	5.3-6.1	20-30	0.5-0.8	5.0-12.0	0.8-3.0	0-0.5	12-25	50-85	Min N
		pH	Olsen P (mg L^{-1})	Potassium ([1]me 100 g^{-1})	Calcium (me 100 g^{-1})	Magnesium (me 100 g^{-1})	Sodium (me 100 g^{-1})	[2]CEC (me 100 g^{-1})	Base Saturation (%)	µg g^{-1} soil
H15		6.6	21	0.2	11.5	1.12	0.2	15	87	15.35
P7		6.1	26	0.47	12.5	0.78	0.19	18	78	57.48
R1		6.4	48	1.07	9.6	1.98	0.42	16	83	36.33

[1]milliequivalents

[2] cation exchange capacity

Fresh and dry weights of both roots and shoots were obtained before and after drying them at 70°C for 24 h. Since water uptake by the plants was also considered to reflect their growth and disease symptoms, the total for each pot over the trial was calculated from the amounts added at each watering time.

3.3.5 Statistical analyses

Statistical analyses of the percent infected roots, root and shoot dry and fresh weights and total water uptake were analysed using two-way Analysis of Variance (ANOVA). ANOVA tables are shown in Appendix 5, A5. 3.

3.4 Results

3.4.1 Disease incidence

Plants grown in soils amended with *Ggt* inoculum had significant increases in mean incidence of infected roots with increasing *Ggt* inoculum levels ($P<0.001$) (Figure 3.1). The highest incidence of root infection (77.4%) occurred in plants grown in soils amended with 4% of *Ggt* inoculum. There was evidence of soil origins causing a significant difference on root infection ($P<0.01$) (Figure 3.2), and of them interacting with the *Ggt* inoculum levels ($P<0.05$) (Figure 3.3). From 0 to 1% added *Ggt* inoculum, incidence of infected roots significantly increased in all the soils. At 1% added *Ggt* inoculum, soil H15 had the highest incidence of infected roots, with soils P7 and R1 having significantly lower incidence. However, at 4% added *Ggt* inoculum, incidence of infected roots was the lowest in soil P7 and highest for soils H15 and R1.

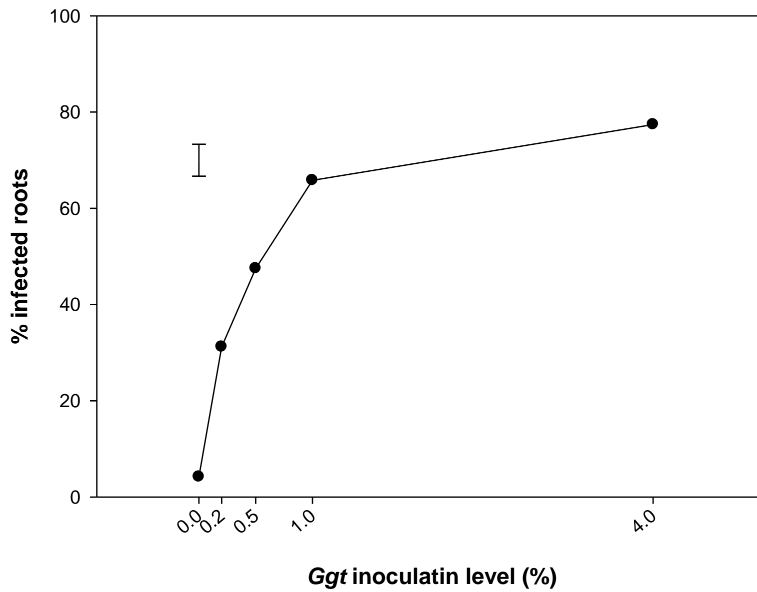

Figure 3.1 Mean incidence of infected wheat roots in soils with different levels of added *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59).

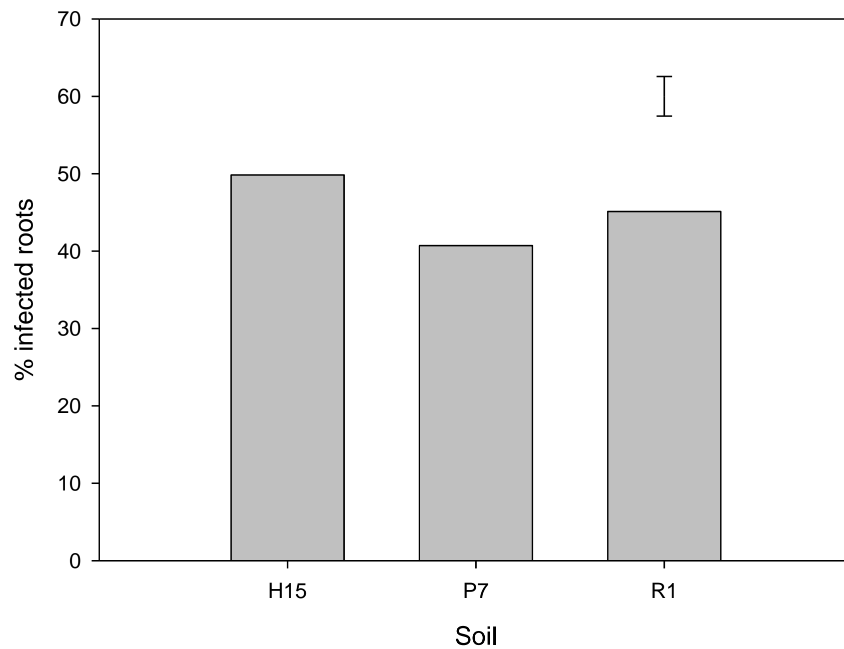

Figure 3.2 Mean incidence of infected wheat roots in the three soils treated with different levels of *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59).

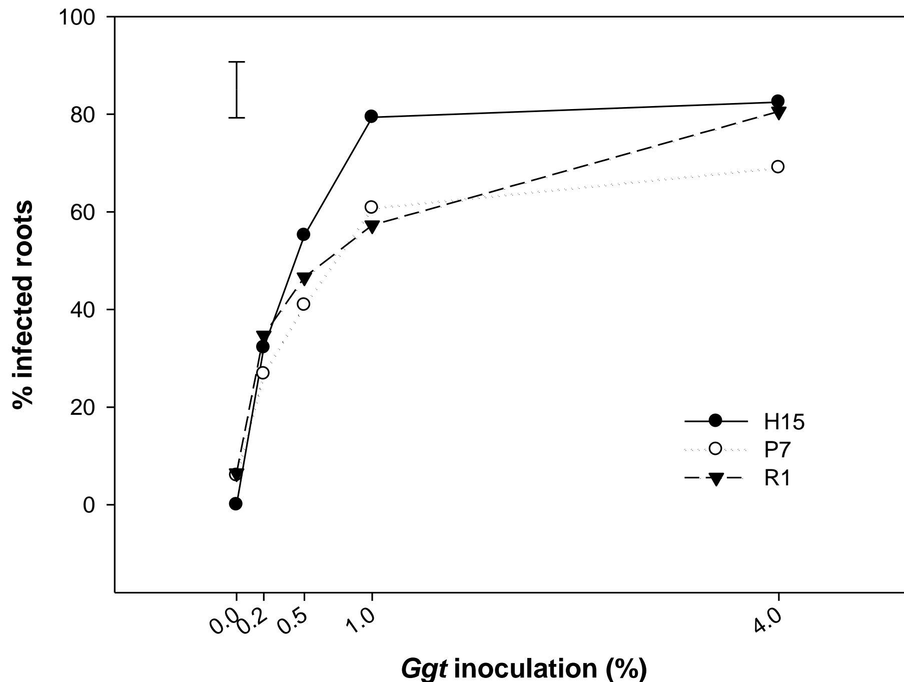

Figure 3.3 Variations in incidence of infected roots on wheat plants grown in the three soils treated with different levels of *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (d.f. = 59).

3.4.2 Plant dry and fresh weights

Overall, root and shoot weights were greater in nil or low inoculum treatments than in high inoculum treatments (Figures 3.4 a and b). However, differences were significant only for shoot dry ($P<0.001$) and root fresh ($P<0.01$) weights with the lowest (0.63 g and 3.1 g, respectively), being at 4% added inoculum.

There was significant variation between different soils in dry and fresh weights of both roots ($P<0.01$ and $P<0.001$, respectively) and shoots ($P<0.001$ for both) (Figure 3.5), with consistent trends of plants in soil R1 having the lowest root and shoot dry and fresh weights, which were significantly different ($P<0.05$) to plants in soil P7, which had the highest weights. There was however no significant difference between plants grown in soils P7 and H15 in their root and shoot dry weights, whereas, plants grown in soils H15 and R1 were similar and lowest in their root and shoot fresh weights. There was no interaction effect on plant dry and fresh weights between the different soils and the levels of added *Ggt* inoculum.

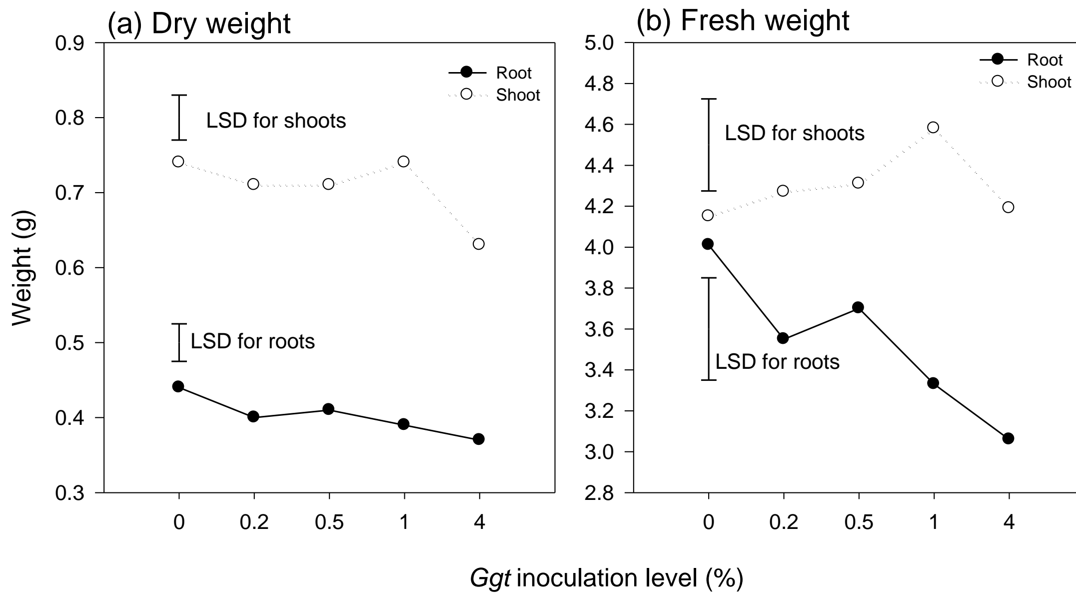

Figure 3.4 Mean weights of plants grown in soils amended with different levels of *Ggt* inoculum (a) root and shoot dry and (b) root and shoot fresh weights. Error bars are the least significant differences at the 5% level (d.f. = 59), and apply only to the individual plant variables.

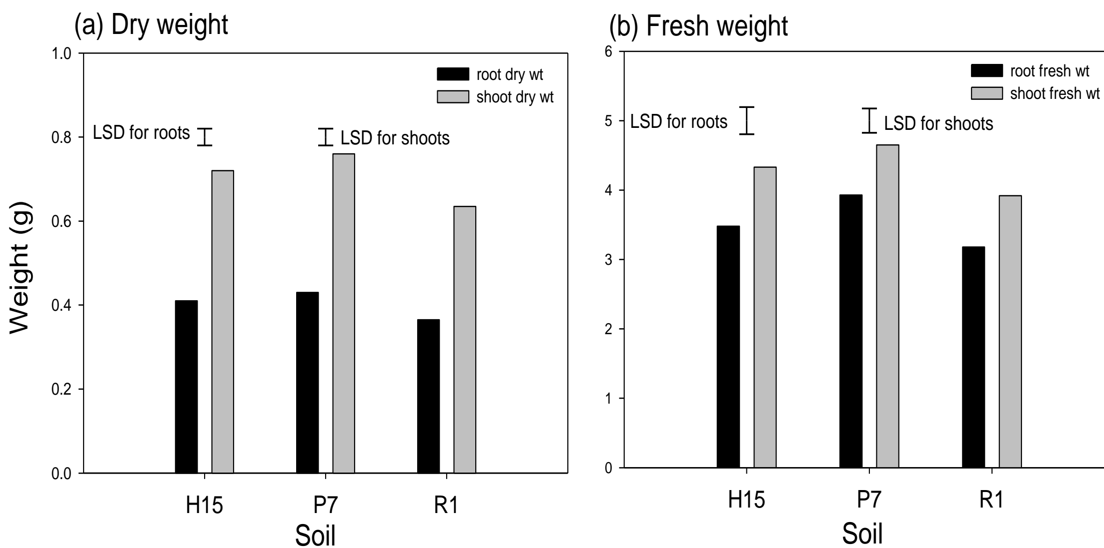

Figure 3.5 Root and shoot (a) dry and (b) fresh weights of plants grown in the three soils amended with different levels of *Ggt* inoculum. Error bars are the least significant differences (LSD) at the 5% level (d.f. = 59), and apply only to the individual plant variables.

3.4.3 Water uptake

Mean total water uptake by the plants decreased significantly with increasing levels of added *Ggt* inoculum ($P<0.001$) (Figure 3.6), with total water uptake being lowest (299 g) for plants grown in soils amended with 4% *Ggt* inoculum. Different soils also varied in their total water uptake ($P<0.01$) (Figure 3.7). Least water was used by plants grown in soil R1 (276 g). There was however, no difference in total water uptake between plants grown in P7 and H15. There was no interaction effect on the amount of total water uptake by the plants between different soils and the levels of added *Ggt* inoculum.

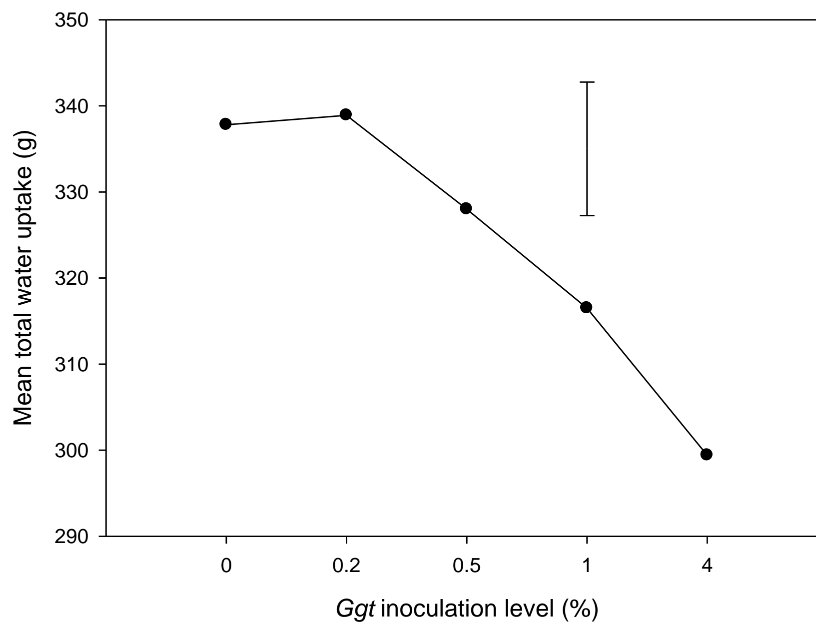

Figure 3.6 Mean total water uptake for plants grown in soils amended with different levels of *Ggt* inoculum. Error bar is the least significant difference at the 5% level (d.f. = 59).

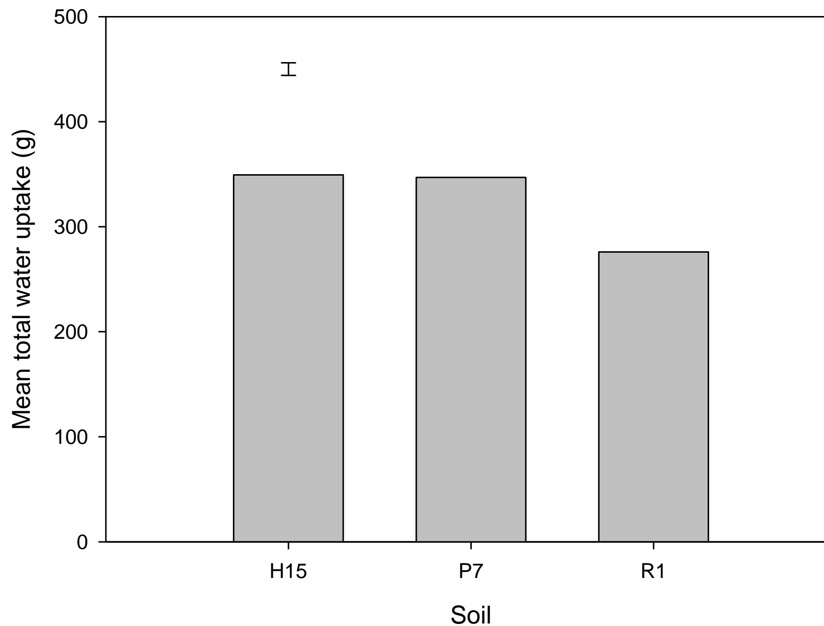

Figure 3.7 Mean total water uptake for plants grown in the three soils amended with different levels of *Ggt* inoculum. Error bar is the least significant difference at the 5% level (d.f. = 59).

3.5 Discussion

Microbial activities in the soils are believed to be responsible for the onset of TAD (Rovira & Wildermuth 1981; Weller *et al.* 2002; Cook 2003), with the magnitude of suppression being largely dependent on the amount of the pathogen relative to the natural antagonists present in the soil (Cook 2003). In the current research, the main objective was to introduce different levels of *Ggt* inoculum into soils with differing magnitudes of putative suppressiveness to develop a pot trial protocol that could determine whether the soils had the capacity to inhibit the pathogenic activity of *Ggt* (Hornby 1983).

The increasing disease incidence found with increasing *Ggt* inoculum levels agreed with results reported by Shipton *et al.* (1973), who compared a range of inoculum levels (4%, 17%, 50% and 91% v/v) in a form of milled oat kernels and sand (1.9 v/v), introduced into three different soils to test for the suppressive properties of the soils. They reported that lowest numbers of infected plants consistently occurred in soils amended with 4% *Ggt* inoculum, and that higher inoculation rates appeared to swamp the antagonists present in the suppressive soils.

In the current study, mean total water uptake was reduced with increasing added *Ggt* inoculum but root dry weight was not affected by the inoculum, suggesting that the function of the roots in water uptake had been impaired (pathogen-induced water stress). The decreasing root fresh weight with increasing *Ggt* inoculum also indicated inhibition of the root system in absorbing water. According to Pillinger *et al.* (2005), either a reduction in the size of the root system, or a reduction in the efficiency of uptake function by the roots can reduce the root system's ability to supply the plants with adequate water and nutrients. However, Asher (1972) showed that infected root systems could translocate a greater proportion of their total assimilates to produce extensive systems of adventitious roots, thereby compensating for infection. As root systems were not partitioned into primary and adventitious roots in this study, the potential increase in numbers of adventitious roots, which may have grown to compensate for the infected root system, was unknown. Besides altering root morphology, infection may also reduce leaf expansion and therefore the total shoot dry weight (Ayres 1978). In this study, this reduction of shoot dry weights was only apparent with 4% added *Ggt*, suggesting that this concentration was the most suitable level for inducing water stress in plants, and for distinguishing the suppressive levels of the different soils (Figure 3.4). However, as inoculum levels higher than 4% were not included in this experiment, the possibility that the higher inoculum levels could have been even better at distinguishing the differing levels of suppressiveness, cannot be dismissed.

Comparison of the three soils, which were amended with different levels of *Ggt* inoculum showed least disease in P7 (Figure 3.2). This soil was previously cropped with 8 y of wheat, and was therefore expected to exhibit greater suppression to take-all than both H15 and R1, possibly due to the greater numbers of natural antagonists in the soil (Cook 2003). Plants in H15 (with 0 pg *Ggt* DNA soil g^{-1}) had similar levels of infection to R1 (with 1126 pg *Ggt* DNA soil g^{-1}), whereas P7 had 200 pg *Ggt* DNA soil g^{-1}. This suggests that the low disease incidence in P7 was not caused by the lower natural *Ggt* inoculum present. The higher root and shoot dry and fresh weights of P7 plants also indicated less effect on plant growth and root functions than from H15 and R1, which were formerly cropped with 6 y of ryegrass and 2 y of wheat, respectively. The disease incidence at 0% *Ggt* inoculum did not reflect the natural *Ggt* DNA concentrations present in the three soils (Figure 3.3). This could be due to the reason that the natural *Ggt* inoculums present in different soils might vary in their virulence and aggressiveness and the subsequent infectivity

(Cunningham 1981). In addition, presenting disease incidence (by counting the numbers of infected roots) might not reflect the actual infectivity of the natural *Ggt* inoculums present in the soils as well. In future experiments, disease severity using a rating system (i.e. by estimating the area of root system covered with lesions) should be considered instead.

Other factors such as soil texture, structural stability, depth, available water capacity, pH and nutritional status of the soil can all affect take-all severity (Hornby *et al.* 1998). The pH (6) of all the soils in this study (Table 3.1), was considered suitable for take-all infection (Cook 1981), and nutrient status, especially N, was never a limiting factor here since the rate of 150 kg ha^{-1} was considered suitable for optimal plant growth (Hornby *et al.* 1998). Therefore, the possibility of these factors affecting disease incidence was dismissed. Mean total water uptake in H15 was similar to that in P7, which may have been due to its higher water content at field capacity (Table 3.1). Soil type is reported to affect the occurrence of take-all mainly because of its effect on soil structure and available water content (Hornby *et al.* 1998). All the soils in this study were silt loams which are mainly made up of clay and sand (McLaren & Cameron 1996), but because the fraction of each aggregate type and the soil water release characteristics were not measured, their available water capacities (i.e. the amount of water which a soil can store for plant growth (McLaren & Cameron 1996)) were unknown.

The three soils also responded differently to the added inoculum concentrations, especially at 1 and 4%. The introduction of 4% of *Ggt* inoculum into the soils was sufficient to distinguish between soils with different wheat cropping histories (P7 against R1 and H15). This result was similar to a report by Lester & Shipton (1967), who also found that on plants grown in a pot assay the lowest levels of root infection were in soils with 5 or more years of consecutive wheat or barley crops. The effective inoculum concentration of 4% found in this study agrees with Shipton *et al*. (1973), who reported that soils demonstrated high levels of suppression when amended with 4% of *Ggt* inoculum. The media used for inoculum growth in both studies were very similar with the current study using 1:7 (w/w) sand and maizemeal mixture, whilst Shipton *et al*. (1973) prepared their inoculum in a milled oat kernels and sand (1:9 volume by volume, v/v) mixture.

Other inoculum concentrations and media have also been reported. Wildermuth (1982b), Andrade *et al.*(1994b), Weller *et al*. (1985), Wilermuth & Rovira (1977), Wildermuth (1980) and Hiddink *et al*. (2005) used much lower inoculum concentrations (0.1 to 0.5%),

whereas Lester and Shipton (1967) used a much higher concentration (20%) in screening for the magnitude of take-all suppressive soils. However, comparison between these two groups is difficult due to their different methods of scoring disease. The inoculum growth medium used by the former group was either whole oat kernels or ground oats, and their mean disease scores, over a 0-8 and a 0-7 range, were 1.6 and 3 respectively (with 2 being lesions present on three or more roots, and with 3 being lesions present on all the roots). The latter group, (Lester & Shipton 1967) used sand and wheat meal mixtures as inoculum growth medium and reported plants in suppressive soils generally had 11 to 14% of their roots infected, although one of the few suppressive soils investigated, had up to 42% of roots infected.

Simon & Rovira (1985) compared plant response to *Ggt* inoculum prepared in ryegrass seeds with those prepared in millet seeds and found that millet seeds produced consistently more severe disease than the ryegrass. They explained that the larger size and weight of the millet seeds (5.3 mg vs. 1.8 mg for ryegrass) contained larger food reserves to support the infective *Ggt*, therefore causing more disease. The inclusion of inert material such as sand in the medium in the current study might dilute the food reserve for *Ggt* growth, therefore resulting in the need to incorporate a greater inoculum concentration to obtain a similar plant disease response. Clearly, the type of substrates used in the production of *Ggt* inoculum, is likely to be one of the factors determining the ultimate infectivity levels.

Other factors such as isolate pathogenicity and aggressiveness, inoculum placement and the properties of the test soil have also been reported to influence the effectiveness of the inoculum (Cunningham 1981). However, in this study, the use of five pooled pathogenic *Ggt* isolates mixed thoroughly into the homogenised soil might have gone some way to prevent such variability. In non-sterilised soils, where artificial inoculum was added, temperatures and moisture potential were also known to affect the efficacy of the assays, mainly due to the modification caused by the interaction between these factors and the microbial antagonisms (Hornby 1981). Future studies could investigate the influence of these two factors in pot bioassays used for screening take-all suppressive soils.

In conclusion, introducing 4% of *Ggt* inoculum into the soils enabled the antagonistic activities in the soils to be reflected through the differing disease incidences, and hence allowed the differentiation of soils with different take-all suppressiveness. This inoculum

concentration will be used in screening soils with differing take-all suppressiveness in future experiments (Chapters 4 and 5).

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Chapter 4
Manipulating controlled environmental conditions to maximise efficacy in screening of take-all suppressive soils

4.1 Abstract

Take-all suppressive soils have been studied worldwide with particular emphasis on the role of the soil microbiota in suppressing the pathogen, *Gaeumannomyces graminis* var. *tritici* (*Ggt*). Under controlled environmental conditions, pot bioassays that use plants as indicators of disease potential can indicate the magnitudes of suppression exerted by the microbiota in these soils. However, the environmental conditions reported to be used for pot bioassays have varied and provided different results, possibly because they were not optimal for the potential antagonistic microbes present in the soil. This research was undertaken to investigate the effects of different temperatures and watering regimes on disease expression and plant growth. The results indicated that with sieved test soils, a pot bioassay conducted in a 16°C growth chamber, with watering twice a week to field capacity (w/w), could be used to distinguish the differing magnitudes of suppressiveness between the test soils.

Keywords: *Gaeumannomyces graminis* var. *tritici*, take-all, successive wheat, DNA, inoculum, suppressive soils, take-all decline, plant growth

4.2 Introduction

Soil suppressiveness can be an important factor in the epidemiology and control of take-all in wheat, caused by the soilborne fungus, *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (*Ggt*). This phenomenon, which appears to be complex, has been the subject of many investigations with much emphasis on the properties of suppressive soils, the causes of suppression and its role in disease management. Assessment on the magnitude of suppression in the field can be difficult, mainly because of the many factors (such as climate, soil and the uneven distribution of inoculum in the soil), which may influence the level and severity of the disease (Rovira & Wildermuth 1981).

The presence of suppressiveness in a soil can be demonstrated in the laboratory by bringing together the soil, a susceptible crop and pathogen (Rovira & Wildermuth 1981; Hornby 1983). Many studies have used pot or tube bio-assays to confirm the existence

and/or magnitude of soil suppressiveness (Lester & Shipton 1967; Shipton *et al.* 1973; Baker & Cook 1974; Wildermuth 1980; Andrade *et al.* 1994a, 1994b; Hiddink *et al.* 2005).

The controlled environment conditions such as temperatures and watering regimes used for pot experiments have varied between studies. For instance, while investigating the effect of mixed and single crops on disease suppressive soils, Hiddink *et al.* (2005) conducted their pot bioassay in a greenhouse at about 20°C with watering as required (not defined). However, Shipton *et al.* (1973) and Wildermuth (1980) conducted pot bioassays in an incubator at 20°C without mentioning the watering regime. Methods for maintaining the water potential at -8 kPa were described by Wildermuth (1982), who ran a bioassay in a controlled environment room at 15°C, with a 12 h photoperiod, to investigate the effects of *Ggt* suppressive soils on other soil fungi. In all the above studies, the soils were mixed and sieved before use, but MacNish *et al.* (1973) used undisturbed soil cores in their bioassays, which were maintained at 15°C and watered to -60 kPa every second day.

Several studies have demonstrated the importance of conditions, such as temperature, water and soil aeration, on the rates of infection by *Ggt* (Grose *et al.* 1984; Wong 1984; Cotterill & Sivasithamparam 1987; Augustin *et al.* 1997). Hence, it is crucial in a pot bioassay that the stress effects (such as retarded growth) and disease symptoms expressed by the plants are not induced by the conditions under which it is conducted, but by the pathogen present in the soil. These conditions must therefore, not restrict plant growth, permit infection to take place and allow other potential antagonists in the soils to react to the introduced pathogen. In order to optimise the efficacy of the pot bioassay used for take-all suppressive soil screening, this study aimed to determine the various environmental conditions most suitable for disease expression and growth of wheat plants.

4.3 Materials and methods

4.3.1 Soil samples

Three soils P7, R1 and R1a were collected from fields with different wheat cropping histories from Methven and Ashburton in the South Island, New Zealand. Soil P7 was from a field cropped with 8 y of wheat and so potentially suppressive to take-all, whereas, soils R1 and R1a were cropped with 2 y of wheat and so represented conducive soils.

Soils P7 and R1 were collected before the 2004/05 crop harvest, in November 2004, when the crop was at booting stage [GS40, (Tottman 1987)]. Sampling, drying, sieving, soil

testing (*Ggt* DNA and physico-chemical analyses) and storage of the two soils were carried out according to the methods described in Chapter 3. Soil R1a was collected from the same field but earlier (September 2004) than R1, for a separate pot bioassay study by a different researcher who used a different growth chamber and unsieved soil. In this separate study, a much higher root infection (average 23% infected roots, unpublished data) was reported than the one reported in Chapter 3, therefore, this soil was included in the current study to investigate the effect of sieving on disease incidence. The soil was collected in much smaller sampling volumes than used for soils P7 and R1 by taking two soil cores (20 mm dia., 100 cm depth) from 25 positions (providing at least 650 cm^3), along a 'W' pattern over the whole field. The soil was placed onto a tray (40 by 25 cm) and partially dried overnight at 25°C, but was not homogenised by sieving. It was stored at room temperature (20°C) and was thoroughly hand mixed before the current pot bioassay. Since both soils were from the same field, their physico-chemical properties were similar. The *Ggt* DNA analysis for this soil was carried out before sowing of wheat crop in the field for the 2004/05 season. Table 4.1 shows the site information, cropping histories, soil types, textures and *Ggt* DNA concentrations of the soils. Soil physico-chemical properties are presented in Table 4.2. The methods on *Ggt* DNA quantification and other physico-chemical properties of the soils were outlined in Chapter 2.

4.3.2 Pot bioassay experiment

The set up of the pot bioassay was similar to that in Chapter 3, with each pot of test soil being amended with 4% (w/w) mixed *Ggt* inoculum to give a final weight of 300 g, and planted with four pre-germinated healthy wheat seedlings (Appendix 3, A3. 3). Detailed accounts on the preparation of inoculum and soil inoculation are in Chapter 3.

All the pots were then watered either by weight to field capacity (FC, at -5 kPa) twice per week (Wt), or saturated with water by continuously sitting pots in separate 2 L containers filled with 1 L of water (F). All the F Pots had four holes (5 mm diameter) drilled at the bottom to allow water uptake to the soil via capillary action. To avoid any confounding effects that nutrient solution might have on the watering treatment, no nutrient solution was applied to the pots in this study.

The experiment was conducted in three growth chambers C16, C19 and MC16. Both C16 and C19 were standard growth chambers (Conviron, Controlled Environments Ltd., Canada) maintained at 16 and 19°C respectively. MC16 was a modified growth chamber

(CoolRite, Refrigeration and Air Conditioning Ltd., Christchurch, New Zealand) maintained at 16°C. This growth chamber was also used in the separate study for soil R1a referred to above (Section 4.3.1). Only C16 and C19 were maintained at 80% relative humidity (RH), since there was no RH control for MC16. However, open containers of water situated within the growth chambers were considered likely to increase the RH in MC16 to 80% or greater. All the growth chambers were maintained at alternate 12 h diurnal light/dark photoperiods. The light intensity in different growth chambers was not measured.

The pots were laid out in randomised complete blocks with each soil by water treatment being replicated four times within each growth chamber, to give 72 pots in total. However, due to limited numbers of available growth chambers, they were not replicated. Sieving was not the main factor in the experiment, but was included for comparison between sieved R1 and non-sieved R1a soils from the same field. Set up of the pot bioassay in MC16 is shown in Figure 4.1. The experiment was conducted for 4 wk and during this period, the amount of water used over time for Wt pots was recorded, but water use over time for F pots were not measured as the pots were not sealed.

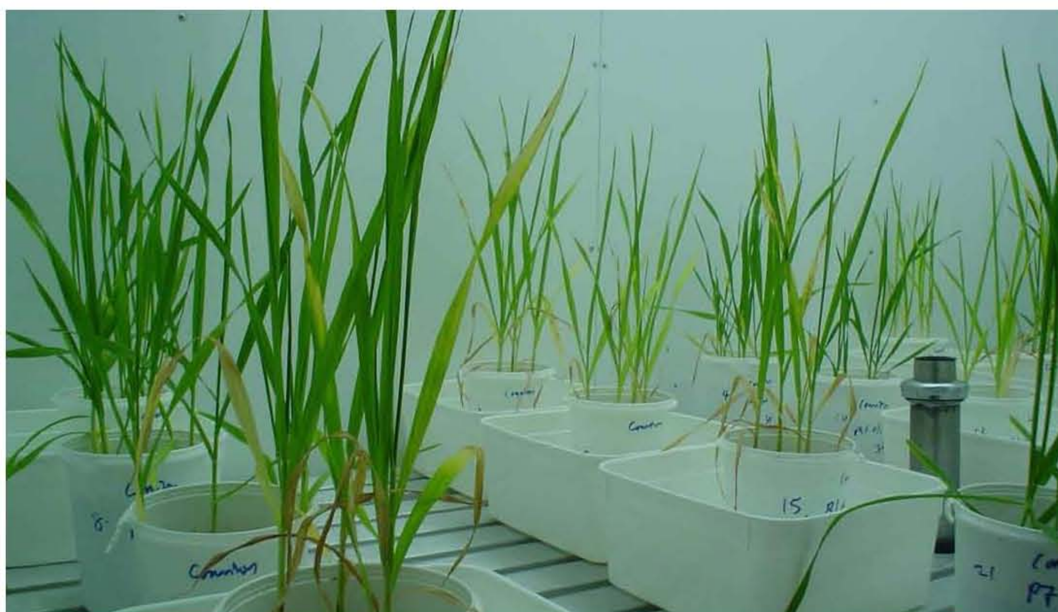

Figure 4.1 Set up of pot experiment in C16.

Table 4.1 Field location and cropping history, as well as some properties of the three soils used in the pot experiment.

Field code	GPS Location	Soil type	Soil texture	[1]Preceding crops 1997/98	1998/99	1999/00	2000/01	2001/02	2002/03	2003/04	2004/05	Years of wheat	*Ggt* DNA pg g^{-1} soil	[2]Risk	Moisture content (% W/W) Original	At FC
P7	S43°44.3', E171°38.6'	Mayfield silt loam	Silt loam	W	W	W	W	W	W	W	W	8	200	Medium	10.85	30.74
R1	S44°02.5', E171°43.3'	Templeton silt loam	Silt loam	-	-	-	P	W	Rg	W	W	2	1126	High	20.49	28.38
R1a	S44°02.5', E171°43.3'	Templeton silt loam	Silt loam	-	-	-	P	W	Rg	W	W	2	1692	High	1.66	29.70

[1]Preceding crops:

Ryegrass (RG)
Wheat (W)
Pea (P)

[2]Take-all risk categories developed by SARDI for Australia at the time of quantitative DNA analysis of *Ggt* in 2003/04:

Low risk: 5-130 pg *Ggt* DNA g^{-1} of soil
Medium risk: 131-325 pg *Ggt* DNA g^{-1} soil
High risk: >325 pg *Ggt* DNA g^{-1} soil

Table 4.2 Physico-chemical properties of the two soils used in the pot experiment.

Field code	pH	Olsen P (mg L^{-1})	Potassium ([1]me 100 g^{-1})	Calcium (me 100 g^{-1})	Magnesium (me 100 g^{-1})	Sodium (me 100 g^{-1})	[2]CEC (me 100 g^{-1})	Base Saturation (%)	Min N (µg g^{-1} soil)
Normal range	5.3-6.1	20-30	0.5-0.8	5.0-12.0	0.8-3.0	0-0.5	12-25	50-85	
P7	6.1	26	0.47	12.5	0.78	0.19	18	78	57.48
R1	6.4	48	1.07	9.6	1.98	0.42	16	83	36.33

[1] milliequivalents
[2] cation exchange capacity

4.3.3 Disease assessment and other measurements

Methods for root washing, disease assessment, calculations on incidence of infected roots (%) and fresh and dry weights of plant growth components were as described in Chapter 3.

4.3.4 Statistical analyses

Statistical analyses of the incidence of infected roots (%) and all the growth components of plants (root and shoot dry and fresh weights) for the general treatments were analysed using analysis of variance (ANOVA). The mean water uptake over time for the plants watered by Wt was analysed with ANOVA, treating 'time' as a split-plot treatment, and adjusting for the repeated measures using the Greenhouse-Geisser epsilon correction factor (Greenhouse & Geisser 1959). All the ANOVA tables of the analyses are presented in Appendix 5, A5. 4.

4.4 Results

4.4.1 Effects of environmental conditions on disease incidence

The analyses showed that the proportions of infected roots on the plants were affected by the different growth chambers, soils, watering regimes and sieving ($P<0.05$, <0.001, <0.001, and <0.05, respectively). The percent infected roots differed significantly between growth chambers MC16 and C19, but not between MC16 and C16, or between C16 and C19. Plants grown in MC16 had the greatest proportion of roots infected (43%) (Figure 4.2a). The highest percent of infected roots (46%) occurred on plants in soil R1, while those in P7 and R1a showed similarly lower infection incidence (35%) (Figure 4.2b). Comparison of the two watering regimes showed that Wt plants had 15.7% more infected roots than F plants (Figure 4.2c). The incidence of infected roots was higher in sieved soil (41%) than in the unsieved soil (35%) (Figure 4.2d).

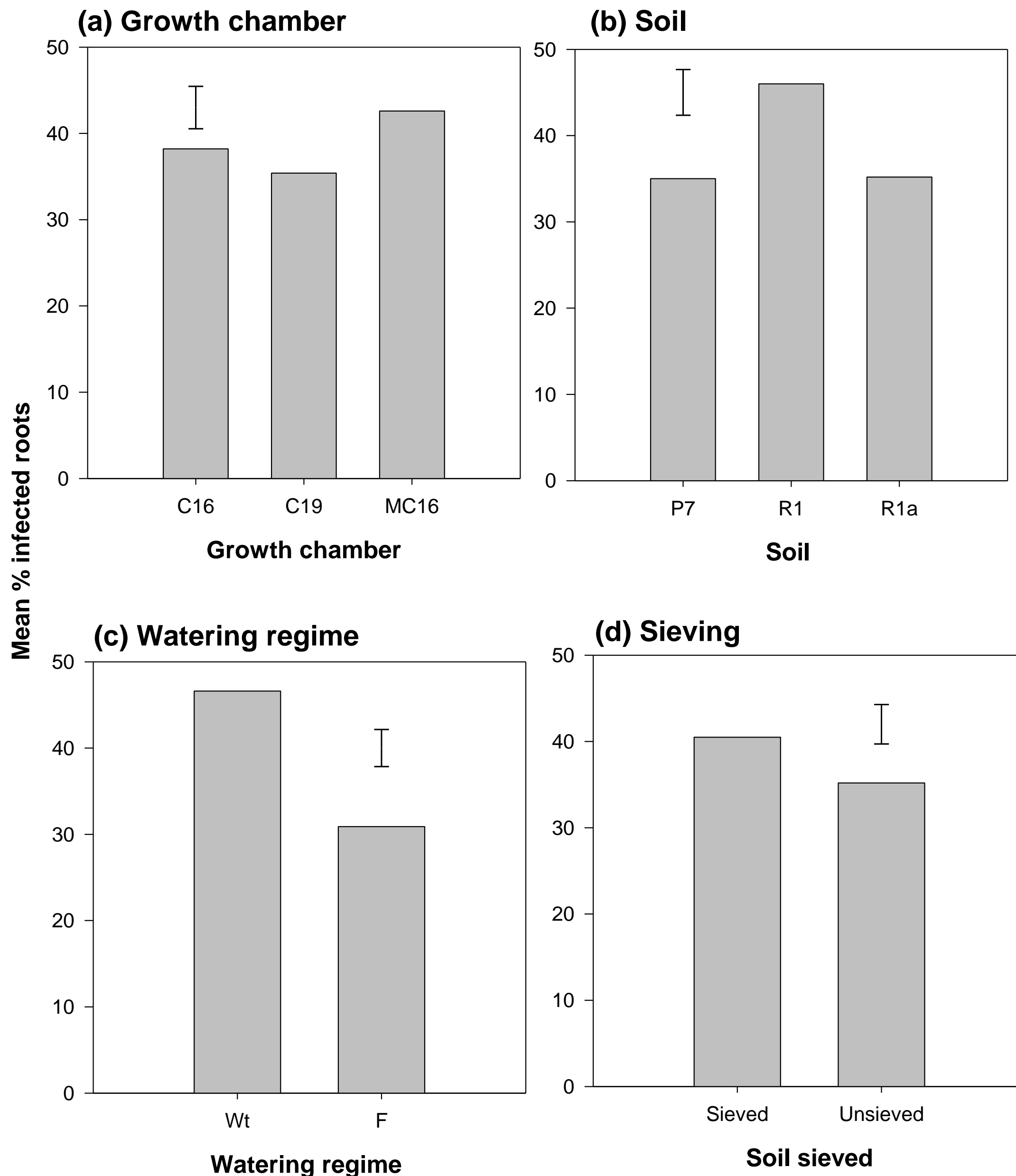

Figure 4.2 Mean incidence of infected wheat roots in (a) different growth chambers and (b) soils, subjected to (c) the two watering regimes and (d) with sieving. Error bars are the least significant differences (LSD) at the 5% level [df = 9 (a) and 45 (b-d)].

There was a marginal 3-way interaction effect for incidence of root infection between growth chambers, soils and watering regimes (P = 0.08) (Figure 4.3). In general, the disease incidence was higher in Wt than F watering regimes, and on plants grown in soil R1. Plants in soils P7 and R1a had similarly low incidence of root infection. Overall, R1 plants watered by Wt maintained in MC16 had the greatest proportion of their roots infected (65%), while those in P7, watered by F maintained in MC16 had the least

proportion of their roots infected (22%). There was also a marginal 3-way interaction for incidence of root infection between growth chambers, sieving and watering regimes (P = 0.07), however, the sieving treatment was shown as a soil type, the results being represented in its interaction with growth chambers and water regime Figure 4.3.

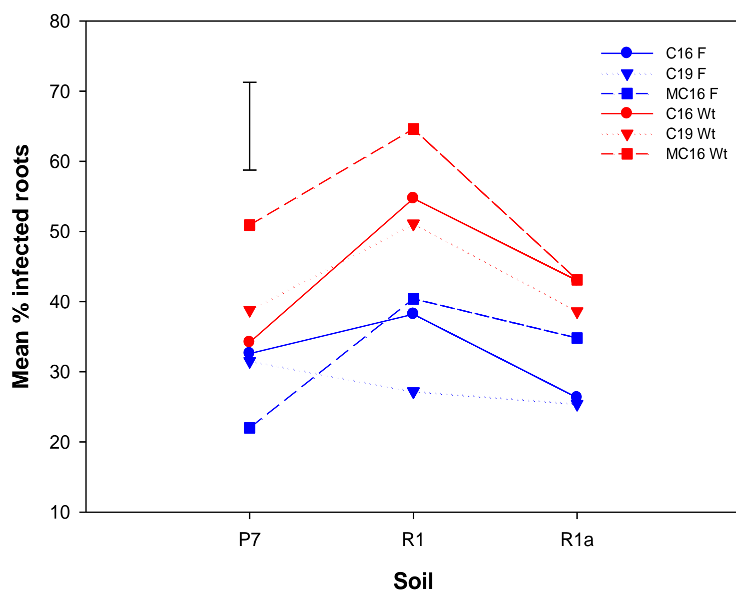

Figure 4.3 Mean incidence of infected wheat roots on plants grown in different growth chambers (C16, C19 and MC16) and soils (P7, R1 and R1a) with different watering regimes (Wt = by weight and F = saturation). Error bar is the least significant difference (LSD) at the 5% level (df = 54).

4.4.2 Effects of environmental conditions on plant dry weights

The growth components of the plants were affected by the different growth chambers, soils and watering regimes in the pot bioassay experiment. The mean root and shoot dry weights of plants differed substantially between the growth chambers ($P<0.001$ for both variables), with plants in MC16 consistently producing the least root and shoot dry weights (0.1 and 0.2 g, respectively) (Figure 4.4a). In contrast, plants in C16 produced the greatest root and shoot dry weights (0.5 and 0.7 g, respectively). Root and shoot dry weights of plants also varied with soils ($P<0.001$ for both variables) (Figure 4.4b), with the least root and shoot dry weights (0.3 and 0.4 g, respectively) being consistently found on R1 plants. On the other hand, R1a plants produced the highest root and shoot dry weights (0.38 and 0.58 g, respectively). The different watering regimes also affected root and shoot dry weights ($P<0.001$ for both variables) (Figure 4.4c), with Wt plants having higher mean root dry weight than F plants (0.4 and 0.3 g, respectively), but significantly decreased shoot dry

weight (0.42 and 0.53 g, respectively). Sieving also affected root and shoot dry weights of plants substantially (P<0.001 and <0.05, respectively), with plants in sieved soils consistently producing lower dry weights of roots and shoot (0.3 and 0.6 g, respectively) (Figure 4.4d).

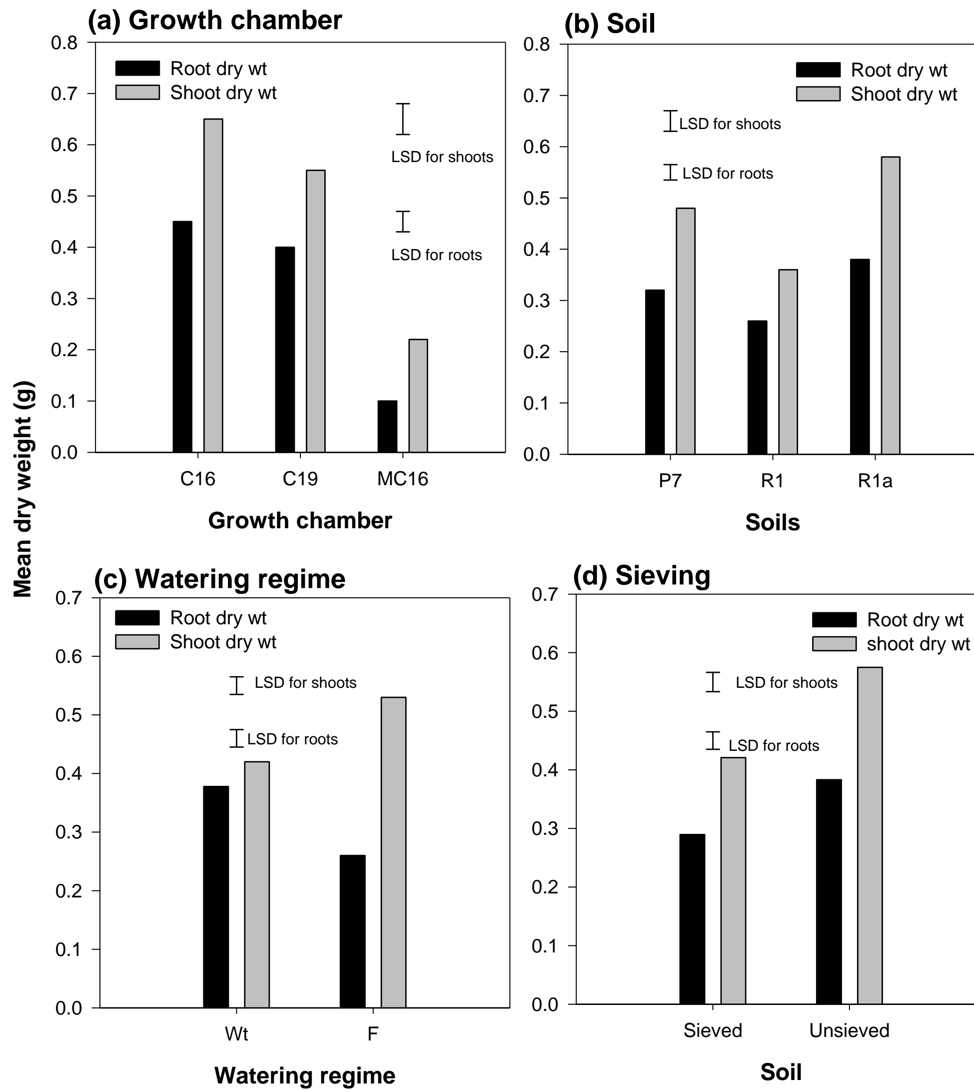

Figure 4.4 Mean dry root and shoot weights of plants grown (a) in different growth chambers, (b) in different soils, (c) with different watering regimes and (d) in soils with sieving treatments. Error bars are the least significant differences (LSD) at the 5% level (df = 9 (a) and 45 (b-d) and apply only to the individual plant variables.

There were also significant 2-way interaction effects on mean root dry weights between growth chambers and soils, and between growth chambers and watering regimes ($P<0.001$ for both) (Figures 4.5a-b). The growth chamber and soil interaction showed that plants in the different soils reacted differently in C16 and C19, but were the same in MC16 (4.5a). In growth chambers C16 and C19, mean root dry weight was the highest in R1a plants followed by P7 and R1 plants. The different watering regimes, also responded differently to the growth chambers, with Wt plants producing higher root dry weights in C16 and C19 than F plants, but not in MC16, where they were the same for the two watering regimes (4.5b). The greatest differences in root dry weights due to soils and watering regimes occurred in C16. Plants grown in the three soils also responded differently to the two watering regimes ($P<0.05$) (4.5c). The greatest root dry weights were in R1a plants, followed by P7 and R1 plants, all watered by Wt. Root dry weights of F plants were not significantly different between P7 and R1, and between P7 and R1a.

From visual assessment, the plants in MC16 consistently had smaller root systems than those in C16 and C19 regardless of soils and watering regimes (Figure 4.6). In addition, R1 plants had much smaller root systems than P7 and R1a plants, especially for those maintained in growth chambers C16 and C19.

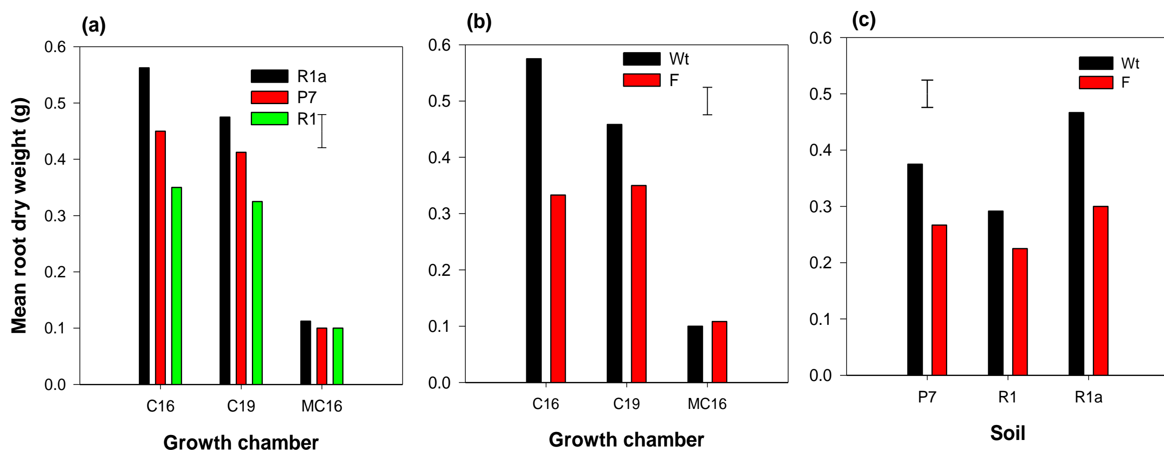

Figure 4.5 Mean root dry weight of plants grown in (a) soils maintained in different growth chambers, (b) watering regimes carried out in different growth chambers and (c) soils with different watering regimes. Error bars are the least significant differences (LSD) at the 5% level (df = 46, 31 and 45, respectively).

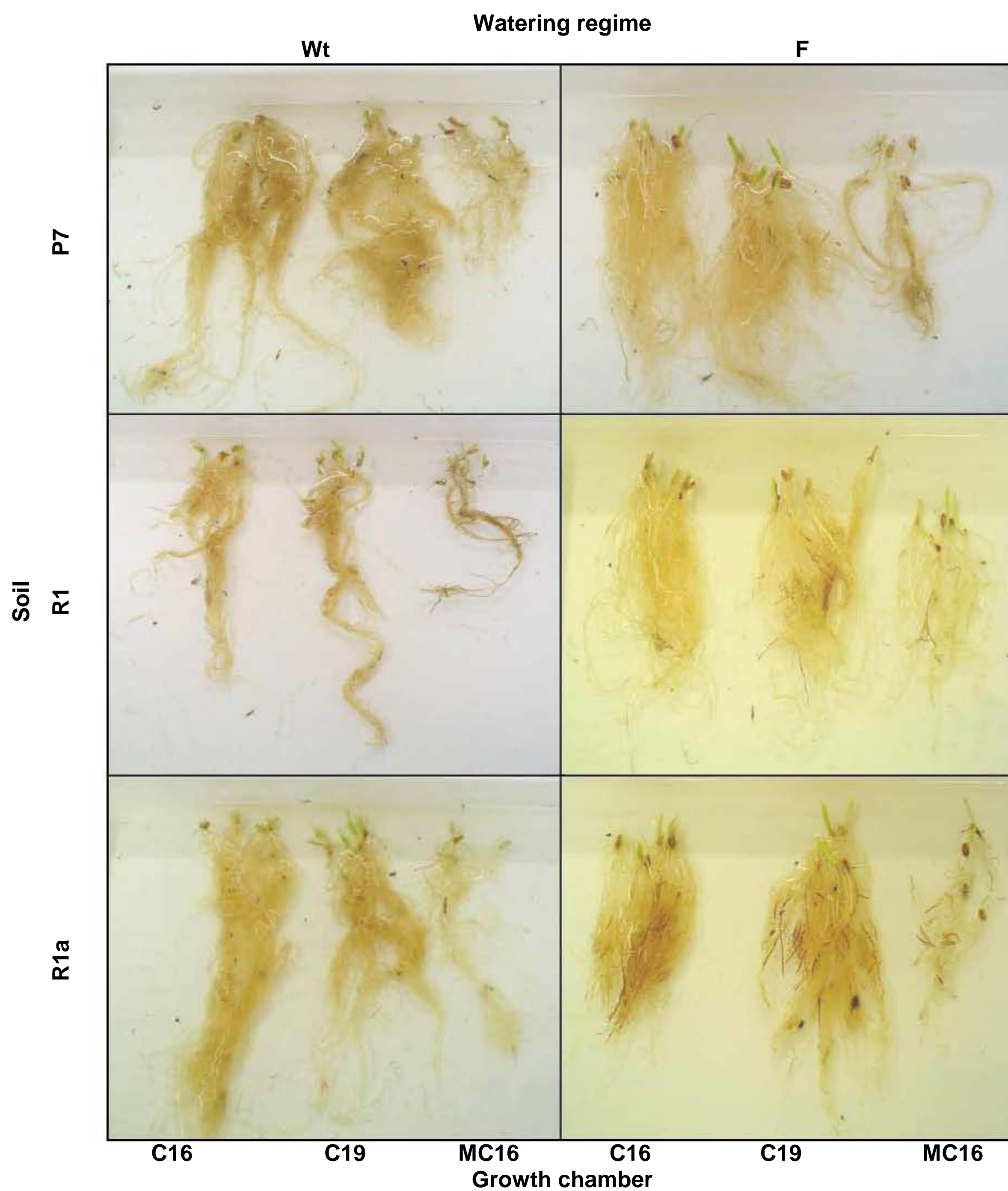

Figure 4.6 Comparative sizes of root systems of plants grown in the three soils subjected to the two watering regimes and maintained in the three growth chambers.

Shoot dry weight was also significantly affected by the 2-way and 3-way interactions of all the main treatments ($P<0.001$and <0.05, respectively). However, only the results on the 3-way interaction (growth chamber, soil and watering regime), which are considered more relevant are presented (Figure 4.7). Similar to the root dry weight (4.5a), plants in the different soils also reacted differently in C16 and C19, but were similar in MC16 (Figure 4.7). Plants in growth chamber C16, however, had greatest shoot weights overall, with greater differences between soils than in C19. Contrary to the root dry weight data, F plants in general, produced greater shoot dry weight than Wt plants. The results on the interaction between sieving and other main factors are not specifically presented here for the same reasons stated in Section 4.4.1.

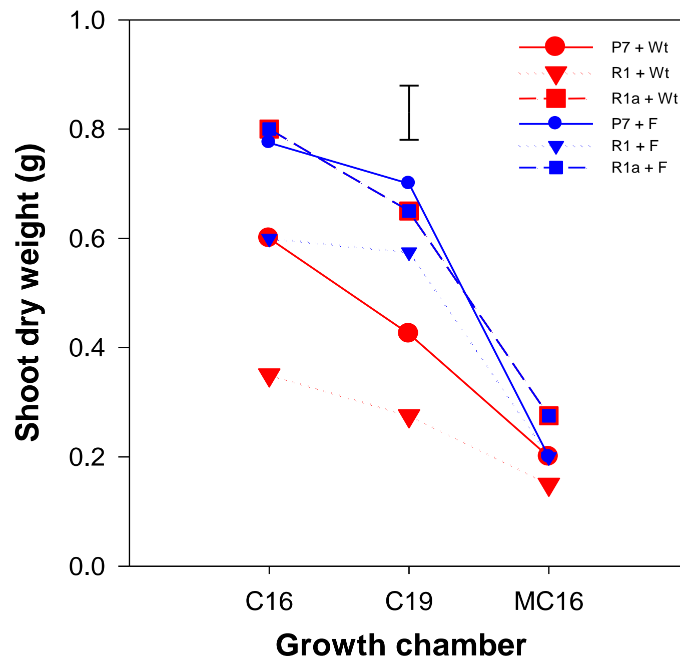

Figure 4.7 Mean shoot dry weights of plants grown in different soils (P7, R1 and R1a) subjected to different watering regimes (Wt = by weight and F = saturation) maintained in growth chambers. Error bar is the least significant difference at the 5% level (df = 50).

4.4.3 Water uptake

Mean biweekly total water uptake per pot in the Wt treatment was significantly affected by the growth chambers, soils and time ($P<0.001$ for all treatments). Mean biweekly water use was the greatest in growth chamber C19 (32 g) followed by C16 (25 g) and MC16 (14 g) (Figure 4.8a), and greater in soils P7 and R1a (26 and 29 g, respectively) than R1 (17 g) (Figure 4.8b). The mean biweekly water uptake by the plants increased over time until day 19 and then remained at about 35 g with fluctuations (Figure 4.9).

The mean biweekly water uptake was also significantly affected by the 2-way and 3-way interactions of the main treatments ($P<0.001$ for the 2-way interaction and $P = 0.001$ for the 3-way interaction). The results of the 3–way interaction (i.e. growth chamber, soil and time), which were considered more relevant are presented in Figure 4.10. In general, R1 plants used significantly less water over time than P7 and R1a plants in C16 and C19, whereas all the plants in MC16 used similar amounts of water over time.

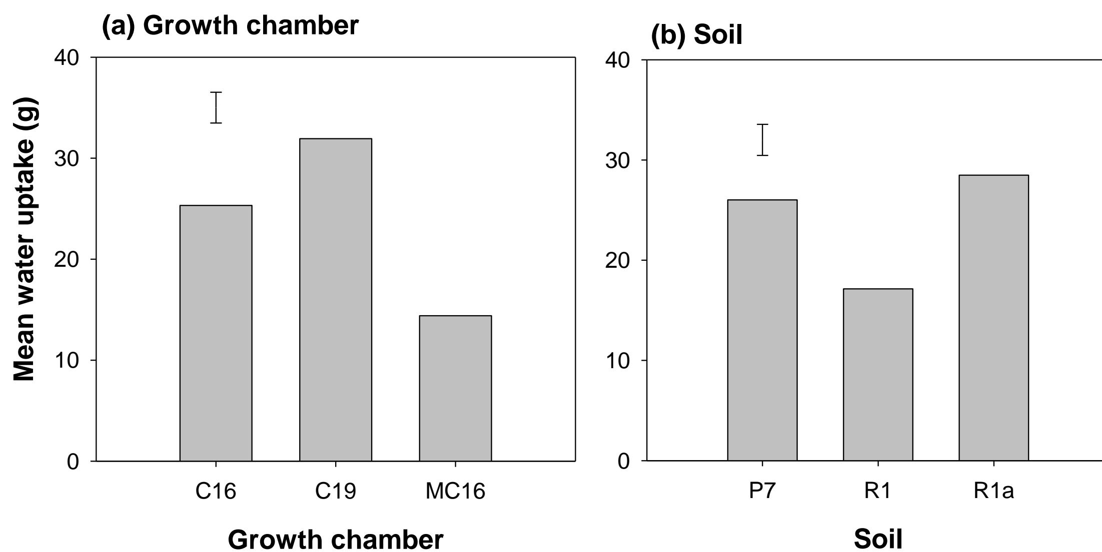

Figure 4.8 Mean water uptake by Wt plants in the three (a) different growth chambers and (b) soils. Error bars are the least significant differences (LSD) at the 5% level (df = 9 and 18, respectively).

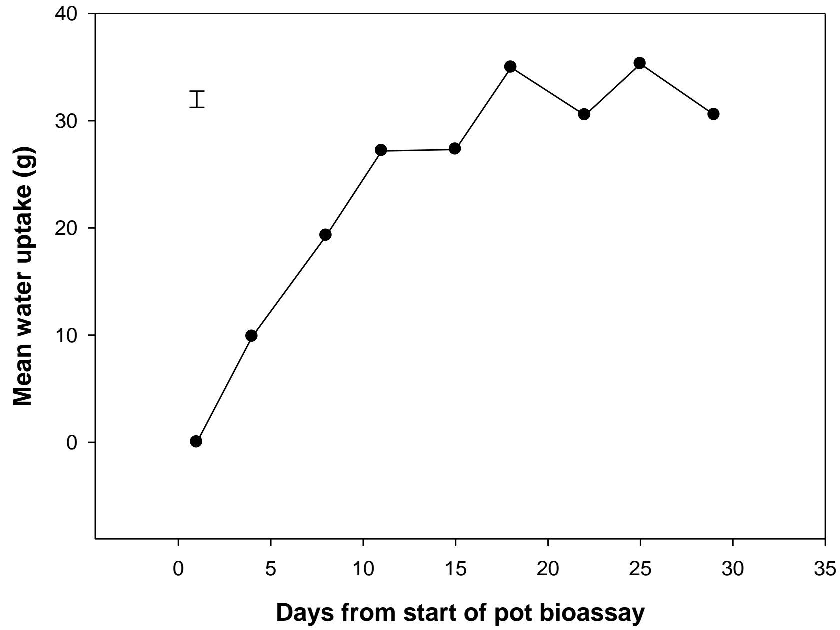

Figure 4.9 Mean biweekly water uptake over time by Wt plants. Error bar is the least significant difference (LSD) at the 5% level (df = 216).

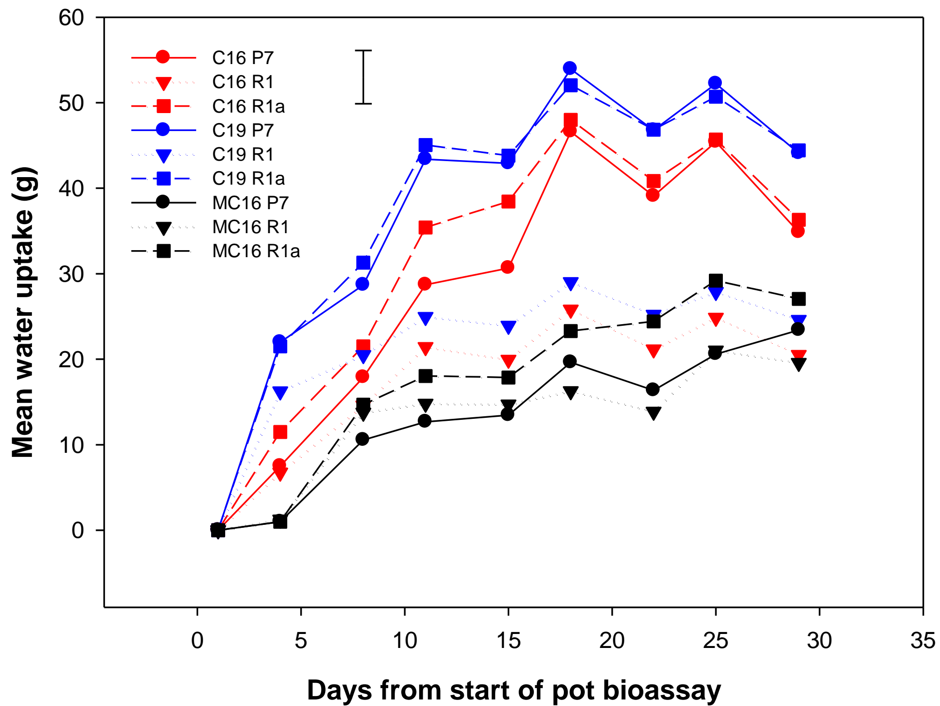

Figure 4.10 Mean water uptake over time by plants watered by Wt during the pot bioassay in the three growth chambers (C16, C19 and MC16) and soils (P7, R1 and R1a). Error bar is the least significant difference (LSD) at the 5% level (df = 216)

4.5 Discussion

The current study aimed to optimise the methods capable of clearly differentiating suppressive from conducive soils, and used P7 and R1, the soils identified as such in Chapter 3 to accomplish this. Although soil R1 was conducive to take-all as expected, soil R1a, which came from the same site and also had a relatively high *Ggt* inoculum concentration, was not as conducive to take-all as soil R1. The plants maintained in soil R1 displayed similarly low levels of take-all as P7 plants. The differences between soils R1 and R1a were that the former soil was collected at a later time, sieved, mixed, and stored at 4°C until use, whereas R1a was collected at an earlier time, not sieved or mixed, and had been stored at room temperature (about 18°C) until use.

The higher incidence of infected roots in sieved R1 soil than unsieved R1a soil disagrees with the results of MacNish (1973), who reported that sieving the soil caused a reduction in the incidence of infected roots. However, because MacNish (1973) discarded all the debris caught in the sieve, it was likely that he had reduced the amount of original inoculum. In this study, there was no or very little change to the original inoculum concentration as all the debris caught in the sieve (4 mm aperture), was returned to the soil. The greater activity of *Ggt* in sieved soil found here was consistent with early work by Garrett (1934; 1936; 1937), who concluded that *Ggt* favoured aerated and light textured soils, as caused by sieving.

Sieving the soils may also have distributed the *Ggt* inoculum more uniformly through the soil, thereby increasing the chances of contact between the roots and the pathogen. For the plant roots to become infected, they need to be in close enough proximity with an inoculum unit for the hyphae of the pathogen to detect and grow towards the roots (Wiese 1998). Thus the more uniformly distributed inoculum may have led to the higher proportion of infected roots and reduced plant growth in R1 soil, compared to plants grown in R1a. Most studies involving the use of bioassays to investigate take-all suppressiveness have incorporated soil sieving and returning of the caught plant material back into the sieved soils, prior to the assays (Lester & Shipton 1967; Shipton *et al.* 1973; Wildermuth 1980; Cook *et al.* 1986; Andrade *et al.* 1994b; Hiddink *et al.* 2005). In addition, the differences in sampling dates, sieving and mixing treatments, as well as storage conditions and durations between soils R1 and R1a, might have led to significant biological differences between the two soils, thereby causing differences in their conduciveness.

It is generally accepted that take-all disease is most severe at soil temperatures of 12-16°C, which is also the optimum temperature range for the host (Baker & Cook 1974). This observation agrees with one by (Deacon 1997) who reported that under natural conditions in non-sterilised soil, the infection declines as the temperature is raised above 18°C. However in the present research, the plants maintained in 16°C (C16) and 19°C (C19) provided similar root disease incidences (Figure 4.2a), which indicates that the New Zealand isolates of *Ggt* survived and were infective under both temperature regimes.

Soil P7 had lower disease incidence than soil R1 (Figure 4.2b), with correspondingly higher shoot and root weights (Figure 4.4b) as well as water uptake (Figure 4.8b), a trend which was consistent in growth chambers C16 and C19. As both soils had very similar water contents at FC (Table 4.1), the higher mean water uptake by P7 plants than R1 plants in both growth chambers could not be due to their differing water holding capacities. Although the amount of available water was unknown (moisture release characteristics of these soils were not analysed), the significant differences in the mean water uptake between the two soils were clearly related to the differing disease incidence.

In the current trial, the F plants consistently had lower incidence of root infection than Wt plants. This result agrees with early work by Wong (1984), Glenn *et al.* (1987) and Heritage *et al*. (1989), who demonstrated that increasing the soil moisture (i.e. water potential) from -10 kPa to -1 kPa (saturation point), reduced the infectivity of *Ggt* in the soils. However, if soil becomes drier than the optimum for *Ggt*, the pathogen's growth and infectivity are also reduced, as reported by Grose *et al*. (1984) and Campbell & Clor (1985), in whose trials, soil moisture potentials ranged from -10 kPa to -108 kPa. In pure culture or in sterilised soil, *Ggt* was also reported to progressively grow more slowly as the water potential was adjusted to below -100 kPa, and to cease growth at -4500 to -5000 kPa (Baker & Cook 1974). Clearly, *Ggt* remains viable under conditions of extreme dryness, and it tolerates moisture levels close to FC but not beyond (such as when waterlogged). Hence, it seems likely that in this study the *Ggt* inoculum (both natural and introduced) in F soils had become less active over the 4 wk experimental period, thereby resulting in less disease incidence than in the Wt soils, making watering by Wt more suitable for *Ggt* infection.

The F plants consistently produced lower root and greater shoot dry weights in the present study. Continuous saturation of soils has been known to impair root growth and

regeneration, by reducing soil aeration, increasing CO_2 levels and reducing oxygen levels in the soils (Baker & Cook 1974). In addition, prolonged oxygen deficiency in the rhizosphere may also lead to accumulation of toxic ions in the roots (Levitt 1972). During an investigation of the effects of different environmental conditions on various *Gaeumannomyces/Phialophora*-complex fungi, wheat shoot growth was reported by Augustin *et al*. (1997) to be affected by moisture levels. They found that when the *Ggt* inoculated soils were kept at 85% of their maximum water holding capacity (close to FC), shoot dry weights were highest (up to 0.13 g) for plants grown in the soils. They suggested that the combined effects of soil moisture and higher temperature (18°C day and 9°C night) conditions might have stimulated plant growth. The authors however, did not assess the plants for *Ggt* infection. In this study, the Wt plants, produced greater root and lower shoot dry weights than F plants (Figure 4.4a). The possibility of infected root systems trans-locating a greater proportion of their total assimilates to produce more adventitious roots to compensate for infection (Asher 1972; Pillinger *et al.* 2005) could account for this response.

In natural soil, it is possible that any change in water potential will place the pathogen at a greater or lesser competitive advantage, relative to the other organisms in the soil (Cook & Papendick 1972). For instance, soil water potential of -1000 to -1500 kPa (i.e. very dry conditions) has been reported to favour the growth of *Fusarium culmorum* (which causes root, crown, and foot rot of wheat) and to reduce the effect of antagonistic soil bacteria, which do not survive well under these conditions (Cook & Papendick 1972). When investigating the suppression of Pythium root rot in bulbous *Iris* spp. in relation to the biomass and activity of the soil microflora in a pot experiment, Van Os & Van Ginkel (2001) found flooding increased the growth rate of the *P. macrosporum* in the soil by 100%, resulting in 40% more root rot than in the untreated soils. In their flooding treatment, the containers (1.4 × 0.85 × 0.85 m) of soils were flooded with water up to 5 cm above the soil surface, while the water levels in the untreated soils were maintained at 60 cm below the soil surface, which resembled the standard groundwater level in ornamental bulb culture on sandy soils. However, Van Os & Van Ginkel (2001) found that the total microbial biomass was unchanged through flooding, and that the microbial activities (e.g. dehydrogenase activity and $^{14}CO_2$ –respiration) in the soil were significantly reduced by it, facts which appear to conflict with the increased growth and pathogenicity of the fungus.

The reaction of an organism to its abiotic environment is not just due to the direct consequence of that environment, but also results from the effects of the environmental conditions on the surrounding microorganisms. For example, it is known that intermittent wetting of soil could increase the decomposition of soil organic matter and the availability of organic substrates, thereby resulting in a flush of microbial activity and increase in the biomass of fungal hyphae and bacteria (Jager & Bruins 1975). In this research project, watering of the plants with Wt could be considered a form of intermittent wetting, potentially allowing the antagonistic microorganisms to be more active. The results of this trial indicated that the Wt watering system is likely to improve the sensitivity of future pot bioassays that aim to evaluate suppressive soils.

In this study, plants in growth chamber MC16 consistently had the highest proportion of their roots infected, produced the lowest root and shoot dry weights, were observed to have the smallest root systems and had the lowest water uptake over time. The possibility of temperature being a contributing factor was dismissed, mainly because plants in MC16 had similar root infection incidence as those in C16, which had higher root and shoot dry weights. Therefore, this strongly implies that the root infection of plants in this chamber was not caused by any of the treatments, but was an effect of the chamber itself. It is therefore, considered inadvisable to conduct further pot bioassay screening for suppressive soils in MC16.

The plants in C19 had lower dry weights than the C16 plants, and thus could not account for the higher mean water uptake over time in C19. The only explanation for this was the possibly greater evapo-transpiration rate being caused by the higher temperature. Although higher transpiration may create root stresses and facilitate infection by pathogens (Baker & Cook 1974), the disease incidence on plants in both chambers was similar. However, due to lower plant dry weights in this chamber, it appeared less suitable for conducting future pot bioassays for soil suppressiveness screening experiments. Even though the growth chamber treatment was not replicated due to limited growth chambers available, the differences on growth, disease incidence and total water uptake for plants maintained in the individual growth chambers were consistently significant.

This research has highlighted the importance of ensuring consistency in treatment of the designated soils and choosing the most appropriate environmental conditions for the pot bioassays that aim to screen take-all suppressive soils. The recommendations for future

screening of take-all suppressive soils would be to subject the test soils to similar soil treatment such as sieving, to conduct the pot bioassay in the Conviron growth chamber maintained at 16°C, alternate 12 h light/dark photoperiod, and to water the plants to FC twice per week by weight (Chapter 5).

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Chapter 5
Take-all decline in New Zealand wheat soils and its potential mechanisms

5.1 Abstract

In many countries, take-all, a root disease caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) has been shown to decline naturally after successive monoculture of wheat for a number of years, a phenomenon called, take-all decline (TAD). This paper describes three pot experiments that investigated the occurrence of TAD in New Zealand wheat soils, and the mechanisms associated with their suppressiveness. The first experiment compared 13 soils, which had different histories of wheat cropping. *Ggt* inoculum (4% w/w, as sand and maizemeal mixtures) was added to the natural and sterilised soils to measure the magnitudes of their suppressiveness, and to determine their levels of biological involvement. From the calculated differences in root disease severity (take-all ratings, TAR) between natural soils ±*Ggt*, and between autoclaved and non-sterilised soils amended with *Ggt*, the soils were grouped into categories 1-4. The results showed that 10 soils were suppressive to take-all; however, for only four soils were the effects related to high levels of biological involvement, as indicated by the effect of soil sterilisation prior to addition of *Ggt*. The experiment was repeated using only five of the soils, and gave similar results (on suppressive levels) to the first experiment, demonstrating the reliability of the methods. However, two of the soils (P7 and M2) fluctuated in levels of biological involvement (TAR in M2 increased by 60 and P7 decreased by 55). In a third experiment, the successful transference of the suppressiveness of two suppressive soils (H1 and H3) into a γ-irradiated base soil indicated that they had specific suppressiveness, which was of a biological nature. Soil P7, which had low suppression, showed characteristics of a specific suppressive soil, but the levels of microbial involvement were somehow impaired by other factors, thereby repressing the expression of the suppressiveness. The suppressiveness in another soil (M2) was not transferable, indicating that it had general suppressiveness, most probably caused by the conditions in the soil being suitable for the microbial biota to compete with *Ggt*. These results collectively indicated that the TAD soils in New Zealand were associated with both specific and general suppressions.

Keywords: *Gaeumannomyces graminis* var. *tritici*, take-all, successive wheat, DNA, inoculum, suppressive soils, take-all decline, screening

5.2 Introduction

Take-all, caused by *Gaeumannomyces graminis* (Sacc.) von Arx & Oliver var. *tritici* Walker, is one of the most important root diseases of wheat (*Triticum aestivum* L.), worldwide (Weller *et al.* 1985; Cook 2003). Control of this disease has proven to be difficult mainly because breeding of take-all resistant wheat cultivars has been unsuccessful and there has been limited effectiveness of chemical control methods (Weller *et al.* 2002). Crop rotation through the use of break crops other than wheat, barley, or other susceptible hosts for 1 or 2 y, depending on the soil and climate of the area, is known to be the most effective method in controlling the disease (Yarham 1981; Cook 2003).

Although take-all was first reported in New Zealand in 1913 (Cockayne 1913), there have been few published reports on its impact on New Zealand wheat production. A survey in the 1995-96 growing season found take-all in 10% of the 157 wheat fields surveyed (Braithwaite *et al.* 1998), but more recent surveys carried out in 167 commercial cereal fields over three growing seasons in 1999-2002 by Cromey *et al.* (2006) found take-all in 45% of the fields. They also found that when the preceding crop was wheat, take-all incidence was high, with 61% of the fields showing average take-all incidence of over 20%. However, 39% of these fields had less than 20% take-all incidence, which led the authors to hypothesise that wheat cropping histories might affect the *Ggt* inoculum concentrations in the soils. They, therefore, conducted quantitative assessment of the soil *Ggt* DNA concentrations (Ophel-Keller & McKay 2001) before and after cereal crops in the 2003/04 growing season (Cromey *et al.* 2004). Their study on 112 soils from fields with varying wheat cropping histories (from non-wheat to 3 y of successive wheat) found that before sowing there were higher *Ggt* DNA concentrations (from 106 pg g^{-1} soil to 1419 pg g^{-1} soil) with increasing years of successive wheat cropping, probably due to inoculum carry-over. However, in some soils which had been cropped with 3 and 4 y of wheat, their relatively high inoculum concentrations (>325 pg g^{-1} soil) and so high risk to take-all, before sowing, were not reflected by the low levels of take-all in the subsequent crop (take-all indices <20) (unpubl. data), indicating that some form of suppression to take-all might have occurred. Since take-all suppression has been reported to occur in wheat monoculture, with the take-all severity reaching a maximum in 2-7 y (depending on soil, environmental conditions and locations) and subsequently declining (Baker & Cook 1974; Rovira & Wildermuth 1981; Deacon 1997; Mazzola 2002; Weller *et al.* 2002), it therefore,

seemed likely that the soils, which had been cropped successively with wheat for a few years, could be experiencing take-all decline (TAD).

Suppressive soils are defined as those soils in which disease development is suppressed even though the pathogen and susceptible host are present (Baker & Cook 1974), and the environmental conditions are suitable for the infection to take place (Cook & Baker 1983). The suppression may be due to a direct effect of the soil on the pathogen (pathogen suppressive soils), or to an indirect effect mediated through the host plant (disease-suppressive soils), and is related to both biotic and abiotic characteristics of the soil (Whipps 1997). In soils cropped successively with 7-8 y of wheat, both characteristics were reported to be associated with the suppression (Simon *et al.* 1987).

The mechanisms reported to be responsible for TAD have so far varied, but most have concluded that TAD is associated with both general and specific suppressions (Gerlagh 1968; Graham & Mitchell 1999; Cook 2003). In general suppression, the infection potential of the pathogen is reduced by competition from other fungi and bacteria in the root zone (Graham & Mitchell 1999). It usually occurs in soils that have conditions ideal for the proliferation of microorganisms (Weller *et al.* 2002; Cook 2003). This type of suppression is not destroyed by heating the soil to 60°C, and is not transferable between soils (Rovira & Wildermuth 1981). In specific suppression however, the infection and secondary spread of the pathogen are limited by the presence of antagonistic microorganisms in the rhizosphere and in young lesions (Graham & Mitchell 1999; Cook 2003). Specific suppression is thought to be due, at least in part, to the effects of an individual or a select group of microoorganisms, and so is transferable between soils (Cook 2003). The suppressive properties can be eliminated by using aerated steam to raise the soil temperature to 60°C or greater for 30 min (Graham & Mitchell 1999).

To date, take-all suppressive soils have not been reported in New Zealand as they have in many countries such as India, the USA, Japan, France, Switzerland and the United Kingdom, but the studies in Chapter 2 on some of the soils from the 2003/04 survey (Cromey *et al.* 2004) indicated that this phenomenon is likely in New Zealand. This study aimed to confirm the suppressive properties of these soils and to investigate the mechanisms associated with the suppressiveness.

5.3 Materials and methods

5.3.1 Soil samples

Of the 13 soils studied, 12 soils were collected after crop harvest in March 2004 and one (P7) in October 2005, when the crop was at GS39 before the booting stage (GS40, Appendix 1, A1. 1). The soils from fields in the South Island, New Zealand, had different histories of wheat cropping and take-all disease. Their selection was based on the results from earlier studies (Chapter 2), which indicated that some of the soils, B6, C12, H1, H2, H3, H7, H10, G2, L9, and M2, may have had take-all suppressiveness, while soils H9 and H15 were non-suppressive, with soil H15 being from a field cropped with 5 y of perennial ryegrass (*Lolium perenne* L.).

Sampling, drying, sieving and storage of the soils were carried out using the methods outlined in Chapter 3. *Ggt* DNA quantification and other physico-chemical properties of all the soils were determined as in the Chapter 2 study. Table 5.1 shows the site information, including cropping histories, soil types, textures, and *Ggt* DNA concentrations, and Table 5.2 shows the physico-chemical properties of the soils. Information on field management practices and crop yield of the four wheat soils, which was provided by the growers, is shown in Appendix 5, A5. 5.

5.3.2 Experiment 1: Screening of soils for suppressiveness

5.3.2.1 Pot bioassay

Each pot (400 mL) contained 300 g of test soil (sterilised or non-sterilised), and was either amended with 4% (w/w) of mixed *Ggt* inoculum (isolates H11T3 R1/3, A3SL4, H9T3 R1/1.2 and Biomill1SC3) prepared in a sand / maizemeal mixture, as outlined in Chapter 3 or uninoculated, being amended with 4% (w/w) of sand / maizemeal mixture. The sterilised soils, packed in bags (36 x 48 cm, capable of withstanding temperatures up to 150°C, Raylab NZ Ltd., Auckland, New Zealand) of 5 kg, were autoclaved twice at 121°C and 15 psi, for 1 h each time (Alef 1995). Prior to use, soil sterility was ensured by making soil dilutions (10^{-1} to 10^{-3}), using 1 g of soil from each bag, and plating 0.1 mL of the dilutions onto nutrient agar and potato dextrose agar (PDA) plates. The plates were maintained at 25°C for 2 wk with frequent checks for bacterial and fungal growth. No bacterial and fungal colonies were found on the PDA plates inoculated with the γ-irradiated soil, hence the base soil was considered free of any organisms contributing to

biological activity prior to conducting the trial. Each pot was planted with four pre-germinated healthy wheat seedlings (Appendix 3, A3. 3), received 20 mL of a nutrient solution and were then watered to the soil's field capacity (FC, at -5 kPa) by weight following the methods in Chapter 3 and Appendix 3, A3. 4.

The pots were laid out in a randomised complete block design, with each treatment replicated four times, giving 208 pots in total. Due to the large size of the experiment, it was conducted in two growth chambers (Conviron, Controlled Environments Ltd., Canada), with each chamber accommodating two replicates. The growth chambers were maintained at 16°C with alternate 12 h light/dark photoperiods and 80% relative humidity for 4 wk. Throughout this period, all the pots were watered to FC by weight twice per week. These controlled conditions and the watering frequency were found in Chapter 4 to be ideal for conducting the pot bioassays that investigated take-all suppressive soils.

5.3.2.2 Disease assessment

For disease assessment of each pot, the roots were washed carefully in a bucket of water and then examined for take-all lesions in water against a white background. However, the amount of disease visible on the root systems were found to vary substantially for plants grown in different soils. Therefore, instead of measuring disease incidence (i.e. by counting the numbers of roots with lesions), disease severity was determined by estimating the percentage area of each root system covered with take-all lesions. The infected plants were categorised as 'slight' (< 25% of root area covered with lesions), 'moderate' (25-75% of root area covered with lesions) and 'severe' (>75% of root area covered with lesions), by using the infection keys (Appendix 3. A3. 6) developed at Rothamsted Research Station (Hornby *et al.* 1998). The take-all rating (TAR) for each pot was then calculated using the formula of Dyke & Slope (1978):

$$\text{TAR} = 1(\text{a}) + 2(\text{b}) + 3(\text{c})$$

Where,

$$\begin{aligned} \text{a} &= \%\ \text{plants with slight infection} \\ \text{b} &= \%\ \text{plants with moderate infection} \\ \text{c} &= \%\ \text{plants with severe infection} \end{aligned}$$

This calculation leads to a maximum TAR score of 300.

Table 5.1 Field locations, cropping histories and some properties of the soils used in the pot experiments.

Field code	GPS Location	Soil type	Soil texture	[1]Preceding crops 1997/98	1998/99	1999/00	2000/01	2001/02	2002/03	2003/04	2004/05	2005/06	Years of wheat	[2]Pre-sowing *Ggt* DNA pg g^{-1} soil	[3]Disease assesment during grain-filling (Take-all index)	[4]Post- harvest *Ggt* DNA pg g^{-1} soil
B6	S45°51.0', E168°34.0'	Kaweku	Silt loam	-	-	Dt	W	W	W	W	-	-	4	1206	5.8	184
C12	S43°45.2', E171°53.8'	Hatfield silt loam + Templeton silt loam	Loamy silt	-	-	B	B	L	W	W	-	-	2	2924	9.3	22
G2	S43°58.5', E170°50.7'	Ashwick stony silt loam	Silt loam	-	-	W	W	Po	W	W+G	-	-	2	2251	17.6	1048
H1	S44°31.2', E171°06.9'	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase	Silt loam	-	-	B	G	W	W	W	-	-	3	1250	17.6	148
H2	S44°31.2', E171°06.8'	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase	Silt loam	-	-	C	Pa	Pa	W	W	-	-	2	687	21.3	686
H3	S44°31.1', E171°06.5'	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase	Silt loam	-	-	Pa	Pa	W	W	W	-	-	3	1526	10.2	150
H7	S44°30.5', E171°06.8'	Claremont silt loam easy rolling phase + Waitohi silt loam	Silt loam	-	-	Pa	W	W	W	W	-	-	4	477	81.2	397
H9	S44°29.2', E171°05.2'	Timaru silt loam	Silt loam	-	-	Pa	Pa	W	W	W	-	-	3	54	30.9	327
H10	S44°28.6', E171°05.6'	Timaru silt loam	Silt loam	-	-	Pa	W	W	W	W	-	-	4	1321	86.1	1365
H15	S44°30.5', E171°06.5'	Claremont silt loam	Silt loam	-	-	Rg	Rg	Rg	Rg	Rg	-	-	0	-	-	0
L9	S44°41.6', E171°04.7'	Timaru silt loam + Claremont silt loam easy rolling phase	Silt loam	-	-	G	Pa	Pa	W	W	-	-	2	1136	14.0	256
M2	S44°44.1', E171°06.5'	Waitohi silt loam	Silt loam	-	-	G	W	W	W	W	-	-	4	2348	27.1	1683
P7	S43°44.3', E171°38.6'	Mayfield silt loam	Silt loam	W	W	W	W	W	W	W	W	W	9	168	44.1	103

[1]Preceding crops:

dogtail (Dt), wheat (W), pea (P), oat (O), clover (C), grass seed production (G), potato (PO), ryegrass (RG), pasture for grazing(PA), barley (b), borage radish (BR), linseed (L).

[2]Pre-sowing *Ggt* in the field was analysed with soil collected from 25 posiitons (two soil cores at each position) along a 'W' pattern over the whole field using soil corers (20 mm dia., 10 cm depth).

[3]Take-all disease index = (0a+10b+30c+60d+100e)/T,

where a, b, c, d and e = number of plants in each of the infection categories below, and T= total number of plants.

Infection categories:

0 no infection
1 slight (1-10% of roots infected)
2 moderate (11-30% of roots infected)
3 high (31-60% of roots infected)
4 severe (61-100% of roots infected)

[4]Post-harvest *Ggt* in the field was analysed from the soils collected after crop harvest for the current study in 2004. An exception was P7, which was collected during crop growth in 2005.

[5]Take-all risk categories developed by SARDI in 2005:

BDL: <5 pg *Ggt* DNA g^{-1} of soil
Low risk: 5.1 - 96 pg *Ggt* DNA g^{-1} of soil
Medium risk: 96.1 - 256 pg *Ggt* DNA g^{-1} of soil
High risk: >256.1 pg *Ggt* DNA g^{-1} of soil

Table 5.2 Physico-chemical properties of the soils used in the pot experiments.

Field code	Normal range	5.3-6.1	20-30	0.5-0.8	5.0-12.0	0.8-3	0-0.5	12-25	50-85	Min N ($\mu g\ g^{-1}$ soil)	Moisture content (% w/w)	
		pH	Olsen P ($mg\ L^{-1}$)	Potassium ([1]me 100 g^{-1})	Calcium (me 100 g^{-1})	Magnesium (me 100 g^{-1})	Sodium (me 100 g^{-1})	[2]CEC (me 100 g^{-1})	Base Saturation (%)		Original	At FC
B6		5.5	21	0.38	6.20	0.22	0.07	17	41	22.13	24.45	34.28
C12		6.0	19	0.37	7.90	0.41	0.09	13	69	43.07	13.60	31.95
G2		6.0	29	0.38	8.70	0.38	0.14	17	58	38.82	22.55	41.97
H1		5.7	28	0.42	11.50	0.93	0.09	20	66	34.11	21.38	35.85
H2		6.0	14	0.34	11.80	1.07	0.10	18	74	25.09	20.45	32.36
H3		6.1	41	0.38	13.40	1.12	0.12	19	78	33.80	20.40	35.84
H7		6.2	36	0.19	11.10	1.07	0.14	16	77	16.05	20.71	34.22
H9		6.5	16	0.27	11.80	1.44	0.13	17	79	21.17	21.49	33.18
H10		6.1	16	0.43	8.60	1.09	0.09	15	67	29.46	25.15	32.98
H15		6.6	21	0.20	11.50	1.12	0.20	15	87	15.35	18.51	34.69
L9		6.2	13	0.22	8.80	1.07	0.12	15	66	16.67	21.41	33.44
M2		5.7	37	0.26	7.40	0.81	0.10	14	62	19.15	21.06	33.63
P7		6.0	31	0.26	11.50	0.40	0.10	16	76	57.48	28.57	30.74
Base soil		6.5	21	0.83	10.20	0.84	0.23	15	82	124.00	14.52	20.16

[1]milliequivalents

[2]Cation exchange capacity

5.3.2.3 Statistical analyses and other calculations

Since no take-all lesions were found on the roots of plants grown in the sterilised soils that were not inoculated with *Ggt* (i.e. Ster+Inoc-), so the TAR data for this treatment were excluded from the statistical analyses. All the other TAR data were analysed with ANOVA (Appendix 5, A5. 5) using two sets of contrasts. One compared *Ggt* inoculation (±) for the non-sterilised soils (i.e. 'Ster- Inoc+' against 'Ster- Inoc-'), and the other compared soil sterilisation (±) for the *Ggt* inoculated treatments (i.e. 'Ster- Inoc+' against 'Ster+ Inoc+').

To provide a better understanding of the analyses, it was necessary to calculate the increases or differences in the mean TAR, in response to the treatments. The principles of this approach were similar to the ones applied by Gilligan *et al.* (1994), Ausgustin *et al.* (1997) and Andrade *et al*. (1994a), who included changes in root infection, shoot dry and fresh weights to the different treatments, such as *Ggt* addition, to effectively differentiate the effects of the different parameters (such as root parts) or soils investigated. In addition, most studies on take-all suppressive soils treated the non-inoculated, sterilised soils as controls (Shipton *et al.* 1973; Cook *et al.* 1986; Andrade *et al.* 1994a, 1994b) and presented the results on all the treatments for comparisons, usually between three to five soils. With 13 soils to investigate for suppressiveness, it was necessary to eliminate confusion by calculating and presenting the increases in TAR after the *Ggt* inoculation and the soil sterilisation treatments. These were calculated from the mean results using the following formulae:

Increase in TAR for each soil in Experiments 1 and 2,

$$\text{Increase due to } Ggt \text{ inoculation} = [\text{Mean TAR of (Ster - Inoc+)}] - [\text{Mean TAR of (Ster - Inoc-)}]$$

$$\text{Increase due to soil sterilisation} = [\text{Mean TAR of (Ster + Inoc+)}] - [\text{Mean TAR of (Ster - Inoc+)}]$$

Where,

Ster - Inoc- = Non-sterilised soil without added *Ggt*
Ster - Inoc+ = Non-sterilised soil with added *Ggt*
Ster + Inoc+ = Sterilised soil with added *Ggt*

Increase in TAR for each soil in Experiment 3,

$$\text{Increase due to } Ggt \text{ inoculation} = \left[\left(\text{Mean TAR of Inoc+}\right) - \left(\text{Mean TAR of Inoc-}\right)\right]$$

Where,

$$\text{Inoc-} = \text{soil without added } Ggt$$
$$\text{Inoc+} = \text{soil with added } Ggt$$

In these cases, the comparisons made were between soil treatments whose plants had a significant increase in TAR after the treatments, with those that had zero or non-significant increases. Hence, the zero or non-significant increases in the TAR of plants (i.e. increase of TAR < the LSD value) in response to the addition of *Ggt* inoculum, would indicate that the soils were suppressive to take-all. In contrast, a significant increase in the TAR (i.e. increase of TAR > LSD value), when the same soil was sterilised prior to inoculation, would indicate that the suppression was probably biological in nature. Based on these principles, the following four categories were developed to separate the suppression properties of the soils:

Category	Description
1	Soils are weakly suppressive with low levels of biological involvement (TAR_{Ggt}>LSD_{Ggt} and TAR_{Ster}<LSD_{Ster})
2	Soils are weakly suppressive with high levels of biological involvement (TAR_{Ggt}>LSD_{Ggt} and $TAR_{Ster>}$>LSD_{Ster})
3	Soils are highly suppressive with low levels of biological involvement (TAR_{Ggt}<LSD_{Ggt} and TAR_{Ster}<LSD_{Ster})
4	Soils are highly suppressive with high levels of biological involvement (TAR_{Ggt}<LSD_{Ggt} and TAR_{Ster}>LSD_{Ster})

Where,

TAR_{Ggt} = the mean increases in TAR of plants grown in non-sterilised soils added with *Ggt* inoculum.

LSD_{Ggt} = the LSD value at 5% level, where the TAR_{Ggt} is significantly higher than zero increase.

TAR_{Ster} = the mean increases in TAR of plants grown in sterilised soils added with *Ggt* inoculum.

LSD_{Ster} = the LSD value at 5% level, where TAR_{Ster} is significantly higher than zero increase.

5.3.3 Experiment 2: Reproducibility of the screening experiment

Based on the results obtained from Experiment 1 (Section 5.3.2), the pot bioassay was repeated with five of the soils using the same sterilisation and inoculation treatments, replications and incubation conditions, as outlined in Experiment 1 (Section 5.3.2.1). Of the five soils chosen, two wheat soils represented Category 4 (highly suppressive with high

levels of biological involvement), one represented Category 3 (highly suppressive with low levels of biological involvement), one in Category 2 (conducive with a lower level of biological involvement), and a non-wheat soil in Category 2 (conducive and had higher levels of biological involvement). For this assay, six pre-germinated wheat seedlings were planted in each pot, with the two extra seedlings being intended for later use in a separate study that investigated the microbial profile within the roots and in the rhizosphere soil (Chapter 6). Disease severity assessment and statistical analyses were carried out as previously described in Section 5.3.2.2.

5.3.4 Experiment 3: Transferability of suppressive properties between soils

5.3.4.1 Inoculum preparation

A mixed *Ggt* inoculum comprising the same four isolates used in Experiments 1 and 2, (Sections 5.3.2 and 5.3.3) was prepared according to the methods described by Weller & Cook (1983) with a few modifications. The growing medium of oat kernels was autoclaved in bags (similar to those used in Experiment 1, Section 5.3.2.1), each inoculated with one of the *Ggt* isolates and incubated for 3 wk as described in Chapter 3. The oat kernels colonised by the different *Ggt* isolates were spread out separately onto trays (45 cm × 35 cm), and air-dried at room temperature (approximately 20°C) for 4 d. The dried cultures were then ground in 100 mL batches using a grinder (model FR15, Girmi, Italy) for four 5 s durations, to reduce the inoculum size to approximately 1 mm diameter. The ground cultures of the four *Ggt* isolates were then thoroughly mixed.

5.3.4.2 Pot bioassay

The pot bioassay was set up as described by Weller & Cook (1983), with the five test soils H1, H3, H15, M2 and P7, as in Experiment 2 to provide potential sources of suppressive microorganisms. A base soil (Templeton silt loam) had been collected from Lincoln, New Zealand (location S43°37.8′, E172°28.8′) in March 2006, from the sides of a field cropped with wheat, and subjected to the same processing treatments (air-drying, sieving and storage) as the test soils (Section 5.3.2). Its physico-chemical properties are in Table 5.2. The soils were packed and sealed into 20 cm × 20 cm polythene bags (each containing 2 kg of soil) and sent to a commercial laboratory (Schering Plough Animal Health Ltd., Upper Hutt, New Zealand) for gamma (γ) irradiation at 2.5 Mrad (a dose rate of 2 Mrad h^{-1}) (Alef 1995). Sterility of the soils was ensured using the procedures described in Experiment 1

(Section 5.3.2.1). No bacterial and fungal colonies were found on the subsequent PDA plates used for checking soil sterility.

The pots were set up by mixing 0.1% (w/w) of the pooled *Ggt* inoculum and 1% (w/w) of the test soil to the base soil to give the mixture a final weight of 300 g. For the non-inoculated treatments, ground oat kernels that had no *Ggt* growing on them were mixed with the soils at the same rate. Each pot was planted with four pre-germinated healthy wheat seedlings, to which a nutrient solution and water was added by weight as described in Experiments 1 and 2. For the base soil, the FC was 20.2% measured at -5 kPa. The final concentrations of the major nutrients in the soils are listed in Chapter 3 and Appendix 3, A3. 4. The pots were laid out in a growth chamber in a randomised complete block design, with four replicates per treatment combination. The chamber conditions and the watering frequency of the trial were similar to those used in Experiments 1 and 2. Disease severity on the roots was assessed with the same methods as in Section 5.3.2.2. The TAR data from Experiment 3 were analysed with ANOVA (Appendix 5, A5. 5) and the increases or differences in the mean TAR, in response to the inoculation treatment were also calculated as described in Experiment 1 (Section 5.3.2.3).

5.4 Results

5.4.1 Occurrence of take-all suppressive soil

5.4.1.1 Experiment 1: Screening of soils for suppressiveness

Disease severity (mean take-all ratings, TAR) in roots differed substantially with different soil origins ($P<0.05$) (Figure 5.1). Plants in soils B6, G2 and M2 were less severely infected (TAR of 110, 102 and 100, respectively) than those from the other soils. Overall, the mean TAR were significantly increased when the soils were sterilised prior to addition of *Ggt* inoculum ($P<0.001$). The highest TAR occurred in Ster+Inoc+ plants followed by the Ster-Inoc+ and the Ster-Inoc- plants (Figure 5.2).

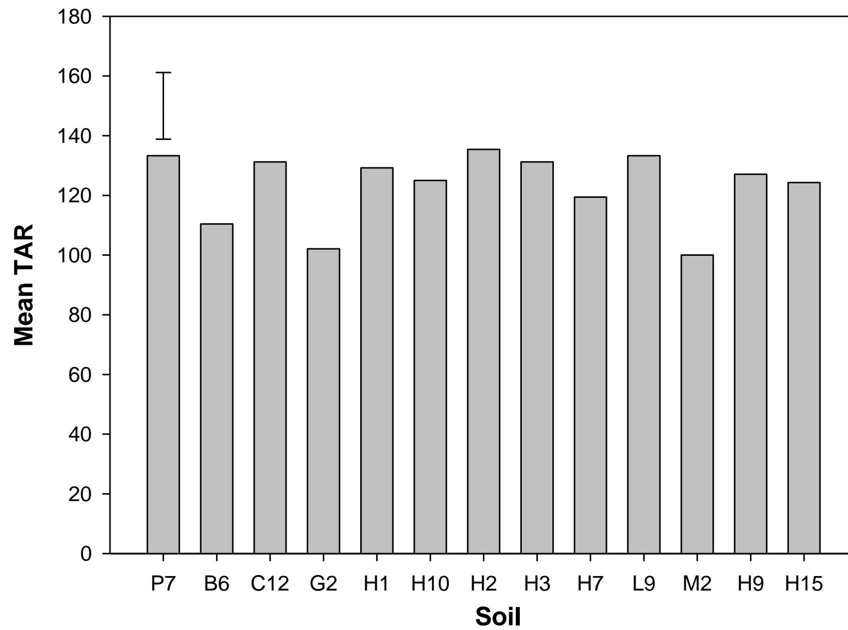

Figure 5.1 The mean TAR (take-all ratings) of plants grown in different soils. Error bar is the least significant difference (LSD) at the 5% level (df = 114).

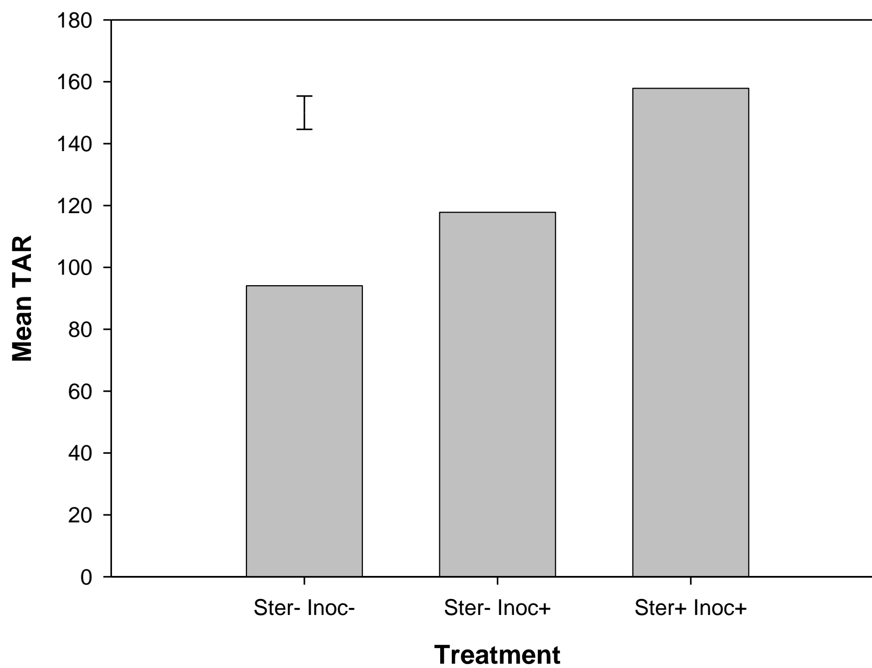

Figure 5.2 The mean TAR (take-all ratings) of plants grown in soils subjected to the three soil sterilisation and *Ggt* inoculation treatments. Error bar is the least significant difference (LSD) at the 5% level (df = 114).

When comparing Ster-Inoc+ with Ster+Inoc+ plants, TAR was significantly affected by a 3-way interaction between the soil origins, soil sterilisation and the *Ggt* inoculation treatments (P = 0.01), with the P7, C12, H1, H3, L9 and H15 plants having increased TAR in soil with sterilisation (Figure 5.3). However, in non-sterilised soils, the TAR showed no significant interaction between *Ggt* inoculation and the soil origins (P>0.01), with the majority of the soils except P7, H2 and H15, having similar TAR (Figure 5.4). Plants in P7, H2 and H15 on the other hand, had increased TAR (by 56, 56 and 52, respectively) due to the addition of *Ggt* inoculum.

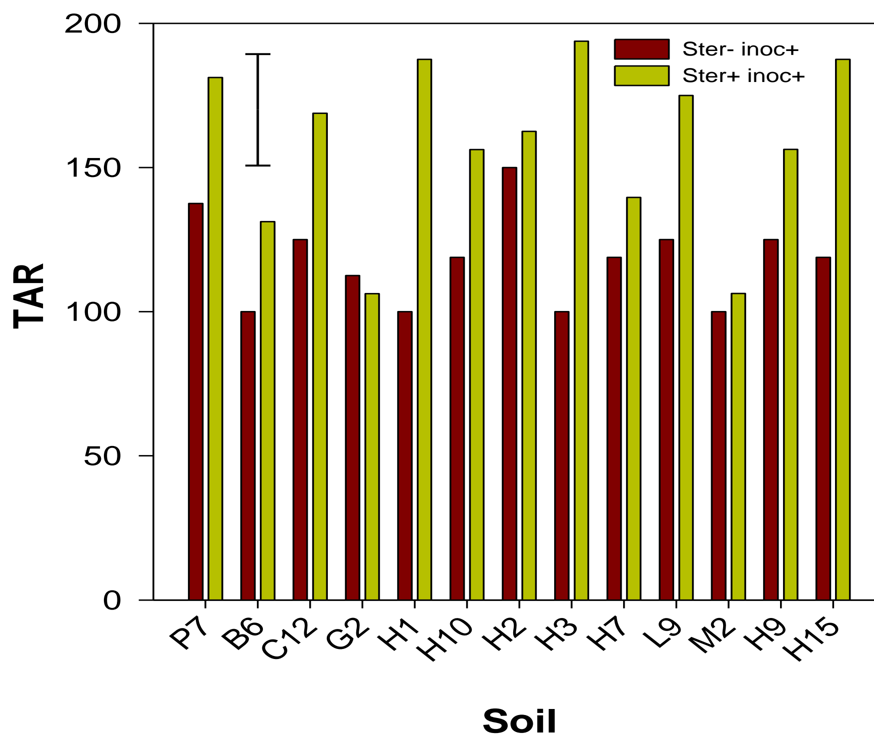

Figure 5.3 TAR (take-all ratings) of plants grown in soils with or without sterilisation prior to adding *Ggt* inoculum. Error bar represents the least significant difference (LSD) at the 5% level (df = 114).

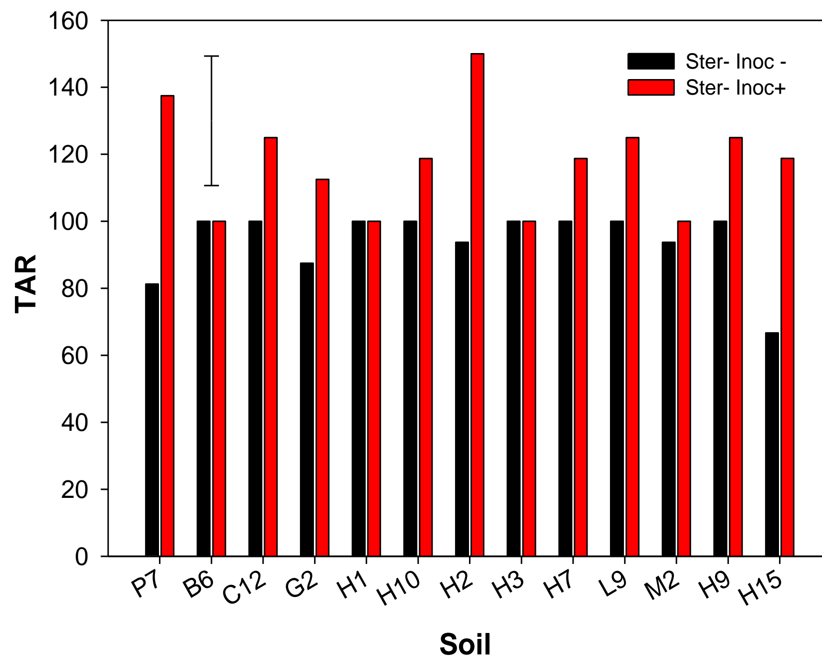

Figure 5.4 The mean TAR (take-all ratings) of plants grown in non-sterilised soils with or without added *Ggt* inoculum. Error bar represents the least significant difference (LSD) at the 5% level (df = 114).

The increases in the mean TAR of plants after adding *Ggt* inoculum to the non-sterilised soils (Figure 5.4), relative to the increases in the mean TAR of plants in response to soil sterilisation before adding *Ggt* inoculum (Figure 5.3), are shown in Figure 5.5. The control soil H15, was conducive to take-all and had a high level of biological involvement (Category 2). Similarly, soil P7, being in Category 2, was also conducive to take-all but with a lower level of biological involvement. Soils G2, M2, B6, H7, H9, H10, C12, L9, H1 and H3 were able to suppress take-all, but had varying levels of biological involvement. Soils C12, L9, H1 and H3 fell into Category 4, being highly suppressive and having high levels of biological involvement. Among these four soils, H1 and H3 were most suppressive to take-all and had the highest levels of biological involvement present.

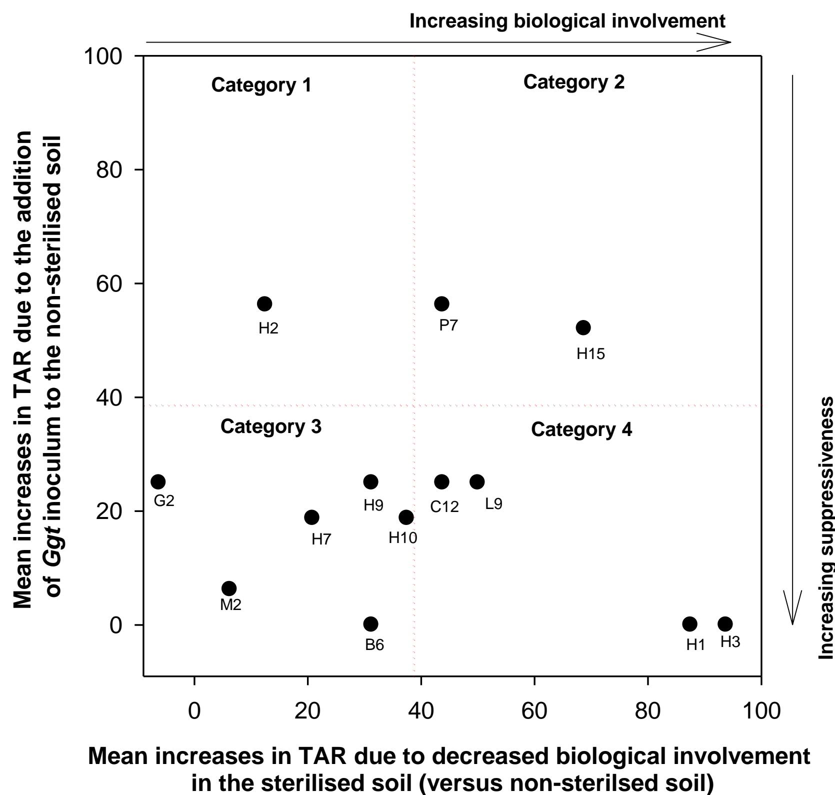

Figure 5.5 Increases in the mean TAR (take-all ratings) of plants, which were grown in non-sterilised versus sterilised soils, both being inoculated with *Ggt* before planting (x-axis), relative to the increases in the mean TAR of plants grown in non-sterilised soils with and without added *Ggt* inoculum (y-axis). The vertical dotted line represents the least significant difference (LSD) at the 5% level compared to zero or non-significant increase in the mean TAR (df = 114) for the x-axis, while the horizontal dotted line is the LSD at the 5% level compared to zero or non-significant increase in the mean TAR (df = 114) for the y-axis.

5.4.1.2 Experiment 2: Reproducibility of the screening experiment

This was a repeated trial of Experiment 1 (Section 5.4.1.1) with five of the soils, H1, H3, M2, P7 and H15. Soils H1 and H3 represented Category 4 (highly suppressive with high involvement of biological component in suppression), M2, Category 3 (highly suppressive with low involvement of biological component in suppression), P7, Category 2 (conducive with a lower involvement of biological component), and H15, a non-wheat soil in Category 2 (conducive and had higher involvement of biological component). The mean TAR of roots differed substantially with the origins of the five soils ($P<0.001$) (Figure 5.6), the plants grown in soil P7 being most severely infected (TAR = 167), followed by H15 (TAR = 142). The effects of soil sterilisation prior to *Ggt* inoculation treatments showed similar

trends to those obtained in Experiment 1, with the roots of Ster+Inoc+ plants being the most severely infected, followed by the Ster-Inoc+ and the Ster-Inoc- plants (P<0.001) (Figure 5.7).

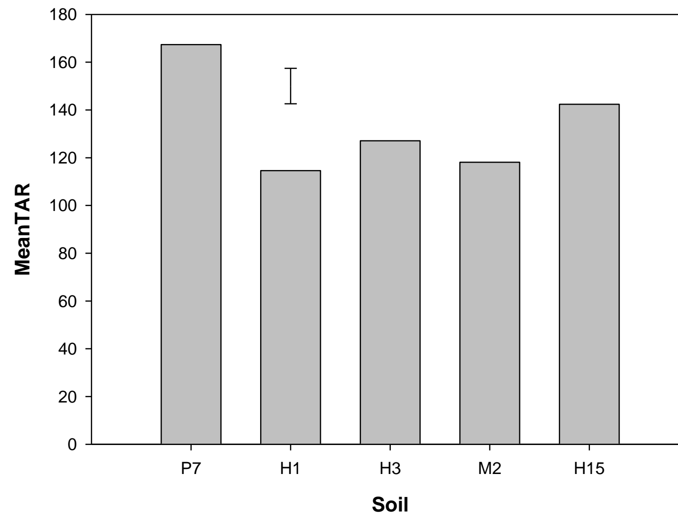

Figure 5.6 Mean TAR (take-all ratings) of plants grown in different soils. Error bar is the least significant difference (LSD) at the 5% level (df = 42).

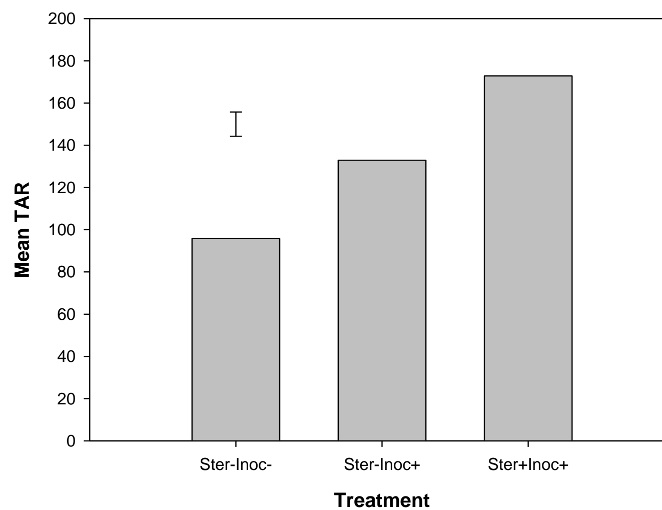

Figure 5.7 Mean TAR (take-all ratings) of plants grown in soils subjected to the three sterilisation and *Ggt* inoculation treatments. Error bar is the least significant difference (LSD) at the 5% level (df = 42).

Figure 5.8 shows the calculated increases in the mean TAR of plants in response to the addition of *Ggt* to non-sterilised soil, relative to the calculated increases in the mean TAR

of plants in response to soil sterilisation before the addition of *Ggt* inoculum. The increases in TAR for the control soil, H15, after soil sterilisation and the addition of *Ggt*, were again in Category 2 (conducive with a high level of biological involvement) as in Experiment 1. Soils H1 and H3, having no change in their mean TAR with the treatments, were also in the same category (4) as in Experiment 1. However, although the mean TAR of soil M2 remained low after the addition of *Ggt* inoculum, it increased by 56 with the sterilisation treatment, moving it from Category 3 into 4. The mean TAR of soil P7 increased by 44 after the addition of *Ggt* inoculum to non-sterilised soil, and was still conducive to take-all. Prior sterilisation of soil P7 however, did not increase the TAR as much as it did in Experiment 1, causing it to be classified in Category 1, not 2 as before.

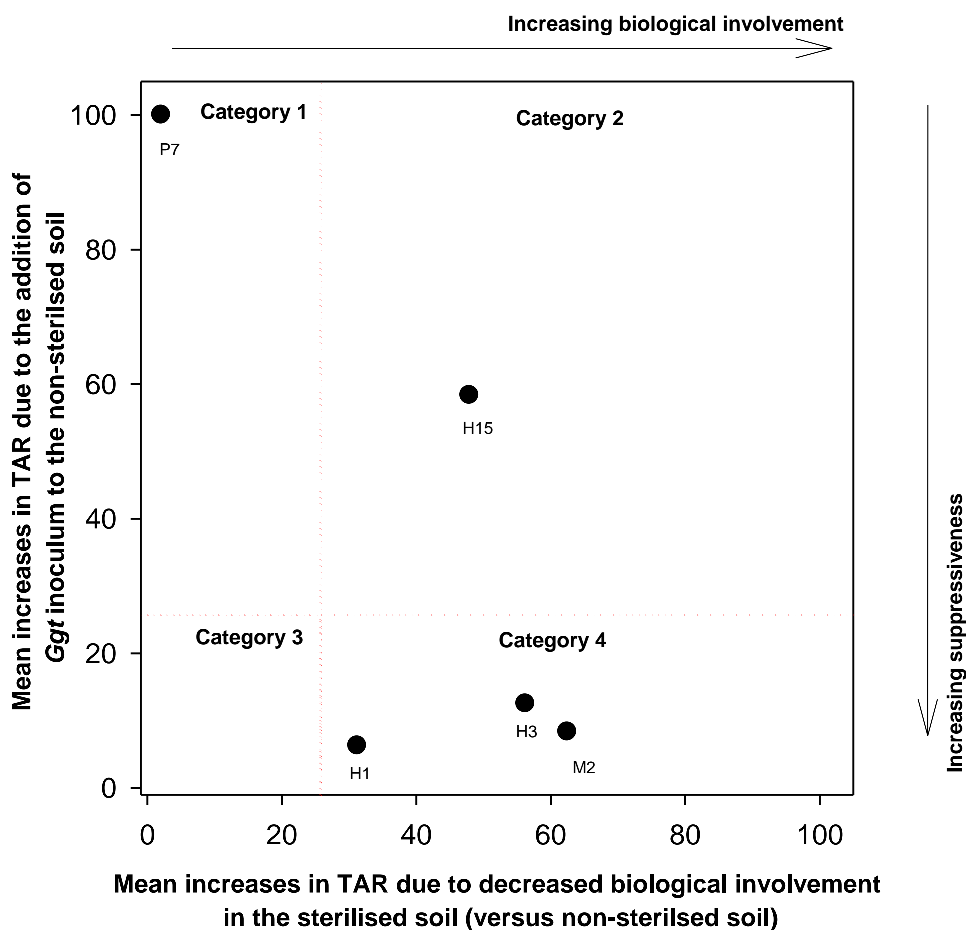

Figure 5.8 Mean increases in the TAR (take-all ratings) of plants, which were grown in non-sterilised versus sterilised soils, both being inoculated with *Ggt* before planting (x-axis), relative to the differences in plants grown in non-sterilised soils with and without added *Ggt* inoculum (y-axis). The vertical dotted line represents the least significant difference (LSD) at the 5% level compared to zero or non-significant increase in TAR (df = 42) for the x-axis, while the horizontal dotted line is the LSD at the 5% level compared to zero or non-significant increase in TAR (df = 42) for the y-axis.

5.4.2 Experiment 3: Transferability of suppressive properties between soils

In inoculated soils, the disease severity (mean TAR) of plants was much greater (P<0.001) (TAR =125) than in uninoculated soils (TAR = 86). The mean TAR also varied with soil origins (P<0.001), being greatest for plants grown in pots of γ-irradiated soil amended with 1% of soil M2 (TAR = 131) (Figure 5.9). There was also a significant interaction effect between *Ggt* inoculation and soil origins on disease severity (P<0.05). Figure 5.10 shows the calculated increases in the TAR of plants in response to the addition of *Ggt* to the γ-irradiated base soils, which already had 1% of the five different test soils added to them. For soils amended with H15 and M2, inoculation significantly increased their mean TAR (by 75 and 63, respectively), whereas soils amended with H1, H3 or P7 had little change in the mean TAR. This indicated that the characteristics of soils H1, H3 and P7 were transferred into the base soil.

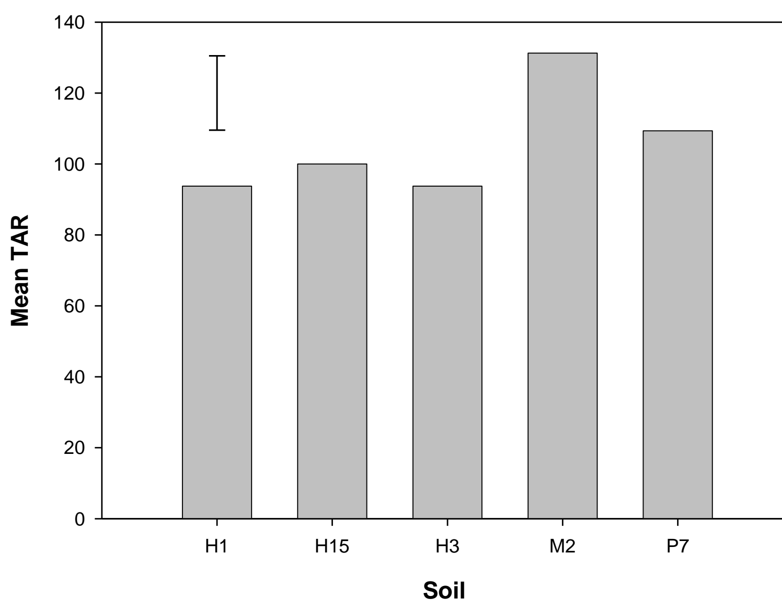

Figure 5.9 Mean TAR (take-all ratings) of plants grown in the γ-irradiated base soil, to which was added 1% of the five different test soils and ± *Ggt* inoculum. Error bar is the least significant difference (LSD) at the 5% level (df = 27).

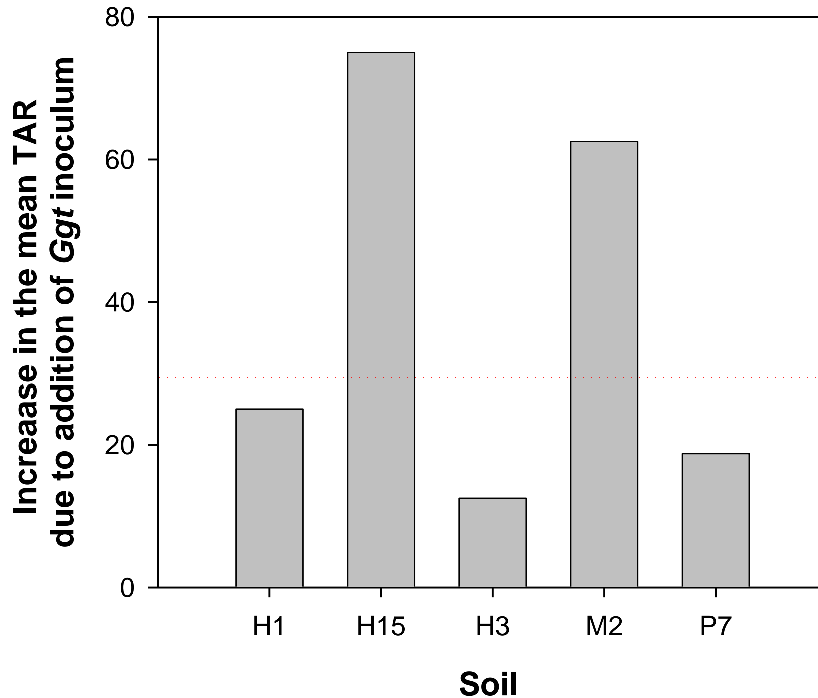

Figure 5.10 Increases in the mean TAR (take-all ratings) of plants due to addition of *Ggt* inoculum to the γ-irradiated base soil, to which was added 1% of the five different test soils. The dotted line is the least significant difference (LSD) at the 5% level compared to ‘0’ or non-significant increase in TAR after the addition of *Ggt* inoculum (df = 27).

5.4.3 Comparative analyses of the suppressiveness of soils between the three pot experiments

Figure 5.11 summarises the increases in the mean TAR of plants due to the addition of *Ggt* inoculum to the five soils for the three pot experiments. The increases in the mean TAR for the control soil H15, and the suppressive soils H1 and H3, were reproducible, showing consistent results throughout the three experiments. However, the apparent suppression of soil M2 in Experiments 1 and 2 was not transferable, and so addition of inoculum increased the mean TAR in Experiment 3 by approximately 60. Soil P7 had a higher TAR (by 44) in Experiment 2 than Experiment 1. However, when a small amount of this soil was transferred into a γ-irradiated base soil with or without *Ggt* inoculum added (Experiment 3), the mean increase in TAR due to the added *Ggt* inoculum, was only 19. Such a low increase, compared to the effect of adding inoculum to the sterilised soils of Experiments 1 and 2, which had increased mean TAR of 56 and 100, indicated that this soil and soils H1 and H3, had transferable properties of suppressiveness.

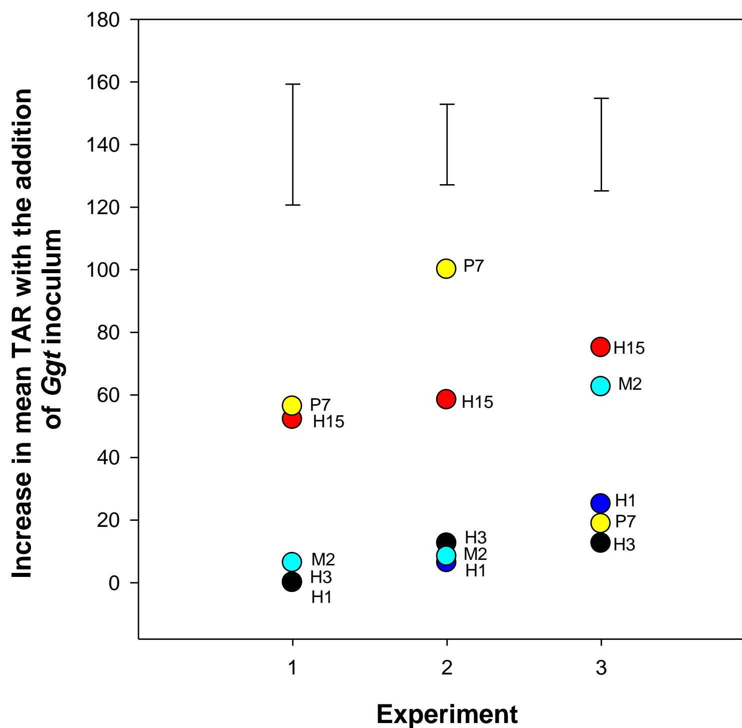

Figure 5.11 Increases in the mean TAR (take-all ratings) of plants due to addition of *Ggt* inoculum to the five soils in the three experiments. Error bars are the least significant differences (LSD) at the 5% levels compared to '0' or non-significant increase in TAR after the addition of *Ggt* inoculum (df = 114, 42 and 27, respectively).

5.5 Discussion

In this research, all the test soils either had low disease severity coupled with high *Ggt* DNA inoculum concentrations in the field prior to sowing, or were in their third, fourth or ninth year of wheat cropping (Table 5.1). Weller *et al.* (2002) considered the monoculture of a susceptible host, the presence of *Ggt*, and at least one severe outbreak of take-all, as the three components required for TAD to eventuate. Whereas Whipps (1997) considered the ideal soils for the initial selection of antagonists are those in which diseases should occur but fail to materialise, mainly because these soils have already provided a range of indigenous potential antagonists. These soils therefore fitted the criteria for selection because TAD could already have begun to happen in them. However, the timing of the possible take-all outbreaks in the fields was unknown at the time of the soil collection during the 2003/04 survey.

For the selected soils of different origins, the initial mean TAR between the treatments with and without the addition of *Ggt* inoculum, could not indicate the presence of a suppressive soil and the mechanisms involved in any suppression (Figures 5.1 and 5.4). This was because the soils had varying amounts of natural inoculum, hence, the addition of *Ggt* inoculum increased the total *Ggt* inoculum and so the disease severity. However, the calculated increases in the mean TAR in the soils, due to the uniform addition of *Ggt* inoculum, indicated the differing magnitudes of suppressiveness among the soils. This pot bioassay (Experiment 1) was therefore, capable of effectively distinguishing the levels of suppressiveness in the New Zealand wheat soils, some of which showed high suppression to take-all (i.e. increase in TAR<LSD of-y-axis in Figure 5.5). The reliability of the method was shown by the repeated pot bioassay (Experiment 2), which demonstrated consistent increases in the mean TAR of the five test soils in response to the addition of *Ggt* inoculum.

The variation in the levels of take-all suppression exhibited by the soils in this study could be attributed in part, to the impact of their different wheat cropping histories on the microbiota in the soils. Field studies have consistently shown that previous cropping histories could affect the extent and the speed of TAD development, and that the number of wheat crops required before its onset, varied with the location of the field, the soil type and the environmental conditions (Shipton 1975). It has also been reported that different degrees of biological buffering in relation to the *Ggt* inoculum in the soil can be achieved by introducing different host crops into the field (Baker & Cook 1974). Cultivation of a rotation of different crops in a given field will maintain the microbiota in a greater state of flux, resulting in different microorganisms dominating the microbiota each year. In contrast, repeated monoculture of a particular crop, will favour the perpetuation of dominating species, eventually leading to a stable microbiota (Baker & Cook 1974). Hence, the dominating microbial populations, or the antagonists associated with TAD, are likely to differ between soils with different durations of wheat monoculture and different rotation crops, resulting in their differing suppression capabilities as well.

Another explanation for the TAD mechanism during wheat monoculture is that there is a gradual reduction in populations of the aggressive *Ggt* strains, which are replaced by less aggressive strains that allow other soil antagonists to compete for colonisation sites on the roots (Shipton 1977). Lebreton and co-workers (2004) investigated the changes in the

population structure of *Ggt* using the RAPD (Random Amplification Polymorphism DNA) markers and AFLP (Amplified Fragment Length Polymorphism) fingerprinting systems. They found that the populations of aggressive *Ggt* strains in the soil increased to reach a peak after three or four consecutive wheat crops and then decreased to the same population as the less aggressive *Ggt* strains by the sixth crop. This shift in the *Ggt* population structure was proven in a later study to correlate positively with a polyetic take-all epidemic in the field (Lebreton *et al.* 2007). When TAD was observed, the population of the aggressive *Ggt* strains was low in the first wheat crop, highest in the fifth wheat crop and decreased to an intermediate level by the tenth consecutive wheat crop. They hypothesised that the aggressive *Ggt* group was sensitive to the antagonistic microflora in the soil during the parasitic phase, therefore resulting in the selection of a less aggressive population.

In this research programme, autoclaving of soils caused TAR to increase when *Ggt* inoculum was also added (Figures 5.2 and 5.6), indicating that sterilisation caused loss of suppressiveness, which is consistent with its known capacity to destroy the soil's biotic characteristics (Whipps 1997). A similar result was reported by Shipton *et al.* (1973), who took three soils from fields, which were previously cropped with 4 and 12 y of irrigated wheat and 22 y of dryland wheat/barley (number of years for each rotation was not specified), and pasteurised each of them at 60, 70, 80 and 121°C for 30 min. The pasteurised soils were then assayed for antagonism by transferring 1% w/w of each soil, which was also inoculated with 0.5% w/w *Ggt*, into a methyl bromide fumigated base soil. They reported that the suppressive effect could be eliminated in all the soils at 60°C. They proposed that the thermal tolerant actinomycetes or spore-forming bacteria, which generally survive at this temperature, would not be responsible for the suppression, and that the heat-killed soil fungi and non-spore forming bacteria were more likely to be responsible for it.

In a similar study, Cook and co-workers (1986) investigated the infection efficiency (i.e. number of lesions/infections per unit weight of inoculum) of *Ggt* inoculum in a TAD soil (20 y of wheat cropping). They found that when the suppressive soil was pasteurised at 60°C before adding the *Ggt* inoculum (oat grain), the infection efficiency of *Ggt* was greater than in a pasteurised conducive soil. They postulated that soil pasteurisation would eliminate the microorganisms that might act as colonists of the *Ggt* inoculum, resulting in a

flush of carbon and energy (i.e. increasing nutrients in the food base), especially in suppressive soils, thereby enhancing the survival and infectivity of *Ggt* and increasing root infection. The effects of potential proliferation of the fewer heat-resistant microorganisms in the pasteurised suppressive soil, were however, not discussed by the authors. These two studies have demonstrated that any biological components of suppression were likely to be altered by heat-treating the soil at temperatures as low as 60°C.

Soils G2, M2, H7, H9, B6 and H10 had no or very little increase in TAR due to autoclaving before adding the *Ggt* inoculum in Experiment 1 (i.e. increase in TAR<LSD of x-axis in Figure 5.5), which may have been because they had a form of 'general suppression'. General suppression may incorporate a continuum of antagonistic effects, including competition for resources (such as carbon and energy in the food base) by the microbiota in the rhizosphere, antagonism in lesions, and stimulation of host defence mechanisms, which can all lower the inoculum potential of *Ggt* (Cook 2003). The level of general suppression provided is, therefore, a characteristic of the soil and probably not caused by the cropping system, but might be associated with organic amendment, fertility build up and soil temperatures (Rovira & Wildermuth 1981; Weller *et al.* 2002). In other words, the suppression is a characteristic of the soil, which by being ideal for the proliferation of many/a diversity of microorganisms, or the stimulation of host defence (Weller *et al.* 2002; Cook 2003) is, hence, not related to any one microorganism (Weller *et al.* 2002). This suppression is not destroyed by 60°C, and the suppression factor is not transferable (Rovira & Wildermuth 1981).

In Experiment 1, soil M2, which was grown with 4 y of consecutive wheat, had low TAR, that did not increase greatly when the soil was autoclaved and inoculated with *Ggt*. However, in Experiment 2, the TAR were low only in non-sterilised soil, being significantly higher in the autoclaved soil treatment. This indicates that there might be a biological basis for the low levels of disease suppression. In Experiment 3, its suppressiveness was shown not to be transferred with a small amount of the soil added to the γ-irradiated base soil, indicating that it might have a general form of suppression to take-all. Gerlach (1968) did a series of studies to demonstrate TAD with wheat monoculture in the Dutch polder soils and reported that although TAD could be due to a specific suppression of the pathogen, general suppression usually occurs before the onset of specific suppression. The relatively low levels of take-all in the field in relation to the

high *Ggt* DNA concentrations, which reduced during the 2004/05 wheat crop, appeared to support this hypothesis (Table 5.1). However, more work is required to validate this hypothesis as other characteristics, including the non-elimination of the suppressiveness by soil fumigation, and the operation of suppressiveness in bulk soil but not in the rhizosphere, are indications of general suppression as well (Hornby *et al.* 1998).

Sterilising the soils prior to inoculation and planting, can indicate the natural levels of possible biological involvement that could be associated with the suppression, which Experiment 3 showed was due to populations of specific microorganisms in soils H1 and H3 (i.e. specific suppression). When small portions of these two soils were transferred into the γ-irradiated base soil that contained added *Ggt* inoculum, they conferred similar levels of suppression as found in the non-sterilised soils in Experiments 1 and 2. In a similar study by Shipton *et al* (1973), 1% (w/w) of a 5 y untreated wheat soil in Lind, eastern Washington was incorporated into a soil plot of the same field which previously had been fumigated with methyl bromide and inoculated with 0.5% (w/w) *Gaeumannomyces graminis* (*Gg*) to a depth of 12-15 cm. They reported that for the subsequent wheat crop in the fumigated soil, addition of the untreated wheat soil was able to reinstate the suppressiveness. In another field study conducted in Puyallup, Washington (Shipton *et al.* 1973), methyl bromide fumigated soil plots were inoculated with 0.5% (w/w) *Gg* before adding 1% (w/w) of either suppressive soils, from 12 and 22 y continuous wheat crops obtained from Quincy and Pullman respectively, or virgin (non-wheat) soils from nearby sites. After 3 mth, they found that the fumigated plots amended with the long-term wheat soils, were able to provide higher levels of suppression to take-all (43 and 59% of crops being infected) than those amended with the virgin soils (74 and 71% of crops being infected). Both of these studies have demonstrated that microorganisms antagonistic to *Ggt* were associated with these examples of TAD, and the suppressiveness was thus transferable into the fumigated soil in the field.

Other soil-borne diseases have been successfully controlled by transferring the suppressive properties from one soil into another. For example, the antagonists in a soil suppressive to *Streptomyces scabies* could be transferred into an untreated conducive soil at 50% (w/w) to give control of scab in potatoes just as well as the suppressive soil itself (Menzies 1959). Scher & Baker (1980) showed that when a suppressive soil was added at 50 or 70% to a conducive soil previously inoculated with 1% (w/w) *Fusarium oxysporum*, it significantly

decreased the incidence of Fusarium wilt disease in flax (*Linum usitatissimum*). In these studies, the suppressiveness was removed from the soils when they were autoclaved at 121°C or steamed at 57°C, indicating a similar characteristic to take-all suppressive soils (Hornby 1983).

The 3 y successive wheat cropping in the H1 and H3 fields, has been considered as sufficient for the development of specific suppression or TAD in soils (Baker & Cook 1974). Their *Ggt* DNA concentrations, which reduced significantly during the season, to 12 and 10% of pre-sowing levels, respectively (Table 5.1) are also consistent with reports of the development of specific suppression. Hornby (1983) considered that a drop in the levels of the natural *Ggt* inoculums in a soil from beginning to the end of the growing season indicated that the pathogen was being suppressed, probably by inhibition of saprophytic growth in the debris or survival in the soil. However, soils B6, C12 and G2 also had similar reductions in *Ggt* DNA during the season without any further evidence of being suppressive, so this phenomenon alone is not conclusive evidence of a suppressive soil developing. Past research has shown that *Ggt* has poor competitive saprophytic ability, and that throughout the saprophytic period in the soil the inoculum continually reduces through degradation of the crop debris (Garrett 1970, 1975, 1981; Shipton 1981; Skou 1981). Future studies should investigate the relationships between the *Ggt* DNA concentrations in/on the roots of the host and disease severity, during the crop cycles of successive wheat monoculture. Such a study would improve understanding of suppression processes/mechanisms on the parasitic and saprophytic interactions of *Ggt* in the soil.

In the present study, soil H15, which was from a field grown with 5 y of perennial ryegrass and had no *Ggt* DNA (Table 5.1), was incorporated as a control soil for all the experiments. Despite the indication of high biological involvement present, it was more conducive to take-all after the addition of *Ggt* than most of the other wheat soils tested. This result disagrees with Hornby (1983), who categorised soils grown with one to several seasons of grass or grass/legume leys, which are not susceptible to take-all, as Type IV suppressive soil capable of suppressing take-all in the field when wheat was cropped. The principle applied here is very similar to that applied in practising crop rotation with non-hosts, to eliminate or reduce the natural *Ggt* inoculum concentrations, by starving the pathogen of nitrogen needed during its saprophytic survival in the host residues (Garrett 1947). As there was no recorded evidence of any *Ggt* in soil H15 prior to the ryegrass crop,

the possibility that *Ggt* had been present, but eliminated from the soil was dismissed. Hence, the TAR obtained from H15 in the current study was thought to indicate only the disease caused by the introduced *Ggt*.

The development of take-all suppression in pasture soils, was also investigated by Wildermuth (1980) in a wheat pot assay study. He used a fumigated soil amended with 1% of field soils, which were cropped with either 10 or 24 y of continuous pasture, and added 0.1% *Ggt* inoculum (grown in ground oat). The study showed that the 10 y pasture soil was non-suppressive to take-all (disease rating = 5.5), while the 24 y pasture soil was able to give similar suppression as that of a suppressive soil (disease rating = 3.1). Wildermuth (1980) proposed that the suppressiveness in the 24 y pasture soil was a form of transferable suppression, caused by a non-pathogenic dark runner-hyphae fungus. However, he did not provide any explanation for the conduciveness of the 10 y pasture soil or the types of pastures previously grown in these fields. Other research studies have shown that the suppressiveness of pasture soils to take-all was due to the increased populations of *Phialophora radicicola* Cain var. *graminicola*, a fungus non-pathogenic to wheat (Garrett 1941; Deacon 1973b, 1973a; Speakman *et al.* 1978; Hornby *et al.* 1998). In the present study, the lack of suppression to take-all by H15 after the addition of *Ggt* inoculum in Experiments 1 and 2, and the high TAR in a γ-irradiated base soil which was then amended with some of H15 soil, suggests that this soil had no transferable suppressive characteristics due to the presences of microorganisms antagonistic to *Ggt*.

The soil expected to have the greatest chances of being suppressive was P7, because it had the longest period of continuous wheat cropping (9 y) and low concentrations of *Ggt* DNA at the start of the trials (Table 5.1). However, in Experiments 1 and 2, the addition of *Ggt* inoculum to this soil caused large increases in disease severity. In Experiment 3, the addition of a small amount of the non-sterilised P7 to the γ-irradiated base soil caused a reversal of the expected decrease in disease levels due to inoculation. These results indicate that the mechanism of the suppressiveness in the soil could be specific, but it was not well expressed in the original bulk soil of Experiments 1 and 2. A similar result was reported by Andrade *et al.* (1994a), who found that one of the three soils investigated for suppressive mechanisms demonstrated a relatively low level of activity in the original soil. However, when they transferred a small amount of it to a chemically and physically different soil (to constitute 1% of the mixture), the mixed soil became suppressive to take-all and increased shoot dry weights. However, the authors did not provide any explanation for this

phenomenon. In this research, no bacterial and fungal colonies was found growing on any of the culture plates of the γ-irradiated base soil when checked for its sterility, and so the likelihood of inadequate or uneven removal of any biologically active microorganisms, which might affect the suppressiveness of soil P7 in the transference trial (Experiment 3) was dismissed. However, most soil microorganisms are not culturable using conventional isolation techniques, and so it was possible that some, which were resistant to the irradiation treatment, could have remained in the soil. A later study that characterised the microbial populations in the roots and rhizosphere of plants grown the five test soils in this PhD programme, however, showed that soil P7 was indeed a conducive soil that had similar microbial profiles as that of the conducive ryegrass soil, H15 (Chapter 6).

Test results indicating low concentrations of soil *Ggt* DNA identified the low levels of inoculum in soil samples. In P7, the initially low concentrations of soil *Ggt* DNA may have been due to the pre-treatment of seeds in 2003/04 with the systemic fungicide, Raxil® (Bayer Crop Science, Auckland, New Zealand), whose active ingredient is tebuconazole, which is of the triazole group (Young 2008). This compound has been reported to reduce the growth of *Ggt* on culture plates (Cotterill 1991), but not to reduce take-all incidence in a pot trial where the fungicide was applied to the *Ggt*-inoculated soils, although it did cause an increase in the dry weights of roots and shoots in the wheat plants (Cotterill *et al.* 1992). A similar field trial that used wheat seeds treated with fluquinconazole (another triazole fungicide), reported decreases in take-all severity while crop yield increased (Dawson & Bateman 2001). In that study, the fungal communities on the roots of wheat plants collected from the field were also identified using culture-based methods. The seed treatment was found to have little effect on the overall fungal community, but did affect the isolation frequencies in some groups, in particular increasing frequency of *Microdochium* sp. (formerly known as *Idriella* sp.) and decreasing frequency of *Fusarium avenaceum*, especially in the months when take-all symptoms were less severe.

The methods employed in this chapter provided ways to measure suppressiveness in different wheat soils, to distinguish some suppressive mechanisms, and have allowed identification of TAD soils in New Zealand. Of the soils tested, H1 and H3 provided specific suppression, which was transferable between soils indicating the role of specific biological factors. Soil M2 appeared to have a general suppression, which was not transferable, but also showed some involvement of biological components in its suppression. However, more investigations involving chemical fumigating and heat-

treating the soils at different temperatures, are required to validate the mechanisms of suppression in these soils. Future work will also attempt to characterise and compare the microbial profiles in the roots of plants grown in soils exhibiting the two forms of suppression and to identify the types of microorganisms associated with them (Chapter 6).

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Chapter 6
Characterisation of the microbial populations in the rhizosphere and the roots of wheat plants grown in soils with different suppressiveness to take-all using denaturing gradient gel electrophoresis

6.1 Abstract

Take-all, caused by the soilborne fungus, *Gaeumannomyces graminis* var. *tritici* (*Ggt*) is an important root disease of wheat. Monoculturing of wheat has been shown to induce take-all decline (TAD), a natural suppression of the disease thought to include the combined effects of both general and specific suppressions. Reports of research trials using a range of microorganisms, including the proteobacteria, ascomycetes and actinobacteria isolated from TAD soils, have demonstrated control of *Ggt* through three-way interactions among the pathogen, the host, and an antagonistic or a competing microorganism, indicating their potential as biocontrol agents. This study used the molecular technique, denaturing gradient gel electrophoresis (DGGE), to investigate the numbers and diversity of the microorganisms in the rhizosphere and roots of wheat plants grown in five New Zealand soils (H15, P7, H1, H3 and M2) which represented different types of take-all suppressiveness. The DGGE analysis of amplified rDNA sequences, which represented four microbial groups, bacteria, fungi, actinomycetes and ascomycetes, revealed consistent banding patterns for soils of different take-all suppressive characteristics, with diversities ranging from 19-30 genera. Since the microbial diversities did not vary between the rhizosphere and roots of wheat plants grown in these soils, the richness of microbial flora was clearly not associated with the suppressiveness. Cluster analyses of the banding profiles of the soils however, showed that the conducive soils, H15 and P7, were most similar in their banding patterns (similarity = 0.76), followed by the specific suppressive soils, H1 and H3 (similarity = 0.67), whereas, the general suppressive soil, M2 (similarity = 0.58), was the most different from the other soils. Principal component analysis indicated that the actinomycetes, *Glycomyces* sp. *Streptomyces* sp., *Actinosynnema violaceoruber*, *Hongia* sp. and *Actinokineospora diospyrosa*, were most commonly associated with take-all conduciveness, and some microorganisms were associated with the suppressiveness in TAD. The actinomycetes *Streptomyces bingchengensis*, *Terrabacter* sp. and *Nocardioides* sp., and ascomycetes *Fusarium lateritium* and *Microdochium bolleyi*, were unique to the take-all suppressive soils (H1, H3 and M2), but not the conducive soils (H15 and P7).

Further differentiation of the specific from the general suppressive soils was associated with presence of the proteobacteria *Pseudomonas putida* and *P. fluorescens*, the actinomycete *Nocardioides oleivorans*, ascomycete *Gibberella zeae*, and basidiomycete *Penicillium allii*, in the specific suppressive soils (H1 and or H3). Nearly all the distinguishing microorganisms identified in these suppressive soils (both specific and general), have been reported to antagonise *Ggt* or other soilborne pathogens to some degree. They are therefore likely to have specific functions in the suppressiveness of these soils to take-all.

Keywords: denaturing gradient gel electrophoresis, DGGE, *Gaeumannomyces graminis* var. *tritici*, take-all, suppressive soils, take-all decline, microbial populations

6.2 Introduction

The occurrence of soils suppressive to take-all, a root disease of wheat caused by the soilborne fungus *Gaeumannomyces graminis* (Sacc.) von Arx & Oliver var. *tritici* Walker, is well documented (Gerlagh 1968; Shipton *et al.* 1973; Cook & Rovira 1976; Andrade *et al.* 1994a, 1994b). In these soils, disease severity is reduced even when the pathogen is present, and the environmental factors are suitable for the development of the disease (Cook & Baker 1983). A severe outbreak of take-all within a 4-6 y monoculture of wheat, can induce development of a natural suppression known as take-all decline (TAD) (McSpadden Gardener & Weller 2001). Past studies have indicated that the mechanisms involved in TAD include the combined effects of both 'general' and 'specific' suppressions (Graham & Mitchell 1999; Cook 2003). General suppression develops when the soil conditions are suitable for many antagonistic activities by the microbiota in the rhizosphere soil and roots, with no single microorganism or a specific group of microorganisms being solely responsible for the suppression (Cook 2003; Janvier *et al.* 2007). Specific suppression operates against a background of general suppression, but is more qualitative, owing to the more specific effects of the individual, or selected groups of antagonistic microorganisms, present in the rhizosphere soil and in young lesions on the infected roots (Graham & Mitchell 1999; Janvier *et al.* 2007).

Many microorganisms representing different taxonomic groups have been isolated from TAD soils, and shown to control *Ggt*. The reported control mechanisms include cross-protection of the roots by the non-pathogenic *Gaeumannomyces graminis* and *Phialophora graminicola* (Deacon 1976; Wong *et al.* 1996; Zriba *et al.* 1999), hyphal lysis of *Ggt* by an

unidentified sterile red fungus (Shankar *et al.* 1994; Aberra *et al.* 1998), antibiosis by a *Trichoderma* sp. (Simon 1989; Duffy *et al.* 1996), a *Bacillus* sp. (Kim *et al.* 1997), a *Pseudomonas* sp. (Weller 1983; Weller & Cook 1983; Weller *et al.* 1988; Raaijmakers & Weller 2001) and a *Penicillium* sp. (Hornby *et al.* 1998), as well as competition by *Microdochium bolleyi* (Kirk & Deacon 1987b, 1987a). Many Actinomycetes, in particular *Streptomyces* spp., are also reported to be associated with TAD soils (Zogg & Jäggi 1974; Sivasithamparam & Parker 1978; Andrade *et al.* 1994b). However, since the TAD characteristics of soils are lost through autoclaving at 121°C and steam pasteurisation at 60°C, it is clear that heat-resistant microorganisms, especially the actinomycetes, are not involved in TAD (Weller *et al.* 2002), leaving the heat-sensitive microorganism(s) (e.g. *Pseudomonas* spp.) as probable agents of the suppressiveness (Cook & Rovira 1976). Since so many different spices of microorganisms have been reported to control *Ggt*, it seems likely that different microorganisms are involved in take-all suppression in different sites.

Most of the studies that attempted to identify the causes of TAD focused on the three-way interaction among the pathogen, the host, and the potential biocontrol agent. They did not consider the roles of other interspecies interaction in the roots and rhizosphere, and their ecological context. Since many studies have reported that the microbial diversity and abundance within the wheat rhizosphere changed with successive years of wheat cropping (Cook & Rovira 1976; Sarniguet & Lucas 1992; Raaijmakers *et al.* 1997; Raaijmakers & Weller 1998), it is relevant to investigate the relationships between soil suppressiveness in TAD and the composition of the microbial communities in the roots and rhizosphere soil.

In the past, the techniques used to study the soil microbial communities have depended upon cultural methods for estimating size and diversity of the microbial populations (Garbeva *et al.* 2004). However, it is widely acknowledged that these methods have underestimated numbers of species responsible, as only 1% of the bacteria and 17% of the fungi known to be present in the soil, can be cultured by common laboratory techniques (Hawksworth 1991; Bridge & Spooner 2001; Kirk *et al.* 2004). Other techniques such as direct plate counts, analysis of carbon utilisation in BIOLOG microplates and fatty acid methyl ester analysis, have also been used to estimate the microbial diversity or their activities (in the case of enzyme production), but cannot identify the microorganism in soil communities (Kirk *et al.* 2004; Mazzola 2004).

Molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), have been able to characterise/profile the soil microbial communities and to identify the dominant microorganisms present (Muyzer *et al.* 1993; Kowalchuk *et al.* 2003; Garbeva *et al.* 2004). DGGE involves total DNA extraction from environmental samples, and subsequent amplification of rDNA segments (most commonly) by using PCR with universal primers, which target the 16S region of bacteria and ITS, 18S or 28S regions of fungi. The double stranded PCR products have a GC clamped end to avoid complete disassociation, and are subjected to a DGGE gel containing a linearly increasing gradient of denaturing chemicals. As the DNA fragments reached higher levels of denaturing chemicals, they begin to disassociate at a point in the gel that is determined by the sequences of the fragments (Muyzer *et al.* 1993). Once the double strands are split completely (but held together by the GC clamp) they are physically prevented from migrating any further in the gel. As the sequences differ for different microbial species, this method can be used with a mixture of numerous species, with each band in the profile representing a taxon (O'Callaghan *et al.* 2003). Subsequent cloning and sequence analysis of the excised bands from the DGGE profiles, can provide information on the identities of the microorganisms through database searches (Muyzer *et al.* 1993; O'Callaghan *et al.* 2006).

In a previous study (Chapter 5), the suppressive soils in New Zealand were found to exhibit both general and specific types of suppression. This chapter aims to use PCR-DGGE to characterise and compare the microbial communities within the roots and rhizosphere of plants grown in soils, which demonstrated conduciveness and suppressiveness (both specific and general suppression) to take-all, and to identify the microorganisms associated with them by addressing the following questions:

1. Do the microbial communities in the rhizosphere and roots, as determined by DGGE, differ between general and specific suppressive soils?

2. Which microorganisms are associated with the specific suppressiveness?

3. Is the whole microbiota responsible for the general suppressiveness?

4. Are soil microorganisms associated with the conduciveness of soils to take-all?

The initial part of this chapter reports the genetic identities of the *Ggt* isolates used as inocula in prior experiments in this PhD programme (Chapters 2 to 5). The second part

reports the similarities and differences of the microbial DGGE fingerprints associated with soils of different take-all suppressiveness, by performing an image analysis of the rDNA fingerprints. Finally, the distinguishing bands that were revealed by the PCR-DGGE analysis were cloned and identified by sequence analysis.

6.3 Materials and methods

6.3.1 Part 1: Confirmation of *Ggt* isolates used in the pot assays

6.3.1.1 *Ggt* isolates

Eleven New Zealand *Ggt* isolates (New Zealand Institute for Crop & Food Research Ltd.) and one isolate from Germany (German National Resource Centre for Biological Material, DSMZ), were included in this study (Table 6.1). Among the New Zealand isolates, 10 were from the roots of wheat and one was from the rhizomes of *Elytrigia repens*. The five isolates used as a mixed inoculum in all the pot experiments in Chapters 2 to 5 (shaded in Table 6.1) were pathogenic to wheat (Appendix 3, A3. 2).

Table 6.1 The origins of the twelve *Ggt* isolates used in this study. The five isolates shaded in grey were used as a mixed inoculum in Chapters 2-5.

Ggt isolate	Country	Plant / host source
A3SL4	New Zealand	Rhizomes of *Elytrigia repens*
H9T3R1/1.2	New Zealand	Roots of wheat
BIOMILL	New Zealand	Roots of wheat
H9T3R3	New Zealand	Roots of wheat
BIOMILLSC3	New Zealand	Roots of wheat
H11T3R1/3	New Zealand	Roots of wheat
BIO3	New Zealand	Roots of wheat
WF99/3	New Zealand	Roots of wheat
BIO4B	New Zealand	Roots of wheat
BIO4A	New Zealand	Roots of wheat
MAL 1/8	New Zealand	Roots of wheat
DSMZ12044	Germany	Roots of wheat

6.3.1.2 DNA extraction from isolates

Potato dextrose agar (PDA) discs (1 mm dia.) from colony edges of 8 d old *Ggt* cultures (maintained at 23°C), were transferred separately to Petri plates (5 cm dia.) containing 5 mL of potato dextrose broth (PDB). After 2 wk incubation at 22°C, mycelial mats were lifted from the PDB with sterile 1 mL pipette tips and gently pressed between wads of 3-4

sterile paper towels, folded in half, to remove the excess liquid. For each isolate, mycelium (100 mg) was transferred into a 1.5 mL microfuge tube (Raylab NZ Ltd., Auckland, New Zealand), and DNA extraction was performed using a Puregene plant DNA extraction kit (Progenz NZ Ltd., Auckland, New Zealand) according to the manufacturer's instructions. The final pellet was suspended in 100 µL of sterile water. The quantity and quality of the extracted genomic DNA (gDNA) fragments of each *Ggt* isolate were then determined via gel electrophoresis of 5 µL aliquots loaded onto a 1% (w/v) agarose gel stained with ethidium bromide (Appendix 4, A4. 5). The gels were then visualised under UV and the bands compared to a high DNA mass ladder (Invitrogen, Auckland, New Zealand). Where necessary, the gDNA suspension was diluted with sterile Millipore filtered water to give a final concentration of 10-50 ng μL^{-1} (i.e. the recommended concentration for a 25 µL PCR reaction mixture).

6.3.1.3 The amplification and sequencing of the ITS-region

The extracted gDNA of the 12 *Ggt* isolates were subjected to PCR using the universal primers ITS4 (5′-TCCT CCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′), which target the amplification of the ITS1, 5.8s and ITS2 regions of the ribosomal DNA (rDNA) complex (White *et al.* 1990). Amplifications were performed in 25 µL reaction mixtures containing the following:

- 1×buffer (Roche diagnostics NZ Ltd., Auckland, New Zealand).
- 200 µM each of dATP, dTTP, dGTP and dCTP (Roche diagnostics NZ Ltd., Auckland, New Zealand).
- 0.2 µM of each primer (Invitrogen New Zealand Ltd, Auckland).
- One unit of Taq DNA polymerase (Roche diagnostics NZ Ltd., Auckland, New Zealand).
- UltraPure™ DNase/RNase-free distilled water (Invitrogen New Zealand Ltd, Auckland).
- 10-50 ng of template DNA.

A reaction, with no template DNA added, was used as a negative control to test for the presence of DNA contaminants in the reaction mixtures. PCR reactions, performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, U.S.A.), consisted of an

initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. After the 35 cycles, a completion step for extension at 72°C for 10 min was performed. Aliquots of 2 μL of the resulting amplicons were then assessed via electrophoresis on a 1% (w/v) agarose gel stained with ethidium bromide, and compared to a low DNA mass ladder (Invitrogen New Zealand Ltd., Auckland) to estimate the quantities. All the amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN, Biolab Ltd., Auckland, New Zealand), suspended in sterile Millipore filtered water to give final concentrations of 10-50 ng μL^{-1}, and sent to the National Centre for Advanced Bio-Protection Technologies, Lincoln University, New Zealand, for sequencing. The nucleotide sequences were compared to those available in the Genbank database (http://www.ncbi.nlm.nih.gov/Genbank/) using the Basic Local Alignment Search Tool (BLAST).

6.3.2 Part 2: Characterisation of the microbial populations associated with take-all suppressiveness

6.3.2.1 DNA extraction from the root/rhizosphere samples

DNA extraction was performed on roots of wheat (*Triticum aestivum* L.) plants grown in five soils (H1, H3, P7, M2 and H15) of varying suppressiveness to take-all. The cropping histories of these five soils and some information on the mechanisms of suppression as determined in the pot experiments previously conducted (Chapter 5, Experiments 1, 2 and 3), are summarised in Table 6.2. H1 and H3 were TAD soils with the characteristics of specific (S) suppression with high levels of biological activities, and the suppressiveness was transferable to another soil. M2 was a general (G) suppressive soil with variable levels of biological activities present, and its suppressiveness was not transferrable to another soil. Soil H15, which was from a field cropped with 5 y of perennial ryegrass (*Lolium perenne* L.), and had high levels of biological activities present, was included as a control (i.e. conducive, C). Soil P7 (C/S), when in bulk, showed similar levels of conduciveness to take-all as soil H15, and had variable levels of biological involvement. However, when it was transferred into the sterile base soil (1% in the base soil), it was able to cause a decrease in disease levels due to inoculation (Chapter 5).

Table 6.2 Characteristics of the five soils, which were determined in the Chapter 5 studies, including their wheat cropping histories, increases in disease severity (TAR) after the addition of *Ggt*, and potential mechanisms of suppressiveness, used in the current DGGE study.

Field code	Years of wheat	[1]Increase in TAR after the addition of *Ggt*	Levels of biological involvement	Transferability of the suppressiveness into another soil (Yes/No)	[2]Mechanism of suppression (abbreviation)
M2	4	+6	Variable	No	General (G)
H1	3	0	High	Yes	Specific (S)
H3	3	0	High	Yes	Specific (S)
P7	9	+56	Variable	Yes	Conducive (C) / specific (S)
H15	0	+52	High	No	Conducive (C)

[1]TAR represents take-all rating, a disease severity measure calculated from the formula of Dyke & Slope (1978):

TAR = 1(a) + 2(b) + 3(c)

Where,

a = % plants with slight infection (<25% of root area covered with lesions)
b = % plants with moderate infection (25-75% of root area covered with lesions)
c = % plants with severe infection (>75% of root area covered with lesions)

[2] The mechanisms of suppression in the soils were determined via investigating the transferability of the suppressive characteristics in these soils to a sterile base soil, and added with *Ggt* inoculum (Chapter 5).
G = the suppressiveness in the general suppressive soil was not transferrable to a sterile base soil.
S = the suppressiveness in the specific suppressive soil was transferable to a sterile base soil.
C = the soil was conducive to take-all, thus when it transferred to a sterile base soil, the base soil remained conducive to the disease.
C/S = the soil was conducive to take-all, but when it was transferred into a sterile base soil, it was able to cause a decrease in disease levels.

DNA was extracted from the roots/rhizosphere (pooled) of the two extra wheat plants grown in each of the non-autoclaved pots of soils, to which *Ggt* had been added (Experiment 2, Chapter 5). The rhizosphere is known to consist of three different regions: endo-rhizosphere which comprises the root tissues; rhizoplane which is the root bi-dimensional surface and ecto-rhizosphere, which represents the adjacent soil (Lynch 1990; Lemanceau *et al.* 1995) (in: Botelho & Mendonca-Hagler 2006). Plants were taken from the *Ggt* inoculated soils because this was the treatment used to measure the differing take-all suppressiveness (Chapter 5). In the Experiment 2 pot assay (Chapter 5), there were four replicates of each soil sample for the *Ggt* treatment, with two plants per replicate pot being carried over into the current study, to give four replicates of roots/DNA samples of each soil. In order to include only the microorganisms in the closely adhered rhizosphere and rhizoplane soils, and the endophytes in the endo-rhizosphere zone for gDNA extraction

(Gobat *et al.* 2004), nearly all the excess soil on the roots of the two plants was shaken off and the roots were not washed.

Extraction was conducted according to the protocols used by Russell and Bulman (2005) with modifications. The roots were cut into small pieces (about 1 cm in length), with scissors that had been wiped with handy towels moistened with 95% alcohol, dipped in 95% alcohol and flame sterilised between pot replicates. Cut roots from each pot were then pooled and mixed on a piece of sterilised aluminium foil and a 100 mg sub-sample placed in a 2 mL screw cap microfuge tube (Raylab NZ Ltd., Auckland, New Zealand) containing 1 g of stainless steel beads (2.3 mm dia.). After addition of 1 mL of cetyltrimethylammonium bromide (CTAB) buffer (20 mM Tris pH 8, 20 mM EDTA, 0.8 M NaCl and 2% CTAB) and 200 µL of 5% sarcosyl (5% w/v aqueous solution of N-lauryl sarcosine sodium salt), the roots were disrupted using a MiniBeadbeater8™ cell disrupter (Biospec Products, BioLab Scientific NZ). In order to prevent over-heating and breaking of the microfuge tubes in the bead-beating motion, disruption was performed twice at 1 min each time, with a 1 min cooling in ice between the two disruptions. The tubes were then incubated at 65°C for 1 h. Impurities were extracted from the gDNA of the disrupted roots by adding 800 µL of chloroform:iso-amyl alcohol (24:1), mixing, centrifuging at 13, 000 rpm for 1 min, and subsequently transferring the supernatant to a clean tube. An equal volume of 100% isopropanol was then added to the supernatant (to precipitate the gDNA) and the tube was centrifuged at 13, 000 rpm for 5 min. The resultant pellet was washed in 500 µL of 70% ethanol, air-dried at room temperature for 30 min, and then suspended in 50 µL of 10 mM TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The quantity and the quality of the extracted DNA were then determined by running an aliquot (2 µL) on a 1% agarose gel and comparing it to a high DNA mass ladder (Invitrogen New Zealand Ltd., Auckland) as described in Section 6.3.1.2. The root/rhizosphere samples each yielded 10 to 30 ng μL^{-1} of gDNA. All gDNA samples were serial diluted prior to PCR amplifications. Samples from soil H3 were diluted by 20 times (i.e. 1:20), while those from soils H1, M2, P7 and H15 were diluted by 10 times (1:10) using molecular grade water.

6.3.2.2 PCR amplification of group targeted rDNA gene fragments for DGGE analysis

Universal primers, that targeted the rDNA sections of the 16S and the ITS regions of the general bacterial (F984GC and L1401 or R1378) and fungal (ITS1FGC and ITS2)

populations (Table 6.3) were used to amplify these regions from the total extracted gDNA template (1 µL) of each root/rhizosphere sample. Group-specific primers targeting the rDNA sections of the actinomycetes (16S) (primers, F243 and R513GC) and the ascomycetes (ITS) (primers, ITS4Asco and ITS3GC) that may have been present in the samples, were also employed (Table 6.3). A GC-rich sequence was attached to the 5′ end of one of a primer set, to prevent complete melting of the PCR products during separation in the denaturing gradient gel (Heuer *et al.* 2001) (Table 6.3). The product sizes yielded by these primer sets (about 300-400 bp), were suitable for separation by DGGE (Nikolcheva & Bärlocher 2004). The rDNA of the four *Ggt* isolates (A3SL4, H9T3R1/1.2, BIOMILLSC3 and H11T3R1/3, Section 6.3.1.3) used as a mixed inoculum in the previous study (Chapter 5), was also amplified (ITS regions) with the same primer set specific for the fungal populations, and these amplicons were later included in the fungal denaturing gradient gel, to act as markers (Section 6.3.2.3). The volume of the reaction mixture and the final concentrations of the reagents were as described in Section 6.3.1.3. PCR reactions, performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, U.S.A.), consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the respective temperatures for each primer set (Table 6.3) for 60 s, and extension at 72°C for 1 min. After the 35 cycles, a final extension step at 72°C for 10 min was performed. The resulting amplicons were then assessed via gel electrophoresis as described in Section 6.3.1.3.

Table 6.3 Primers used to target and amplify the rDNA gene regions of bacteria, fungi, actinomycetes and ascomycetes.

rDNA target	[1]Primer	[2]Sequence (5′→3′)	Annealing temp	Reference
Bacteria (16S)	F984GC L1401 or R1378	gc-AACGCGAAGAACCTTAC C*GGT*GTGTACAAGGCCCGGGAAGG	60°C	Heuer & Smalla (1997) and Hiddink *et al.* (2005b)
Fungi (ITS)	ITS1FGC ITS2	gc-CTT*GGT*CATTTAGAGGAAGTAA GCTGCGTTCTTCATCGATGC	55°C	White *et al.* (1990) Gardes & Bruns (1993)
Actinomycetes (16S)	F243 R513GC	GGATGAGCCCGCGGCCTA gc-CGGCCGCGGCTGCTGGCACGTA	63°C	Heuer *et al.* (1997)
Ascomycetes (ITS)	ITS4Asco ITS3GC	[3]CGTTACTRRGGCAATCCCTGTTG gc-GCATCGATGAAGAACGCAGC	55°C	Nikolcheva & Bärlocher (2004); White *et al.* (1990)

[1]F and R indicate forward and reverse primers, respectively.
[2]gc indicate the G+C-rich sequence (cgcccgccgcgcgcggcgggcggggcgggggcacgggggg) attached at the 5′ end.
[3]Nucleotides AA were used in place of RR in this study.

6.3.2.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed using the DCode Mutation Detection System (BioRad, Life Science Research, Hercules, California). The polyacrylamide gel (BioRad, Life Science Research, Hercules, California) was made to the concentration of 8% w/v (using 40% acrylamide:bisacrylamide 37.5:1) as recommended by the manufacturers for separating double stranded rDNA sized 200-400 bp. The amplified rDNA products (in aliquots of 8 μL) were separated with the respective denaturing gradients specific for the four microbial groups as outlined in Table 6.4. These gradients were modified from those references listed in Table 6.3 and were found to produce good resolutions of separation. Hence, they were the most suitable for the current study. The 100% denaturant consisted of 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in 1×TAE buffer at 58°C, and constant voltage of 85 V for 16 h. The gels were silver-stained according to the protocols of Heuer *et al.* (2001), sandwiched between a Gelbond^{TM} sheet (BioRad, Life Science Research, Hercules, California) and a sheet of cellophane, and dried overnight at 60°C in an oven to allow long term storage of the gels.

Table 6.4 Vertical denaturing gradient of the 8% polyacrylamide gels used in DGGE to separate the double stranded rDNA of the targeted microbial groups.

Microbial group	[1]Vertical denaturing gradient
Bacteria	45-55%
Fungi	30-38%
Actinomycetes	55-70%
Ascomycetes	35-50%

[1] 100% consists of 7 M urea and 40% formamide

6.3.2.4 Band scoring and statistical analyses

The computer software package GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyse the DGGE banding profiles. To compare band profiles across

soils, gels were first normalised using selected bands present on a single gel. Each band was assigned a number with increasing order, representing its distance of migration (expressed as percent) from the top of the gel. For each sample replicate, bands at each migration point were scored as either present or absent. The resulting binomial data was then subject to cluster analysis using the Jaccard similarity coefficient, and the un-weighted paired-group method with arithmetic mean (UPGMA) (Manly 2005). For each microbial group, the distinguishing bands that differentiated the soils from one another were determined by principal component analyses (PCA). In this comparison, a band was considered present only when it was in the same position in at least three of the four replicates in one soil, but was treated as absent if it was only present in one or two replicates of the soil. A summary of the bands present and the assigned band numbers in the individual soils for the four microbial groups are in Appendix 5, A6. 2. All analyses were performed with the statistical package GenStat Release 9.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2007).

6.3.2.5 Recovery, re-amplification, cloning and sequencing of the distinguishing DGGE bands

The distinguishing DGGE bands, as determined from PCA, were excised from the dried gels, re-amplified, cloned and sequenced. Excision was done by cutting the innermost portion (about 2 mm in length) of each band with a new sterile scalpel to avoid contamination of other DNA bands. DNA was eluted from the polyacrylamde matrix, by placing the excised band in a 1.5 mL microfuge tube (Raylab NZ Ltd., Auckland, New Zealand) containing 50 µL of 1×TAE buffer (pH 8), and breaking the gel matrix with a disposable sterile loop before incubating at 50°C for 2 h with occasional vortexing. Re-amplification of the recovered band was performed using 1 µL of this mixture as template and the same thermal cycler conditions as for the original amplification for DGGE analysis (Section 6.3.2.2) with the exception of replacing the GC-clamped primer with the same primer minus the clamp sequence. Sub-samples of amplified products of the bacterial and fungal populations were initially sequenced (National Centre for Advanced Bio-Protection Technologies, Lincoln University, New Zealand). However, these resulting sequences revealed the presence of more than one microorganism in many of the products. Hence, a decision was made to clone all the amplified products using either the PCR®4-TOPO® or the TOPO TA Cloning® Kits (Invitrogen, Auckland, New Zealand), following the methods of the manufacturer. Five clones were randomly selected from each amplified product

(representing one DGGE band), purified using QIAquick PCR Purification Kit (QIAGEN, Biolab Ltd., Auckland, New Zealand) and sequenced again (Macrogen sequencing services, Macrogen Ltd. Seoul, Korea). Sequences were compared to those available in the Genbank using the Blastn search to identify the microorganisms represented by the bands. The microorganism, which represented the majority of the five clones from each band, was considered the dominant specie that made up that band.

6.4 Results

6.4.1 Identity confirmation of the *Ggt* isolates

Using primers ITS4 and ITS5, the rDNA at the ITS regions of the 12 *Ggt* isolates were successfully amplified, consistently yielding fragment sizes of 600 bp and DNA concentrations of 15 ng μL^{-1}. The sequences of the 11 New Zealand *Ggt* isolates, including the ones used in a mixed inoculum in the pot bioassays in this PhD research, matched *Gaeumannomyce graminis* var. *tritici* in Genbank (Table 6.5). The sequence of the German isolate, DSMZ12044, was closest to an isolate of *Phialophora* sp., which is the anamorph of *Gaeumannomyces graminis* (Table 6.5). The nucleotide sequences of all the 12 *Ggt* isolates are in Appendix 5, A6. 1.

Table 6.5 Genbank ITS sequence comparisons for the 12 *Ggt* isolates showing their nearest relatives in Genbank.

Ggt isolate	Matched nucleotides (%)	Nearest relative (Blast) on queried sequence	Genbank accession No.
A3SL4	99	*Gaeumannomyces graminis* var. *tritici*	AF508155
H9T3R1/1.2	99	*Gaeumannomyces graminis* var. *tritici*	AF508155
BIOMILL	99	*Gaeumannomyces graminis* var. *tritici*	AF508155
H9T3R3	100	*Gaeumannomyces graminis* var. *tritici*	U17222
BIOMILLSC3	99	*Gaeumannomyces graminis* var. *tritici*	AF508155
H11T3R1/3	99	*Gaeumannomyces graminis* var. *tritici*	U17222
BIO3	100	*Gaeumannomyces graminis* var. *tritici*	U17220
WF99/3	99	*Gaeumannomyces graminis* var. *tritici*	AF508155
BIO4B	99	*Gaeumannomyces graminis* var. *tritici*	U17222
BIO4A	99	*Gaeumannomyces graminis* var. *tritici*	U17222
MAL 1/8	99	*Gaeumannomyces graminis* var. *tritici*	U17220
DSMZ12044	99	*Phialophora sp.*	U17216

6.4.2 Microbial communities in the rhizosphere and roots of wheat plants grown in soils of different suppressiveness

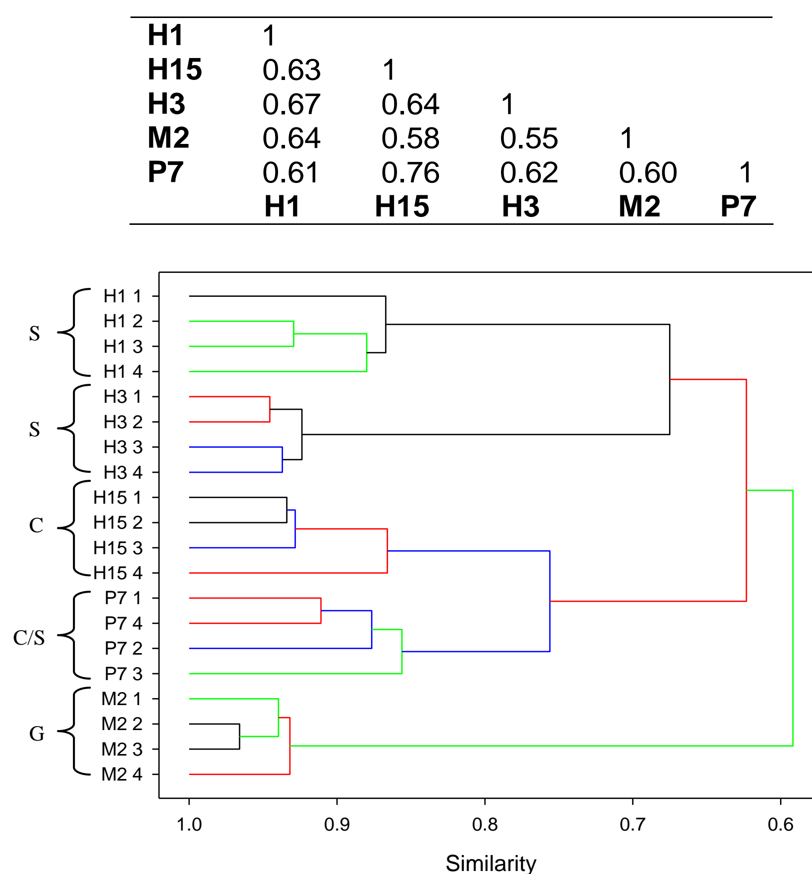
The banding patterns generated on the DGGE gels revealed 40, 44, 28 and 40 individual bands for the respective four microbial groups, bacteria, fungi, actinomycetes and ascomycetes. Table 6.6 shows the number of bands, which were present in at least three replicates of each of the soils. In general, there were large differences between individual soils, with the general bacterial population being the most diverse within the roots/rhizosphere. Soil H3 (S = specific suppression) had 31 bands, while soil H1 (S) produced the lowest number of bands (19 bands) (Table 6.6). Soils H15 (C = conducive), M2 (G = general suppression) and P7 (C/S) were similar in the diversities of their bacterial populations (about 23 bands). For the fungal population, soil H3 (S) also had the highest numbers of bands. Soils H1 (S) and H15 (C) produced a similar numbers of bands (25 and 24, respectively), whereas P7 (C/S) and M2 (G) produced similar numbers of bands (18 and 19, respectively). For actinomycetes, soil H3 (S) had the least number of bands (16), while the rest of the soils had similar numbers of bands. For ascomycetes, soil M2 (G) had the fewest bands (19) (Table 6.6).

Table 6.6 Number of bands present in at least three replicates of each soil for the four microbial groups. Data in parentheses are the total number of bands separated by DGGE for that particular soil.

	Number of bands present				
Type of suppression (abbreviation)	**Specific (S)**	**Specific (S)**	**General (G)**	**Conducive/ Specific (C/S)**	**Conducive (C)**
Microbial group soil	**H1**	**H3**	**M2**	**P7**	**H15**
Bacteria	19 (23)	31 (38)	24 (25)	23 (28)	23 (26)
Fungi	25 (33)	34 (39)	18 (22)	19 (29)	24 (34)
Actinomycetes	25 (33)	16 (16)	25 (29)	30 (32)	30 (32)
Ascomycetes	23 (23)	22 (23)	19 (19)	22 (23)	24 (25)

6.4.3 Similarities among the five soils in the banding patterns derived from DGGE analyses

Similarity analysis on the total 152 bands of the four DGGE gels showed greater differences between the banding patterns / profiles of different soils (range = 0.21), than between the replicates for a single soil (range = 0.03 to 0.09). The similarity matrix, showing the calculated means of each soil, and the resulting UPGMA dendogram, are presented in Figure 6.1. Cluster analysis of the soils showed that H15 (C) and P7 (C/S) were most similar in their banding patterns, followed by soils H1 (S) and H3 (S), whereas M2 (G) was the most different from the other four soils (Figure 6.1). The complete similarity matrix is shown in Appendix 5, A6.3a.



H1	1				
H15	0.63	1			
H3	0.67	0.64	1		
M2	0.64	0.58	0.55	1	
P7	0.61	0.76	0.62	0.60	1
	H1	**H15**	**H3**	**M2**	**P7**

Figure 6.1 The mean similarity matrix and dendogram showing the relationships among the five soils from cluster analysis of the 152 bands separated on the four DGGE gels specific for bacteria, fungi, actinomycetes and ascomycetes. Similarity is expressed as a value of the Jaccard correlation coefficient with a value of '0' indicating the soils had no bands in common (i.e. completely different), whereas a value of '1' indicated the soils had the same bands present (i.e. completely identical). The letters S, C and G represent specific suppressive, conducive, general suppressive soils, respectively. The numbers next to the soil codes are the replicate numbers.

6.4.4 Identification of bands that differentiated the soils

6.4.4.1 Principal component analyses

Principal component analyses (PCA) of all the bands present in all four microbial groups, showed that between the five soils, 88% of the variation was accounted for by the first three principal components (36, 34 and 18%, respectively). A scatter plot matrix of the first three principal components is shown in Figure 6.2. The plots represent three side views of a single cube formed by plotting the data with the three axes (components) in three dimensions. In this illustration, the cube is presented in two dimensions. In order to visualise the cube, the three faces have to be folded in. In the first principal component, the soils were differentiated into two groups, H1, H3 & M2 (suppressive soils) and P7 & H15 (conducive soils) (best represented in Figure 6.2, face a). In the second component, soil H3 (S) was separated from H15 & P7 (C and C/S, respectively), and M2 (G) was further separated from H1 & H3 (S) (best represented in Figure 6.2, face b). In the third component, soil H1 (S) was separated even further from H3, and H15 (C) was separated slightly from P7 (C/S) (best represented in Figure 6.2, face c).

For the four microbial groups, the bands responsible for distinguishing the soils in PCA are summarised in Table 6.7. The larger component coefficient (ignoring the negative sign) represents greater importance of that band class in the separation. As the aim was to identify key microorganisms that distinguished the different suppressive soil types (Chapter 5), only bands derived from the PCA (Table 6.7) which also met the following criteria were sequenced:

1) Band classes present only in H1 and/or H3, which were both specific suppressive soils with high levels of biological involvement.

2) Band classes present only in M2, a general suppressive soil by which the suppressiveness was non-transferable to another soil, and had fluctuating levels of biological component.

3) Band classes present only in H1, H3 and M2, which were all suppressive soils, but varied in their mechanisms of suppression.

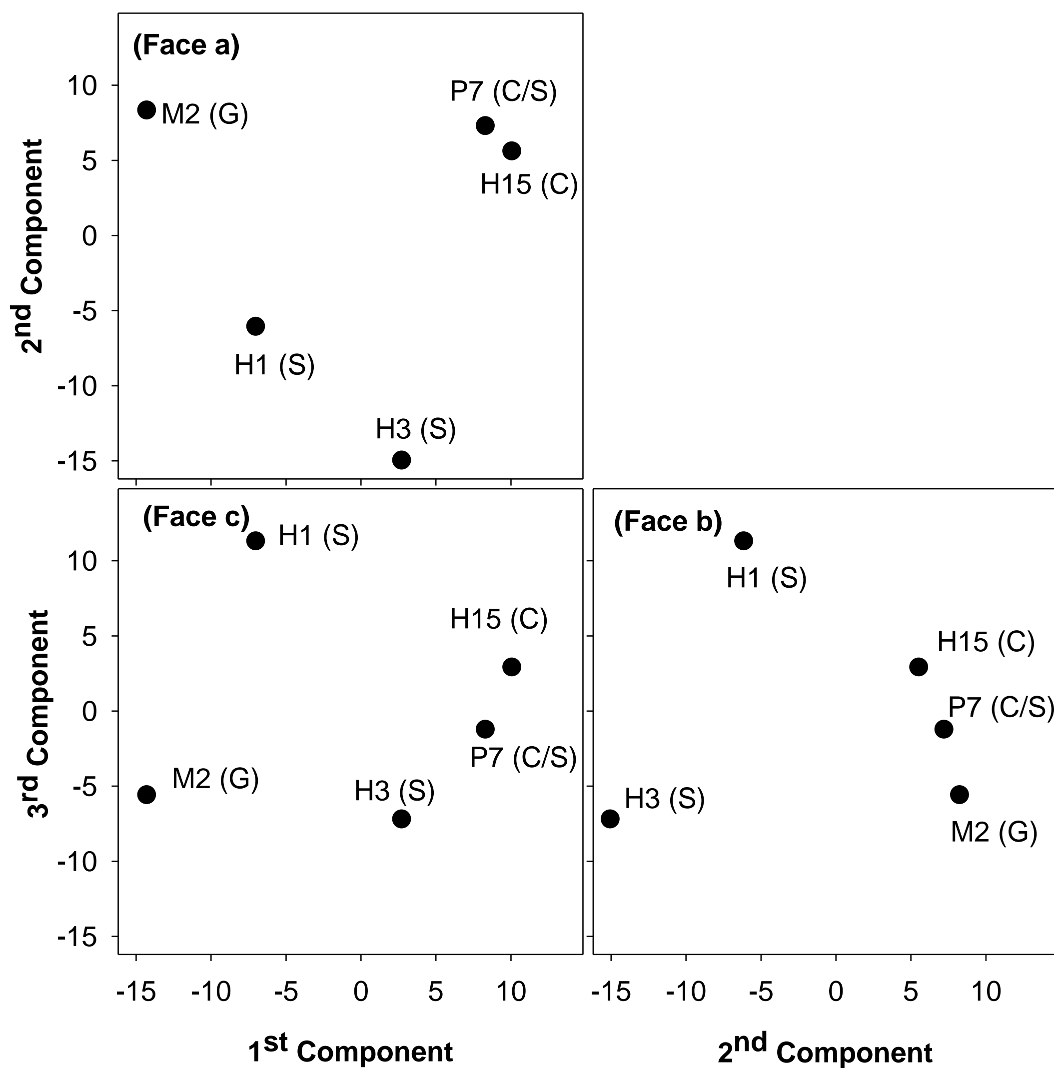


Figure 6.2 A scatter plot matrix of the first three principal components showing the separation of the five soils from the analyses on the bands separated on the four DGGE gels specific for bacteria, fungi, actinomycetes and ascomycetes, respectively. The plots represent three side views (faces a, b and c) of a single cube formed by plotting the data using the three axes (components) in three dimensions. The letters, C, S and G in parentheses, represent conducive, specific and general suppressive soils, respectively. The component coefficient refers to the length of the vector in relation to the principal component of interest.

4) Band classes present only in H15 (conducive) and P7 (conducive in the bulk soil, but found to be suppressive when a small amount of it was transferred to another soil).

5) Band classes present only in H15, P7 and M2 (conducive and general suppressive soils).

The number of distinguishing bands selected from the DGGE gels of bacteria, fungi, actinomycetes and ascomycetes were, seven, seven, six and four, respectively (highlighted in Table 6.7). When the four microbial groups were analysed separately, similar distinguishing bands were found responsible for the differentiation of soils as well, hence, the results of these are not presented. The DGGE band profiles of the four microbial groups, and the positions of the excised bands, are shown in Figures 6.3 and 6.4.

Table 6.7 a-c Results of the principal component analysis, showing the first three principal components and the bands responsible for the separation of the five soils. The number of replicates, in which the distinguishing bands occurred, is also included. Highlighted bands were excised, re-amplified, cloned and sequenced.

(a) 1st principal component

[1]Component coefficient	Band class no.	Microbial group	Present in no. of replicates				
			Soil				
			H1	H15	H3	M2	P7
0.197	16	Bacteria	0	4	4	0	4
-0.171	33	Bacteria	4	0	4	4	0
-0.171	36	Bacteria	4	0	4	4	0
-0.171	37	Bacteria	4	0	4	4	0
0.132	39	Bacteria	4	4	4	0	4
0.165	21	Fungi	2	4	4	0	4
-0.148	9	Fungi	4	1	4	4	0
-0.148	12	Fungi	4	1	4	4	0
-0.132	1	Fungi	0	0	0	4	0
-0.132	6	Fungi	0	0	0	4	0
0.135	5	Fungi	0	3	4	0	2
0.132	11	Fungi	4	4	4	0	4
0.132	13	Fungi	4	4	4	0	4
0.132	27	Fungi	4	4	4	0	4
0.132	28	Fungi	4	4	4	0	4
-0.197	33	Actinomycetes	4	0	0	4	0
0.171	10	Actinomycetes	0	4	0	0	4
0.171	26	Actinomycetes	0	4	0	0	4
0.171	29	Actinomycetes	0	4	0	0	4
0.171	30	Actinomycetes	0	4	0	0	4
0.152	22	Actinomycetes	0	4	0	0	3
-0.131	37	Actinomycetes	4	0	0	2	0
0.129	8	Actinomycetes	3	4	4	0	3
0.313	3	Ascomycetes	4	4	4	0	0
0.306	4	Ascomycetes	4	4	4	0	1
0.284	27	Ascomycetes	4	4	4	0	4
-0.284	1	Ascomycetes	0	0	0	4	0
0.284	5	Ascomycetes	4	4	4	0	4
0.284	10	Ascomycetes	4	4	4	0	4
0.284	11	Ascomycetes	4	4	4	0	4
0.284	19	Ascomycetes	4	4	4	0	4
0.269	17	Ascomycetes	4	0	4	0	0
-0.269	26	Ascomycetes	0	4	0	4	4
-0.261	12	Ascomycetes	0	1	1	4	4

(b) 2nd principal component

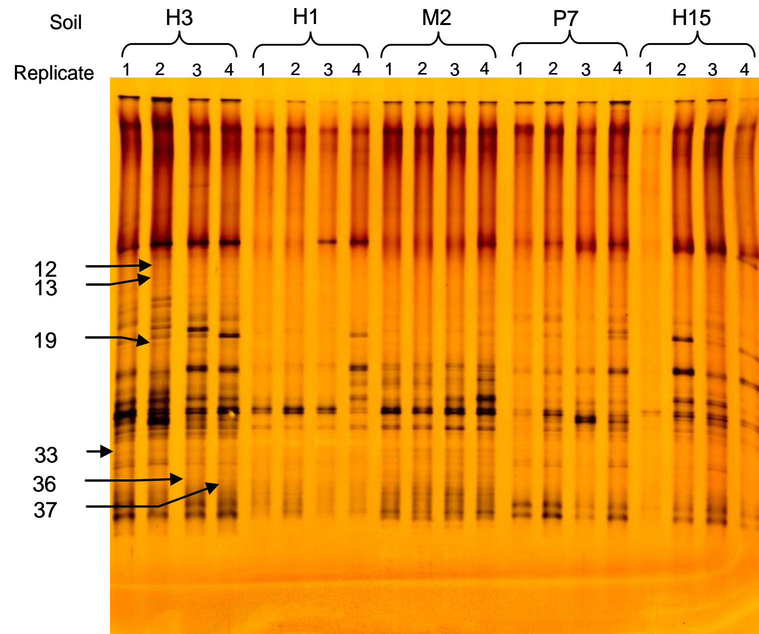
[1]Component coefficient	Band class no.	Microbial group	Present in no. of replicates				
			Soil				
			H1	H15	H3	M2	P7
-0.186	19	Bacteria	4	0	4	0	1
-0.159	13	Bacteria	1	0	4	0	0
-0.145	12	Bacteria	0	0	4	0	0
-0.124	33	Bacteria	4	0	4	4	0
-0.124	36	Bacteria	4	0	4	4	0
-0.124	37	Bacteria	4	0	4	4	0
-0.203	25	Fungi	4	0	4	0	0
0.184	35	Fungi	0	4	0	3	4
-0.139	34	Fungi	1	0	4	1	0
-0.132	14	Fungi	4	4	4	0	1
-0.122	33	Fungi	1	0	4	1	1
-0.120	10	Fungi	4	1	4	0	4
0.189	24	Actinomycetes	1	4	0	4	4
0.157	18	Actinomycetes	2	4	0	4	3
0.145	9	Actinomycetes	4	4	0	4	4
0.145	17	Actinomycetes	4	4	0	4	4
0.145	21	Actinomycetes	4	4	0	4	4
0.127	19	Actinomycetes	4	4	0	4	3
0.124	10	Actinomycetes	0	4	0	0	4
0.124	26	Actinomycetes	0	4	0	0	4
0.124	29	Actinomycetes	0	4	0	0	4
0.124	30	Actinomycetes	0	4	0	0	4
-0.203	17	Ascomycetes	4	0	4	0	0
0.203	26	Ascomycetes	0	4	0	4	4
0.145	16	Ascomycetes	4	4	0	4	4
0.145	24	Ascomycetes	4	4	0	4	4
-0.150	3	Ascomycetes	4	4	4	0	0
-0.132	4	Ascomycetes	4	4	4	0	1
0.127	12	Ascomycetes	4	4	0	4	3
-0.124	21	Ascomycetes	4	0	4	4	0

(c) 3rd principal component

[1]Component coefficient	Band class no.	Microbial group	Present in no. of replicates				
			Soil				
			H1	H15	H3	M2	P7
-0.131	12	Bacteria	0	0	4	0	0
-0.143	15	Bacteria	0	0	4	0	2
-0.153	25	Bacteria	1	4	4	4	4
-0.166	29	Bacteria	1	3	4	4	4
-0.204	17	Bacteria	0	4	4	4	4
-0.204	20	Bacteria	0	4	4	4	4
-0.204	24	Bacteria	0	4	4	4	4
-0.204	31	Bacteria	0	4	4	4	4
-0.204	34	Bacteria	0	4	4	4	4
-0.154	44	Fungi	0	0	4	0	4
0.233	11	Actinomycetes	4	4	0	0	4
0.153	37	Actinomycetes	4	0	0	2	0
0.137	19	Actinomycetes	4	4	0	4	3
0.131	9	Actinomycetes	4	4	0	4	4
0.131	17	Actinomycetes	4	4	0	4	4
0.131	21	Actinomycetes	4	4	0	4	4
-0.499	2	Ascomycetes	4	4	0	4	0
-0.446	24	Ascomycetes	4	4	0	4	4
-0.446	16	Ascomycetes	4	4	0	4	4
0.305	7	Ascomycetes	0	4	4	4	4
0.232	21	Ascomycetes	4	0	4	4	0
0.214	8	Ascomycetes	0	4	4	0	4
0.185	12	Ascomycetes	0	1	1	4	4

[1] The component coefficient in negative refers to the length of the vector in the opposite direction in relation to the principal component of interest.

(a) Bacteria



(b) Fungi

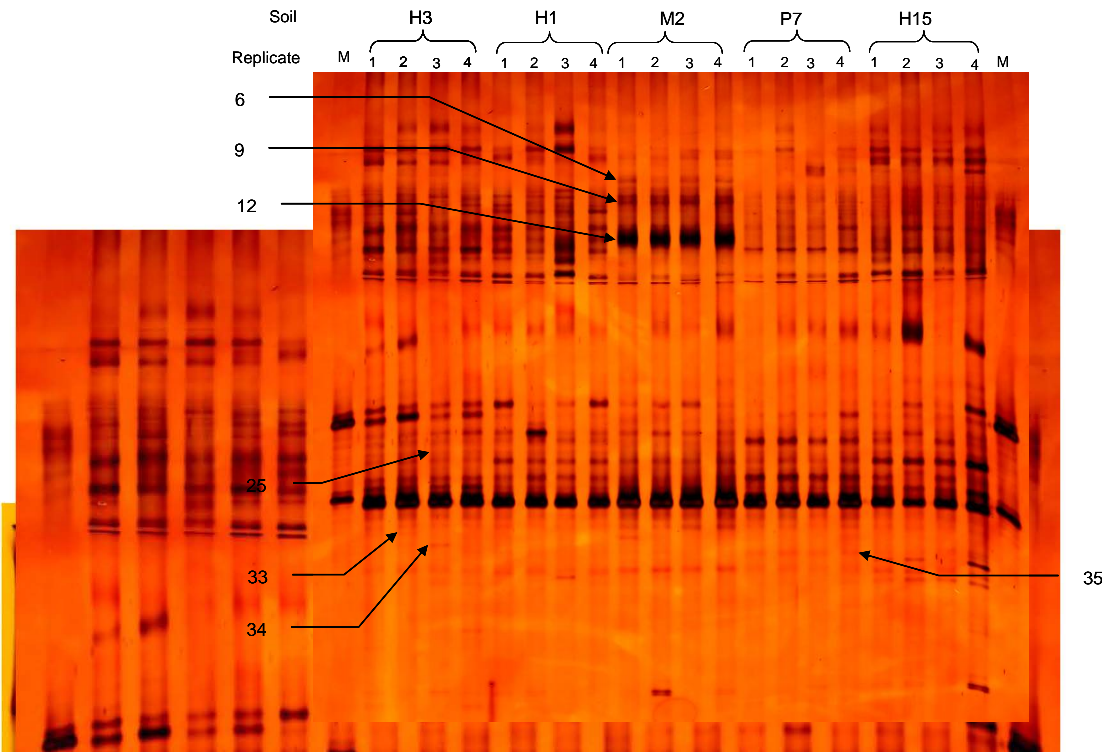
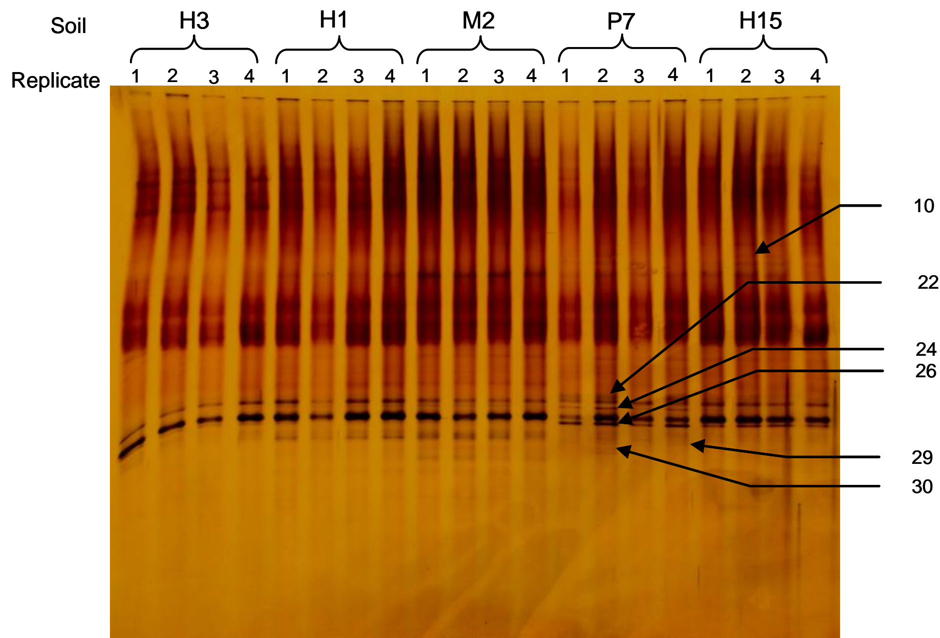
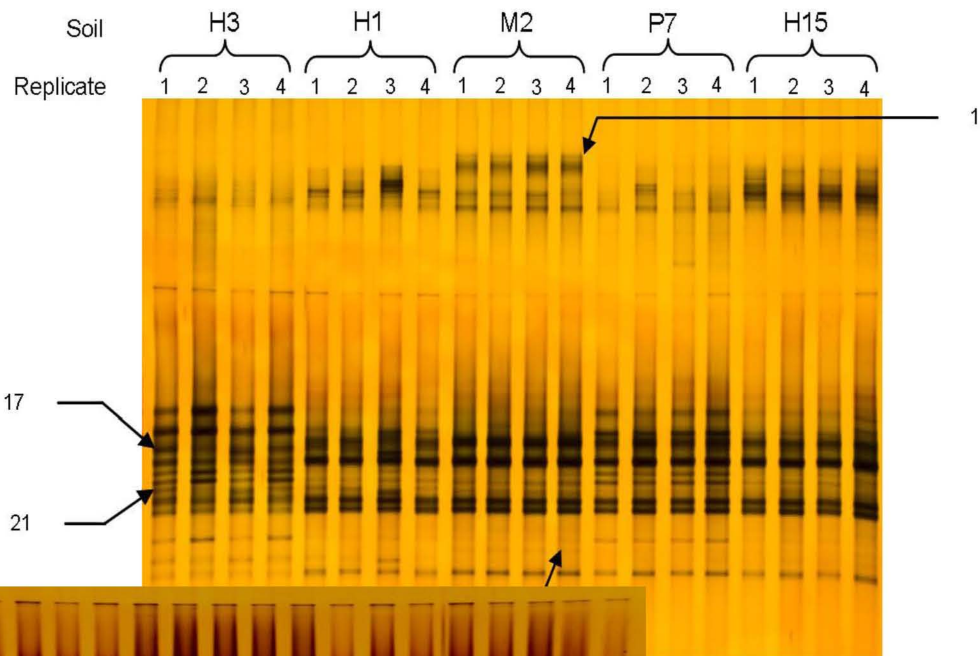


Figure 6.3 DGGE analyses with the primers specific for the general (a) bacterial and (b) fungal populations in the rhizosphere/roots of wheat plants grown in the five soils. M represents the marker lane, which comprised the four *Ggt* isolates. The numbers 1-4 in both gels represent the replicate number of the soil. Other numbers with arrows indicate the bands excised.

(a) Actinomycetes



(b) Ascomycetes



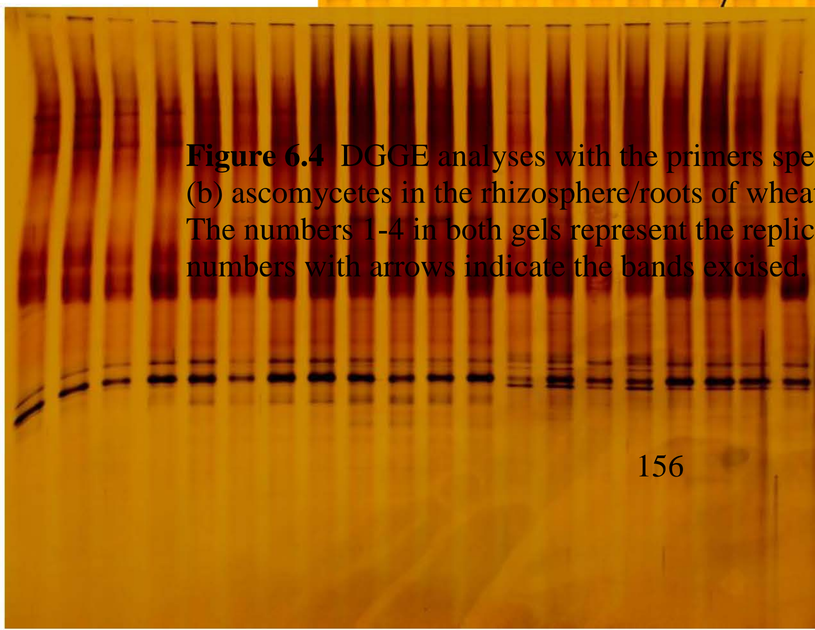


Figure 6.4 DGGE analyses with the primers specific for (a) actinomycetes and (b) ascomycetes in the rhizosphere/roots of wheat plants grown in the five soils. The numbers 1-4 in both gels represent the replicate number of the soil. Other numbers with arrows indicate the bands excised.

6.4.4.2 Sequence analysis of the distinguishing DGGE bands

Table 6.8 shows the microorganisms found in Genbank, whose sequences most closely matched those of the distinguishing DGGE bands. All the nucleotide sequences of the distinguishing bands blast searched in Genbank are in Appendix 5, A6. 4. Within the roots and rhizosphere of wheat plants grown in soils differing in their suppressiveness, there were four, five, five and seven distinguishing genera for the respective microbial groups, bacterial, fungal, actinomycetes and ascomycetes. In general, the dominating distinguishing bacterial genus was *Pseudomonas*, which was most common in the roots and rhizosphere of plants from specific suppressive soil, H3 (S). The genus, *Penicillium*, dominated among the distinguishing bands in the fungal DGGE profiles, while in the actinomycete DGGE profiles, most of the distinguishing bands belonged to the genus, *Streptomyces*. The remaining distinguishing bands from the ascomycete DGGE profiles were made up of various genera (Table 6.8).

The group of three suppressive soils (i.e. H1, H3 and M2) had six distinguishing bands in common, which were only present in the roots and rhizosphere of plants from these soils. Their sequences matched *Streptomyces bingchengensis*, *Terrabacter* sp., *Niocardioides* sp., *Fusarium lateritium*, *Microdochium bolleyi* and an uncultured fungal clone, whose next closest matched relative was *Mortierella elongata*.

When the soils were grouped according to their suppressive mechanisms, the specific suppressive soils, H1 and H3, shared two distinguishing microorganisms, *Nocardioides oleivorans* and *Gibberella zeae*. The sequences from the four distinguishing bands generated only in H3 soils, were identified as *Pseudomonas putida*, *P. fluorescens*, and *Penicillium echinulatum* and *P. allii*. As for the general suppressive soil, M2, sequences of the distinguishing bands matched those of *Penicillium echinulatum*. However, as soil H3 (S) also shared *P. echinulatum* as one of its distinguishing microorganisms, *P. echinulatum*, was not unique to soil M2 (G). Soil H1 did not produce any bands that would distinguish it from the other soils.

The similarity between the band profiles of soils H15 (C) and P7 (C/S) was largely due to five bands, which represented mainly actinomycetes. The nucleotide sequences of these bands, which were found only in these two soils, matched those of *Glycomyces* sp. *Streptomyces* sp., *Actinosynnema violaceoruber*, *Hongia* sp. and *Actinokineospora diospyrosa*.

Table 6.8 Identities of microorganisms, indicated by matching Genbank database sequences to the sequences derived from the distinguishing bands generated on the DGGE profiles specific for the bacterial, fungal, actinomycete and ascomycete rDNA.

DGGE band no.	Present in soil	[1]Mechanism of suppression	[2]Most closely related microbial sequence	Sequence similarity (%)	Accession no.
Bacterial 16S					
12	H3	S	*Pseudomonas putida*	100	DQ48475.1
13	H3	S	*Pseudomonas fluorescens*	100	EF672049.1
19	H1 and H3	S	*Nocardioides oleivorans*	94	AJ698724.1
33	H1, H3 and M2	S, S and G	*Streptomyces bingchengensis*	100	DQ449953.1
36	H1, H3 and M2	S, S and G	*Terrabacter* sp.	95	AF408951.1
37	H1, H3 and M2	S, S and G	*Nocardioides* sp.	96	EF466121.1
Fungal ITS					
6	M2	G	*Penicillium echinulatum*	100	AJ246146
9	H1, H3 and M2	S, S and G	*Fusarium lateritium*	100	AF310979
12	H1, H3 and M2	S, S and G	Uncultured soil fungus clone / *Mortierella elongata*	96 / 91	DQ420868.1 / AJ878534.1
25	H1 and H3	S	*Gibberella zeae*	100	AB250414.1
33	H3	S	*Penicillium echinulatum*	100	AJ246146
34	H3	S	*Penicillium allii*	99	AF218787
35	H15, M2, P7	C, G and C/S	*Pleospora herbarum*	97	DQ491516.1
Actinomycetes					
10	H15 and P7	C and C/S	*Glycomyces* sp.	98	EF212018.1
22	H15 and P7	C and C/S	*Streptomyces* sp.	100	EU216730.1
24	H15, M2 and P7	C, G and C/S	*Streptomyces globosus*	99	EU196532.1
26	H15 and P7	C and C/S	*Actinosynnema violaceoruber*	100	AB28426.1
29	H15 and P7	C and C/S	*Hongia* sp.	100	AB124389.1
30	H15 and P7	C and C/S	*Actinokineospora diospyrosa*	100	AF114797.1
Ascomycetes					
1	M2	G	*Penicillium echinulatum*	98	AF033473.1
17	H1 and H3	S	*Gibberella zeae*	99	AY188924.1
21	H1, H3 and M2	S, S and G	*Microdochium bolleyi*	99	AJ279454.1
26	H15, M2 and P7	C, G and C/S	*Penicillium dipodomyicola*	100	DQ339570.1

[1]Mechanism of suppression represented by the soil
S Specific suppression
G General suppression
C Conducive
C/S A conducive soil, which was able to cause decrease in disease levels due to inoculation, when a very small amout of it was transferred into a sterile soil.

[2]In the case where the closest match was from an unidentified / uncultured microorganism, the next closest known microorganism is also listed.

6.5 Discussion

Many studies have indicated that the onset of TAD is attributed to the microbial activities in soils (Rovira & Wildermuth 1981; Weller *et al.* 2002; Cook 2003). In previous studies associated with this research programme (Chapter 5), high levels of biological involvement were indicated in New Zealand suppressive soils, suggesting the possible involvement of microorganisms in the suppressiveness. The present study used the molecular method of PCR-DGGE to compare the microbial communities in the rhizosphere and roots of wheat plants grown in the soils, either suppressive or conducive to take-all. From these

comparisons, key microorganisms that might be responsible for the suppression were identified. Primer sets and PCR conditions were successfully employed to amplify the targeted regions specific for the four respective microbial groups, bacteria (16S), fungi (ITS), actinomycetes (16S) and ascomycetes (ITS). The band data generated by DGGE, were highly reproducible between replicates, demonstrating the suitability of using the additional pot replications set up within the pot assays into the current study.

In this study, the total number of bands, which represented the numbers of microorganisms, varied largely between soils with different types of suppression (Table 6.6). This suggests that soil suppressiveness is not dependent on the diversity of microorganisms in each microbial community structure, but on the presence of specific groups of microorganisms. Past studies using conventional isolation techniques to compare the microbial populations in TAD and conducive soils, have had varying results. For instance, over a 3 y experiment, Bateman & Hornby (1999) found no clear relationships between the total number of soil fungal species or abundance of individual fungal species, and the differing successive wheat cropping histories (1, 3, 9 and 39 y). Pope (1972) reported that populations in bulk soils were 60-75% greater in TAD soils than in the non-decline soils for bacteria and 12% greater for actinomycetes, but similar for the fungal population. He therefore concluded that specific suppression was due principally to bacteria and general suppression to fungi. Vojinović (1972; 1973) (in: Baker & Cook 1974) on the other hand, found no difference in the total numbers of microorganisms isolated from the bulk TAD and conducive soils. However, greater numbers of microorganisms were isolated from the roots of wheat plants grown in TAD soils, with actinomycetes and bacteria being isolated three times more often, than in the conducive soils. Of all the microorganisms they isolated, 33% of the actinomycetes, 1% of the bacteria and 10% of the fungi, tested effective in inhibiting the growth of *Ggt* in sterile conditions (i.e. *in vitro*), but they were less effective in non-sterile soil (in: Baker & Cook 1974; Shipton 1975). These results suggest that some microflora in TAD soils, were able to antagonise and reduce the inoculum potential of *Ggt*, and that the antagonistic activity against the parasitic phase of the pathogen (i.e. in or on the roots), was relatively more important than that against the saprophytic phase (i.e. in the debris, which decomposed over time) (Shipton 1975). Given that *Ggt* infects only living roots (Cook 2003), and the development of TAD in wheat was disrupted when other susceptible hosts, such as *Holcus lanatus*, were grown as break crops (Hornby *et al.* 1998), it suggests that the antagonistic

microflora may be specific to wheat. Hence, all the above examples suggest that the microbial populations associated with TAD probably inhabit the rhizosphere/ roots of wheat.

This study reports the first use of PCR-DGGE to investigate microbial communities in the rhizosphere/roots of wheat plants grown in soils with different take-all suppressiveness (specific and general suppressiveness), although it has been reported for investigations of bulk soils, which had been subjected to different cropping systems. For instance, Hiddink and co-workers (2005b) used DGGE to investigate the effects of growing single and mixed crops (brussels sprouts, barley and the mix of these crops, or triticale, white clover and the mix of these crops) in different field soils on the bacterial and fungal communities. Their results did not show any difference in the microbial diversities between soils grown with different crops in different locations. However, after conducting principal component analyses of the numbers of bands and intensity data for the bacterial and fungal communities, they were able to cluster the soils according to their location origins, suggesting that the types of microorganisms inhabiting the soils were specific to the soils/crops. However, as not all DNA templates amplify equally due to occurrence of anomalies with PCR (Garbeva *et al.* 2004), the results derived from the band intensity data in their study may be considered dubious. In a different study, Hiddink *et al*. (2005a) compared the bacterial communities and the suppressiveness to *Ggt* between organic and conventionally managed soils grown with barley, wheat or triticale. They found greater take-all suppression in organic soils grown with barley and wheat, than in the conventionally managed soils. Since the organic soils also had twice the microbial activity of the conventionally managed soils (by CO_2 respiration assessments), and higher bacterial diversity (Shannon-Weaver index = 0.5), they postulated that the microbial populations in these soils might be responsible for the disease suppression. However, the authors did not specify the key microorganisms that might be responsible for the suppression. Collectively, both studies conducted by Hiddink *et al*. (2005a; 2005b) showed that the types of crops grown and locations of the fields, may influence the microbial diversity and activity in the soil.

In the current study, the banding patterns, generated by DGGE, of soils H15 (C) and P7 (C/S) were highly similar (Figure 6.1). This suggests that P7 was indeed conducive to take-all despite its longer wheat cropping history (9 y) and expression in specific

suppressiveness, when a small amount of the soil was transferred into a sterile (γ-irradiated) soil (to constitute 1% of the mixture), with added *Ggt* inoculum (Chapter 5). Furthermore, cluster (Figure 6.1) and principal component (2nd component, Figure 6.2) analyses of the banding patterns of the four microbial groups (bacteria, fungi, actinomycetes and ascomycetes, respectively), clustered the soils into three distinctive groups according to their types of suppressiveness: specific suppressive soils (H1 and H3), general suppressive soil (M2) and conducive soils (H15 and P7). This implies that the microorganisms present in the rhizosphere/roots of wheat plants, are specific to soils of similar suppressive properties. It is, therefore, likely that they are associated with the take-all suppressiveness and conduciveness of these soils.

Some limitations are known to be specific for DGGE. For instance, one rDNA sequence may produce two or more bands on the gel due to heterogeneities of some rDNA sequences (Nübel *et al.* 1996) (in: Heuer & Smalla 1997). Alternatively, similar electrophoretic mobilities or only one band, may be observed for closely related and even for phylogenetically unrelated rDNA sequences of different microbial species (Heuer & Smalla 1997; Kowalchuk *et al.* 2003). These limitations were also encountered in the current study. The effects caused by the latter problem were reduced by randomly picking out five clones for re-amplification and sequencing. However, not all bands gave the same identity for all five clones. Therefore, the most occurring identities of the five were recorded (Section 6.3.2.5). Results might have been more reliable if more clones (e.g. 10) were selected for amplification and sequencing, which was not possible in the current study due to financial and time constraints. The DGGE method used in the current study was capable of profiling the microbial communities in the root/rhizosphere of plants grown in soils exhibiting different suppressive activities. However, there was no guarantee that all the distinguishing microorganisms present in the root/rhizosphere samples were detected by this method. In addition, the fungal and the bacterial primers yielded similar or fewer bands than the actinomycete and ascomycete primers for most of the five soils tested (Table 6.6), suggesting that either the ascomycetes or actinomycetes were most dominant in the rhizosphere/roots of wheat plants in these soils, or that the general fungal and bacterial primers were not as universal/sensitive as they should have been. Future experiments involving profiling the microbial community in the rhizosphere/roots of plants should use group-specific primers (e.g. those specific to oomycetes, basidiomyces, pseudomonads, and *Streptomyces* spp.) to discount the latter possibility. Another factor

which may have affected the specificity of the method is the short rDNA fragments (300-550 bp) targeted by the primers used in this study. Short fragments are generally known to provide better resolution on the DGGE but yield less sequence information (Kowalchuk *et al.* 2003), although some authors have reported high quality DGGE resolution using DNA fragments in excess of 1 kb (Vainio & Hantula 2000). Due to the above limitations, the results obtained from DGGE should be interpreted with caution.

In the current study, the clustering of the five soils into conducive (H15 and P7) and suppressive (H1, H3 and M2) soils was due, in part, to the presence of some distinguishing actinomycetes in the conducive soils, but not the suppressive soils (Table 6.8). This has ruled out the involvement of these actinomycetes, *Glycomyces* sp., *Streptomyces* sp., *Actinosynnema violaceoruber*, *Hongia* sp. and *Actinokineospora diospyrosa*, in the suppressiveness of TAD soils. With the exception of *Streptomyces* sp., there have been no reports on the interaction of these actinomycetes with *Ggt*. However, *Actinosynnema* sp. and *Hongia* sp. were consistently isolated from the roots of wheat plants grown in soils conducive to take-all in an Australian study investigating the molecular interactions of endophytic actinobacteria in wheat and arabidopsis (Conn 2005). The absence of most of these distinguishing actinobacteria (excluding *Streptomyces* sp.) in the suppressive soils also raised the question of whether these indigenous species could have inhibited the activities of microorganisms potentially responsible for suppressiveness, and thus the expression of TAD, in soil P7. Future studies, such as plate assays, could investigate their roles in inhibiting other antagonists isolated from the suppressive soils and in pot trial, when introduced into the suppressive soils. The presence of different species of *Streptomyces* in the rhizosphere and the roots of plants grown in all the five soils (Table 6.8), suggests that this bacterium could be a common microorganism in the soils, and thus, may play no particular role in TAD. indeed, Coombs and co-workers (2004), showed that not all *Streptomyces* spp. are able to suppress *Ggt*. They screened 34 *Streptomyces* isolates for their anti-fungal activities against *Ggt* in a plate assay, and reported that only 64% of the *Streptomyces* isolates inhibited the growth of *Ggt*. Some *Streptomyces* spp. may even play a role in take-all conduciveness.

In this study, the differentiation between the conducive soils (H1 and H15) and the suppressive soils (H1, H3 and M2), was also partly due to the presence of some other microorganisms, which were present in the suppressive soils (H1, H3 and M2), but not the

conducive soils (H15 and P7) (Table 6.8). This suggests that these microorganisms, which included *Streptomyces bingchengensis*, *Terrabacter* sp., *Nocardioides* sp., *Fusarium lateritium*, *Microdochium bolleyi* and an unidentified soil fungus (Table 6.8), could be responsible for the suppressiveness. The microorganisms, *Streptomyces* spp., *Nocardiodes* spp., and *Microdochium bolleyi*, have been shown to suppress *Ggt* in both plate and plant assays (Lascaris & Deacon 1991; Coombs & Franco 2003; Conn & Franco 2004). In Australia, *Nocardiodes* sp., *S. argenteolus* and *S. caviscabies* were demonstrated to control *Ggt* and reduce root infection by up to 25, 37 and 41%, respectively, in a pot assay using field soils (Coombs *et al.* 2004). In glasshouse trials conducted by Kirk & Deacon (1987b; Kirk & Deacon 1987a), *Microdochium bolleyi*, which is frequently isolated from the roots and stem bases of cereals and grasses (Lascaris & Deacon 1991), when used as a seed-applied inoculum was able to control *Ggt* and reduce take-all. However, Deacon & Berry (1993) concluded that effective control of take-all only occurred if the population of *M. bolleyi* greatly exceeded that of *Ggt*, and this was most likely when *Ggt* had to infect from a critically low food base (i.e. continuously fragmenting and decomposing crop residues).

To date, there is no recorded evidence of *Fusarium lateritium*'s capability in controlling *Ggt*, but the efficacy of *F. lateritium* as a biocontrol agent to other soil pathogens is widely documented. For instance, *F. lateritium* was demonstrated to reduce infection by *Sclerotinia sclerotiorum* in lettuce, by inhibiting ascospore germination in the soil (Sitepu & Wallace 1984), and to reduce infection of grapevine pruning wounds by *Eutypa lata* (Ho *et al.* 2005; John *et al.* 2005), by transforming the toxin, Eutypine, secreted by the pathogen, into a non-toxic form (Christen *et al.* 2005).

The other two actinobacteria, *Streptomyces bingchengensis* and *Terrabacter* sp., have no previously reported activity against *Ggt* or any other soilborne pathogens. *Streptomyces bingchengensis* was identified in this study by matching with a partial nucleotide sequence submitted to Genbank from China, the only other identification (Gao *et al.* 2006). A *Terrabacter* sp. has also been identified from wheat roots using T-RFLP methods in Australia (Conn & Franco 2004; Conn 2005). In New Zealand, a *Terrabacter* sp. was isolated from soils contaminated with the insecticide 1, 1, 1-trichloro-2-2-bis (4-chlorophenyl) ethane (DDT), once used extensively to control both agricultural pests and disease vectors, and was able to degrade the toxic residual compounds in the soil (Aislabie *et al.* 1999; Aislabie 2000).

In the current study, further differentiation of the suppressive soils (H1, H3 and M2) into specific and general suppressiveness (Figure 6.2), was partly due to the presence of some distinguishing microorganisms in the soils that had specific suppression (H1 and/or H3), but not in the soil that had general suppression (M2). The presence of the distinguishing microorganisms, *Pseudomonas putida*, *P. fluorescens*, *Nocardioides oleivorans*, *Gibberella zeae* and *Penicillium allii* in the specific suppressive soils, suggests that they could be responsible for the specific suppressiveness. However, the 94% sequence match of the bacterial DGGE band 19 to *Nocardioides oleivorans* has brought about the question of whether this percentage match was sufficient to claim the identity of the band.

The antagonistic effects of *Pseudomonas* spp. on *Ggt* are widely documented. For instance *P. fluorescens*, *P. aureofaciens* and *P. chloraphis* have been reported to antagonise *Ggt* through the productions of the antibiotic, phenazin-1carboxylic acid (PC acid) and other metabolites (Thomashow & Weller 1988; Hornby *et al.* 1998; Delaney *et al.* 2001). Fluorescent *Pseudomonas* spp., such as *P. putid*a, are also known to produce siderophores, which are high-affinity Fe^{3+} chelators that enhance microbial acquisition of iron (Fe) in Fe deficient environments (Scher & Baker 1982). For instance, Kloepper *et al*. (1980) inoculated a take-all conducive soil with *Ggt* and a fluorescent *Pseudomonas* sp. (isolated from a suppressive soil), or its siderophore pseudobactin, and were able to cause the soil to become suppressive. They postulated that the microbial siderophores efficiently chelated Fe^{3+} in soils, making it unavailable to pathogens, thus inhibiting their growth. For this reason, amendment of the suppressive soils with exogenous Fe, could presumably convert them to conducive soils by repressing the production of siderophores (Kloepper *et al.* 1980). When the ethylenediaminedi-*O*-hydroxyphenylacetic acid (EDDHA) or the ferrated form (FeEDDHA) of *Pseudomonas putida* (another fluorescent pseudomonad), which was isolated from a fusarium wilt suppressive soil, was added to a conducive soil, the soil became suppressive to the fusarium wilt pathogen (*F. oxysporum* f. sp. *lini*) of flax, cucumber and radish in plant assays (Scher & Baker 1982). Conversely, Brisbane & Rovira (1988) did not find any effect of the addition of the Fe chelators (such as FeNaEDTA) on the inhibition of *Ggt* by *Pseudomonas* spp. in tube assays, but they found that a yellow crystalline compound, most probably a PC acid (phenazin-1carboxylic acid), was responsible for the suppression of the fungus. In Switzerland, hydrogen cyanide (HCN), produced by a certain fluorescent *Pseudomonas* sp., was able to suppress take-all (Hornby *et al.* 1998). The effectiveness of PC acid depends on the soil and rhizosphere pH,

while the production of siderophores depends on the level of available iron in the soil, which is also dependent upon pH (Brisbane & Rovira 1988), and soil type (i.e. type of clay mineral in the soil) (Hornby *et al.* 1998). Another antibiotic, 2,-4-diacetylphloro-glucinol (DAPG or *phl*) produced by *Pseudomonas fluorescens*, has been implicated in the development of TAD (Thomashow & Weller 1988; Harrison *et al.* 1993; Raaijmakers & Weller 1998; Coombs *et al.* 2004). It seems that many fluorescent *Pseudomonas* spp. are capable of antagonising *Ggt*, but the mechanisms may vary with the species. In this study, attempts were made to amplify the 16S regions using primers [S-G-Psmn-028-a-S-20 (PS-for) and S-G-Psmn-1258-a-A-18 (PS rev-GC)] specific for the *Pseudomonas* genus (Widmer *et al.* 1998), but these primers were not successful (results not presented).

Studies on the interaction of *Gibberella zeae* and *Ggt* are restricted to work done by Wildermuth (1982a; 1982b). Wildermuth's studies (1982a; 1982b) have shown that similar levels of take-all suppression to that provided by the take-all suppressive soil (disease ratings = 2.1 and 2.3, respectively), could be obtained when a take-all suppressive soil (1%) was introduced into a fumigated soil prior to inoculation with *G. zeae* (0.5 g g^{-1} soil as colonised oat inoculum). The severity of root rot (caused by *G. zeae*) on wheat plants grown in the amended soil was also reduced (disease rating decreased from 4.8 to 1.8). The author hypothesised that the specific suppression in the introduced soil inhibited the pathogenicity of *G. zeae* resulting in reduced head blight on plants. As there has been no reported antagonistic activity of *G. zeae* against *Ggt* or any other soilborne pathogens, the role of *G. zeae* in the specific suppressiveness of TAD soil is therefore unknown. In addition, at the time of Wildermuth studies (1982a; 1982b), *G. zeae* was known to be the teleomorph of *Fusarium graminearum*, a causal agent of fusarium head blight and fusarium crown rot. Since then, a different species of *Fusarium, pseudograminearum*, was identified to be the causal agent of fusarium crown rot. Hence, it was highly probable that the *G. zeae*, which Wildermuth (1982a; 1982b) was referring to, was actually *F. pseudograminearum*. Future work will have to investigate the interaction between *G. zeae* and *Ggt* in a plate assay and a pot trial.

In vitro antagonism to *Ggt* has been demonstrated by many *Penicillium* spp. isolated from wheat soils (Sivasithamparam & Parker 1980; Dewan & Sivasithamparam 1988; Hornby *et al.* 1998), but this has not been reported specifically for the two *Penicillium* spp. (*P. echinulatum* and *P. allii*) identified in this study (Table 6.8). Both bands 6 (from M2) and

33 (from H3) on the fungal DGGE gel matched *P. echinulatum*, indicating that two migratory positions are possible for this fungus. According to Kisand & Wikner (2003), it is possible that a single DNA sequence (individual microorganism) can have more than one migration point on a DGGE gel (i.e. having multiple melting domains). As a result, several bands can be generated from a single sequence. Since soils H3 (S) and M2 (G) shared *Penicillium echinulatum* as a distinguishing microorganisms, it was not unique to soil M2 (G). This suggests that the general suppressive soil, M2, had no distinguishing microorganisms that would differentiate it from the specific suppressive soils.

The molecular method, DGGE, used in this study was able to characterise the microbial populations in the rhizosphere and the roots of plants grown in soils of different suppressiveness. The five wheat soils representing different take-all suppressiveness were clustered into conducive and suppressive types, with the latter being further clustered into specific and general suppressive soils. The clustering was not dependent on the microbial diversities in the rhizosphere and the roots, but was due to the presence of specific microorganisms. The actinomycetes (*Glycomyces* sp. *Streptomyces* sp., *Actinosynnema violaceoruber*, *Hongia* sp. and *Actinokineospora diospyrosa*) were associated with take-all conduciveness, but no specific groups were associated with take-all suppressiveness. While some actinomycetes (*Streptomyces bingchengensis*, *Terrabacter* sp. and *Nocardioides* sp.) and ascomycetes (*Fusarium lateritium* and *Microdochium bolleyi*) were shown to associate with suppressive soils in general, others, such as the proteobacteria (*Pseudomonas putida* and *P. fluorescens*), an actinomycete (*Nocardioides oleivorans*), ascomycete (*Gibberella zeae*), and basidiomycete (*Penicillium allii*), were unique in the specific suppressiveness. In contrast, the general suppressive soil had no distinguishing microorganism that would differentiate it from the specific suppressive soils. As nearly all the distinguishing microorganisms identified in this study, have been shown to control *Ggt* or other soilborne pathogens to some degree, they are likely to be associated to the suppressiveness of these soils to take-all.

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6.7 References

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Chapter 7
Attempted induction of soil suppressiveness to take-all in a pot experiment

7.1 Abstract

The complex interaction between take-all decline (TAD) and *Pseudomonas* spp. has been widely reported, but specific information on their interaction with the development of TAD during successive wheat monoculture and soil nutrition is limited. This research was undertaken to induce TAD under controlled conditions by growing wheat successively seven times to simulate seven growth seasons, in bins of soils naturally infested with the pathogen, *Gaeumannomyces graminis* var. *tritici* (*Ggt*). The interaction between disease incidence, concentrations of the pathogen's DNA, the numbers of *P. fluorescens* and some physico-chemical properties of the soils was also investigated during the course of the study. The results showed fluctuations in disease incidence (30-55%) and *Ggt* DNA concentrations (45-289 pg g^{-1} soil) with successive wheat crops ($P<0.005$ and <0.001, respectively), indicating unsuccessful induction of TAD, possibly due to the varying *Ggt* inoculum concentrations and other conditions between the soils. However, the strong correlation between disease incidence and *Ggt* DNA concentrations ($r = 0.88$) from seasons 1-5 until nutrient solution was added before season 6, indicated that soil nutrition might play a role in the control of take-all. When the mean available N and Olsen P concentrations were at their maxima (127 kg g^{-1} soil and 78 mg L^{-1} soil, respectively) at season 6, disease incidence declined from 57 to 35% in all the soils, while *Ggt* DNA concentration remained high (289 pg g^{-1} soil). Although both nutrients could have reduced incidence of the disease by promoting root growth, the available N could also have enhanced the survival of the pathogen. The numbers of *P. fluorescens* increased from 4.14 to 6.92 (log_{10}) CFU g^{-1} roots with increasing successive wheat crops ($P<0.001$), but was not correlated to disease incidence and so may have been a natural event. More work is required to validate this and to investigate the tolerance of the bacteria to Na in the soil.

Keywords: *Gaeumannomyces graminis* var. *tritici*, take-all, take-all decline, *Pseudomonas fluorescens*, soil nutrition

7.2 Introduction

Take-all decline (TAD), a natural process by which soil becomes suppressive to take-all, which is caused by *Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (*Ggt*), has been observed worldwide when wheat monoculture occurs continuously for 4-6 y (McSpadden Gardener & Weller 2001; Mazzola 2002). It begins, in the season after a severe outbreak of the disease, usually during the second or third year of monoculture, with a spontaneous decline in disease severity that results in increased yield (Weller *et al.* 2002). Many studies have provided evidence that this suppressiveness is attributed to the increased populations and activities of the 2,4-diacetylphloroglucinol (2,4-DAPG)-producing fluorescent pseudomonads (Cook & Rovira 1976; Simon & Sivasithamparam 1989; Raaijmakers *et al.* 1997; Raaijmakers & Weller 1998; Raaijmakers *et al.* 1999; McSpadden Gardener *et al.* 2000; McSpadden Gardener & Weller 2001; Mazzola 2002). Since an investigation using denaturing gradient gel electrophoresis techniques (Chapter 6) found two *Pseudomonas* spp. including *P. fluorescens* in a New Zealand TAD soil, this study investigated natural incidence of take-all and the populations of *P. fluorescens* in soils that had increasing seasons of successive wheat cropping.

Soil suppressiveness is also known to be affected by some physico-chemical properties, such as nutrition and soil pH, which may act to favour the activity of a specific antagonist to the pathogen (Höper & Alabouvette 1996). In pure culture, *Ggt* is capable of growth over a wide range of pH values, with pH 6-7 being optimum for its growth (Sivasithamparam & Parker 1981). However, in natural soil conditions, severe take-all has been reported in both alkaline (Yarham 1981; Reis *et al.* 1983) and acidic (Hornby *et al.* 1998) soils, at pH levels above and below the optimal for growth of the pathogen. This could be due to the great variability amongst strains of *Ggt* and their tolerance of pH ranges (Sivasithamparam & Parker 1981).

The influence of nutrition on take-all has been reviewed by Huber (1981; 1989) and Hornby (1985). They reported that disease incidence was reduced by high concentrations of N, P, K (in the form of potassium chloride), S, Mg and Cl, while Ca and K (in the form of potassium nitrate) were reported to increase disease severity. However, the availability of these nutrients to the plants depends on soil pH. For instance, P is maximally available at pH 6.5 and less available above and below this pH (Cook 1981); liming has been reported to increase take-all if it increases the pH beyond the optimal range, and to

predispose the host to nutrient deficiencies for elements such as Cu, Mn, Fe, Zn and Mg, which become less available in alkaline conditions.

This paper describes an attempt to induce TAD experimentally by growing wheat successively in soils naturally infested with *Ggt* within a controlled environment, and to investigate the complex interaction between take-all, populations of *P. fluorescens*, natural *Ggt* inoculum concentrations and some physico-chemical properties of the soils during the successive wheat crops.

7.3 Materials and methods

7.3.1 Soil sampling

The four soils P2, P5, P6 and R1 were collected in Feb 2005 from fields with different wheat cropping histories, in the Methven and Ashburton regions, South Island, New Zealand. These fields, which had been cropped with 2-4 y of wheat, had high concentrations of natural *Ggt* DNA inoculum and were conducive to take-all, thus making the soils ideal for inducing TAD. About 40 L of soil was collected from each field by sampling four soil blocks (10 cm × 10 cm × 10 cm each) from each of 10 sampling points. The 10 sampling points were spaced evenly within a 1 ha zone of the field along a 'W' pattern (Van Elsas *et al.* 2002). Collected soils were processed and stored using the methods outlined in Chapter 3. *Ggt* DNA quantifications and other soil physico-chemical properties of all the soils were determined as in the Chapter 2 study. The site information, including cropping histories, soil types, *Ggt* DNA concentrations and the physico-chemical properties of the soils are shown in Tables 7.1 and 7.2 , respectively.

Table 7.1 Field locations, cropping histories and some properties of the soils used in the Experiment 1.

Field code	GPS Location	Soil type	Soil texture	Preceding crops[1] 2000/01	2001/02	2002/03	2003/04	2004/05	Years of wheat	[2]Pre-sowing *Ggt* DNA concentrations in the 2004/05 season (pg g^{-1} soil)	[3]Risk to take-all for the subsequent crop	[4]Disease assesment (Oct 2004) (Take-all index)	*Ggt* DNA concentrations in soils collected in Feb 2005 for the pot experiment (pg g^{-1} soil)
P2	S43°43.9′ E171°38.2′	Mayfield silt loam	Medium loam	W	GS	W	W	W	3	533	High	32.0	678
P5	S43°43.3′ E171°37.9′	Mayfield silt loam	Medium loam	B	W	W	W	W	4	511	High	37.5	199
P6	S43°43.3′ E171°38.3′	Mayfield silt loam	Medium loam	FS	FS	W	w	W	3	688	High	40.5	357
R1	S44°02.5′ E171°49.3′	Templeton shallow silt loam	Heavy medium light clay silt	-	W	RS	W	W	2	1692	High	28.0	205

[1]Preceding crops:

- W Wheat
- GS Non-specified grass for seed production
- FS Fescue for seed production
- RS Ryegrass for seed production

[2] Analysis was carried out with soil collected from 25 posiitons (two soil cores at each position) along a 'W' pattern over the whole field using soil borers (20 mm dia., 10 cm depth).

[3]Take-all risk categories developed by SARDI at the time of quantitative DNA analysis in 2004/05:

- Low risk: 5 - 130 pg *Ggt* DNA g^{-1} of soil
- Medium risk: 131 - 325 pg *Ggt* DNA g^{-1} of soil
- High risk: >325 pg *Ggt* DNA g^{-1} of soil

[4]Take-all disease index = (0a+10b+30c+60d+100e)/T,
where a, b, c, d and e = number of plants in each of the infection categories below, and T= total number of plants.
Infection categories:

- 0 no infection
- 1 slight (1-10% of roots infected)
- 2 moderate (11-30% of roots infected)
- 3 high (31-60% of roots infected)
- 4 severe (61-100% of roots infected)

Table 7.2 Physico-chemical properties of the soils used in Experiment 1.

Field code	Normal range	5.3-6.1	20-30	0.5-0.8	5.0-12.0	0.8-3	0-0.5	12-25	50-85	Mineral N	Moisture content (% w/w)	
		pH	Olsen P (mg L^{-1})	Potassium ([1]me 100 g^{-1})	Calcium (me 100 g^{-1})	Magnesium (me 100 g^{-1})	Sodium (me 100 g^{-1})	[2]CEC (me 100 g^{-1})	Base Saturation (%)	(µg g^{-1} soil)	Original	At FC
P2		6.1	34	0.50	9.5	0.68	0.17	14	77	19.2	17.8	33.7
P5		5.7	28	0.57	10.4	0.77	0.19	18	66	19.5	17.4	38.0
P6		5.8	25	0.52	11.9	0.93	0.19	20	69	44.5	12.5	30.9
R1		6.4	48	1.07	9.6	1.98	0.42	16	83	36.3	13.3	34.5

[1]milliequivalents

[2]Cation exchange capacity

7.3.2 Pot Experiment-Induction of TAD

Each 20 L bin (25 × 40 × 30 cm) of test soil and was planted with 60 pre-germinated wheat plants (cultivar Regency, line 03/W/01P) (Appendix 3, A3. 3). Each bin received 1.15 L of nutrient solution to give final concentrations for N, P, K and S of 150, 50, 100 and 20 $\mu g\ g^{-1}$ soil, respectively, before topping up with water to the soil's field capacity (FC, at -5 kPa) by weight (Chapters 2 and 3 and Appendix 3, A3. 4).

The bins were laid out in a randomised complete block design consisting of four soils, which were replicated four times to give 16 bins in total. The bins were successively replanted with 60 pre-germinated wheat plants to simulate seven growth 'seasons', with each season being 4 wk. The experiment was conducted in a growth chamber (Conviron, Controlled Environments Ltd., Canada) maintained at 16°C with 12 h light/dark photoperiods and 80% relative humidity (Figure 7.1). Throughout each season, all the pots received water twice per week to the soils' FC. These controlled conditions and the watering frequency were found in Chapter 4 to be suitable for investigating take-all suppressiveness in the pot bioassays used in the current PhD research.

At the end of each season, the soil was carefully loosened from the plant roots by hand. For each bin, about 300 g of the loosened soil was sent to SARDI[1] for *Ggt* DNA quantification, and 150 g to a commercial laboratory (R.J. Hill Laboratories Ltd., Hamilton, New Zealand) for basic soil physico-chemical analyses, while the rest was returned to the original bin. The physico-chemical analyses included soil pH, available N, Olsen P, K, Mg, Ca and Na. The roots of 50 randomly selected plants were washed carefully in bucket of water and assessed for take-all lesions (Section 7.3.3), and the unwashed roots of the other 10 plants were used to investigate the population of *Pseudomonas fluorescens* (Section 7.3.4). Each bin of soil was re-planted with wheat and subject to the same management conditions described above. To avoid development of nutrient depletion, at the sixth season, each bin of soil received the same nutrients as at the time of setting up.

[1] South Australia Root Disease Testing Institute

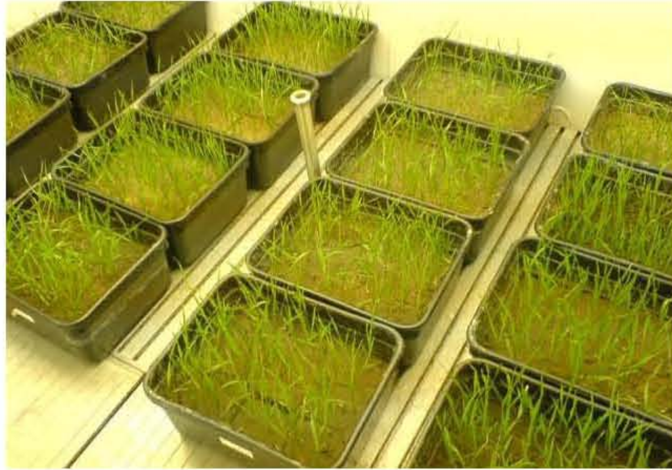

Figure 7.1 Set up of the pot experiment in the growth chamber.

7.3.3 Disease assessment

The washed seminal and nodal roots were assessed for infection incidence, being classed as infected if they had at least one take-all lesion per root axis. This study was set up at the same time as the Chapters 3 and 4 studies, which at the time, had shown that 4 wk growth allowed for development of primary infection but was unlikely to provide time for lesions to increase significantly in size. Hence, disease incidence was considered a more appropriate feature to measure than disease severity. The percent of infected roots per bin was then calculated using the formula:

$$\text{Infected roots (\%) per bin} = \left(\frac{\sum(\text{Number of infected root axes per plant})}{\sum(\text{Number of root axes per plant})} \right) \times 100\%$$

7.3.4 Numbers of *Pseudomonas fluorescens* CFU in the rhizosphere and roots of wheat plants

From the 10 randomly chosen plants from each bin at harvest, 5 g of roots with adhering rhizosphere soil was placed in separate Erlenmeyer flasks, which contained 50 mL of 0.1 M magnesium sulphate (i.e. 10^{-1} dilution), and shaken for 5 min at 200 rpm on an orbital shaker (Chiltern Scientific, Auckland, New Zealand). Using 1 mL of the root/soil suspension, serial dilutions (10^{-2} to 10^{-5}) were then made with sterile water. The numbers of *Pseudomonas fluorescens*, were determined by spreading 0.1 mL of each suspension onto three plates (i.e. three replicates) of King's B agar, amended with 20 mg L^{-1} of Trimethoprim to prevent the growth of other non-fluorescent pseudomonads (Appendix 4,

A4. 3) (Xu & Gross 1986). The sterile water used in the dilution was also plated to check for background contamination by the bacterium. The plates were incubated at 25°C and checked for fluorescent colonies after 48 and 72 h on a UV transilluminator light (Ultra-violet products Inc. San Gabriel, California USA). From the fluorescent colonies, which were suspected to be *P. fluorescens* (Figure 7.2), 10 were randomly chosen and sent to MAF [Plant Pest Information Network (PPIN), Ministry of Agriculture and Forestry, Lincoln, New Zealand] for further identification using Biolog GN plates (Biolog, Inc., USA) and conventional isolation techniques. The numbers of *P. fluorescens* CFU (colony forming unit) in each root sample were estimated from the plates, which had 3-30 fluorescent colonies growing on each, using the following formula:

$$\text{CFU g}^{-1}\text{ root rhizosphere sample} = \left(\frac{\text{A} \times \text{D} \times \text{P}}{\text{T}}\right)$$

Where,

CFU = colony forming unit

A = number of *P. fluorescens* colonies per plate

D = dilution factor

P = 10 (i.e. 0.1 mL of diluted suspension plated)

T = 5 (i.e. 5 g of total root rhizosphere used for isolation)

Figure 7.2 Fluorescent colonies of *Pseudomans fluorescens* at 10^{-4} dilution under UV light.

7.3.5 Data analysis

Data on the disease incidence, *Ggt* DNA concentrations, numbers of *P. fluorescens* CFU (log transformed) and nutrient status were analysed with ANOVA by treating the successive wheat seasons as a split plot factor and adjusting the analysis for the repeated

measurements using the Greenhouse Geisser epsilon correction factor (Greenhouse & Geisser 1959) on GenStat, 9th Ed (2005) (VSN International Ltd, Oxford). The correlations between disease incidence, *Ggt* DNA concentrations and numbers of *P. fluorescens* CFU, and between each of these variables with pH and nutrient status were also analysed.

7.4 Results

7.4.1 Disease incidence

The analyses showed that the percent infected roots on the plants were significantly affected by the successive wheat seasons and soil origins (P<0.005 for both). The mean percent infected roots initially decreased over seasons 1-3 (from 43 to 32%), then increased up to 55% after season 5, and finally dropped to 35% after seasons 6 and 7 (Figure 7.3a). Overall, plants grown in soil P2 had the highest incidence of infected roots (46%) followed by soil R1 (42%), while plants in soils P5 and P6 had significantly lower incidences (36 and 37%, respectively) (Figure 7.3b). The same trend across seasons was observed for all soils, as there was no significant interaction between the successive seasons of wheat and soil origins on the disease incidence.

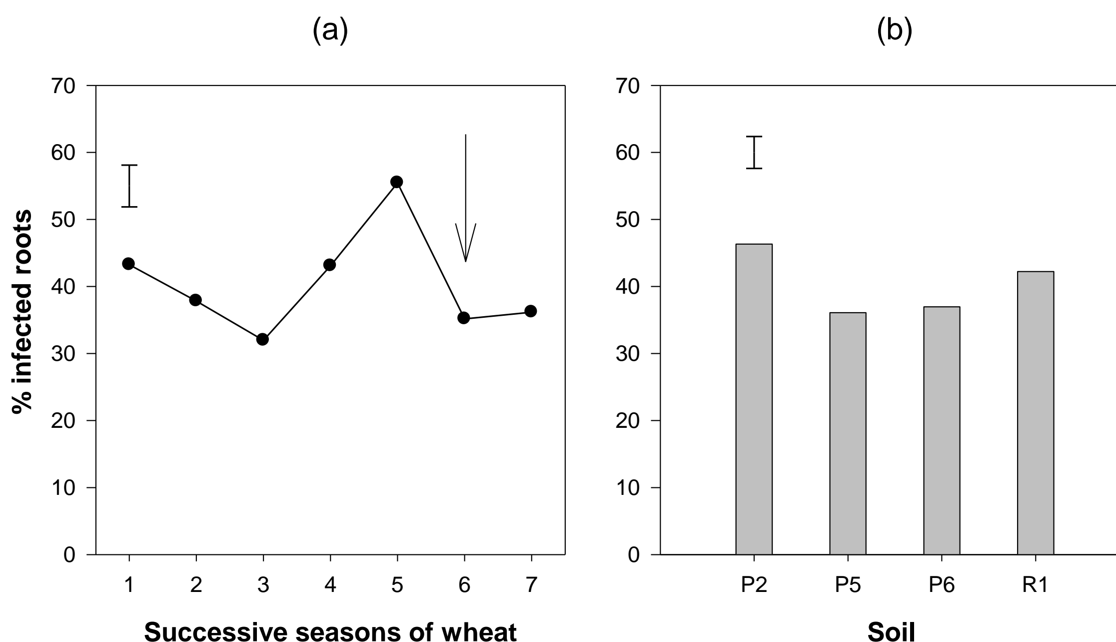

Figure 7.3 Mean incidence of take-all infected roots on wheat plants grown in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111).

7.4.2 *Ggt* DNA concentrations

Overall, the successive seasons of wheat caused a significant effect on the soil *Ggt* DNA concentrations (P<0.001). Initially, the mean *Ggt* DNA concentration decreased (from 161 to 45 pg g^{-1} soil (P<0.005) in a similar trend to that of the disease incidence for seasons 1-3 (Figure 7.3a). However, the mean DNA concentration then increased, peaking after season 6 (289 pg g^{-1} soil), not season 5 as with disease incidence (Figure 7.3a), and then decreased to 194 pg g^{-1} soil after season 7 as also shown in disease incidence (Figure 7.3a). There was evidence of soil origins causing a significant difference on the *Ggt* DNA concentrations (P<0.05) with the lowest *Ggt* DNA concentration (95 pg g^{-1} soil) being in soil P5, while soil P2 had the highest (195 pg g^{-1} soil) (P<0.05) (7.4b). There was no significant interaction between the successive seasons of wheat and soil origins on the *Ggt* DNA concentrations.

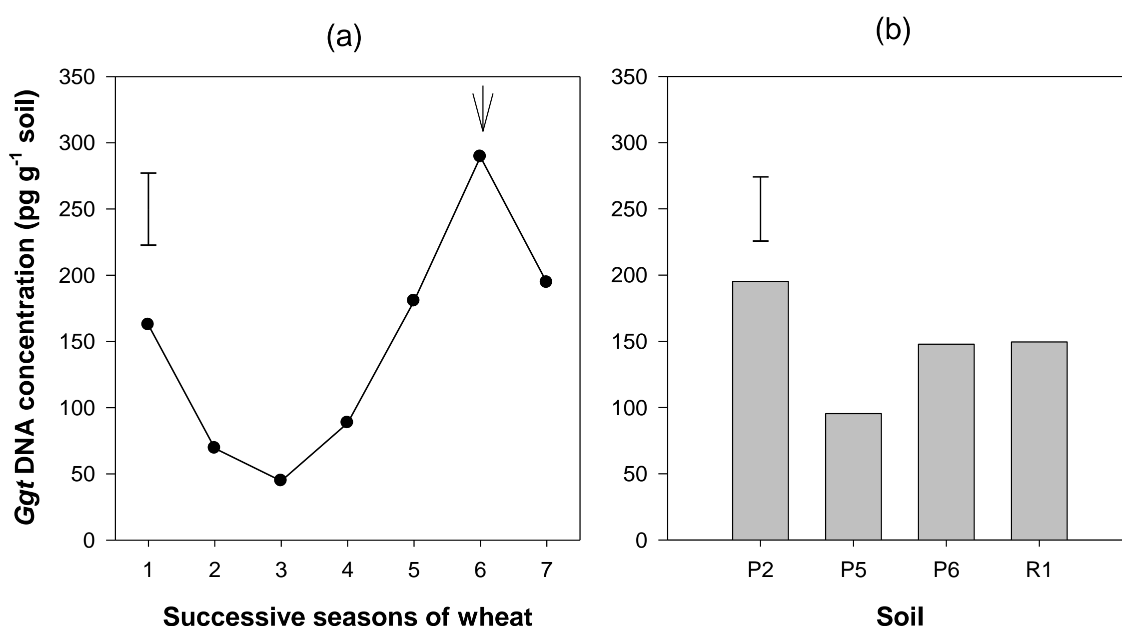

Figure 7.4 Mean *Ggt* DNA concentrations in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111).

7.4.3 Numbers of *Pseudomonas fluorescens* colonies in the roots of wheat plants

The fluorescent colonies growing on King's B agar that were selected (10 each time) for identification were all identified as *Pseudomonas fluorescens*. In general, the mean

numbers of *P. fluorescens* CFU (colony forming units) were significantly affected by the successive wheat seasons and soil origins (P<0.001 and <0.05, respectively). The mean numbers of *P. fluorescens* CFU gradually increased with increasing successive wheat seasons, reaching a maximum of 6.92 (log_{10}) CFU g^{-1} roots after season 7 (Figure 7.5a). Of the four soils tested, P5 produced the lowest numbers of *P. fluorescens* CFU, being 5.01 (log_{10}) CFU g^{-1} roots, whereas the other three soils produced similarly higher numbers of *P. fluorescens* CFU, being 5.7, 5.7 and 6 (log_{10}) CFU g^{-1} roots (Figure 7.5b). There was no significant interaction between the successive wheat seasons and soil treatments on the numbers of *P. fluorescens* CFU.

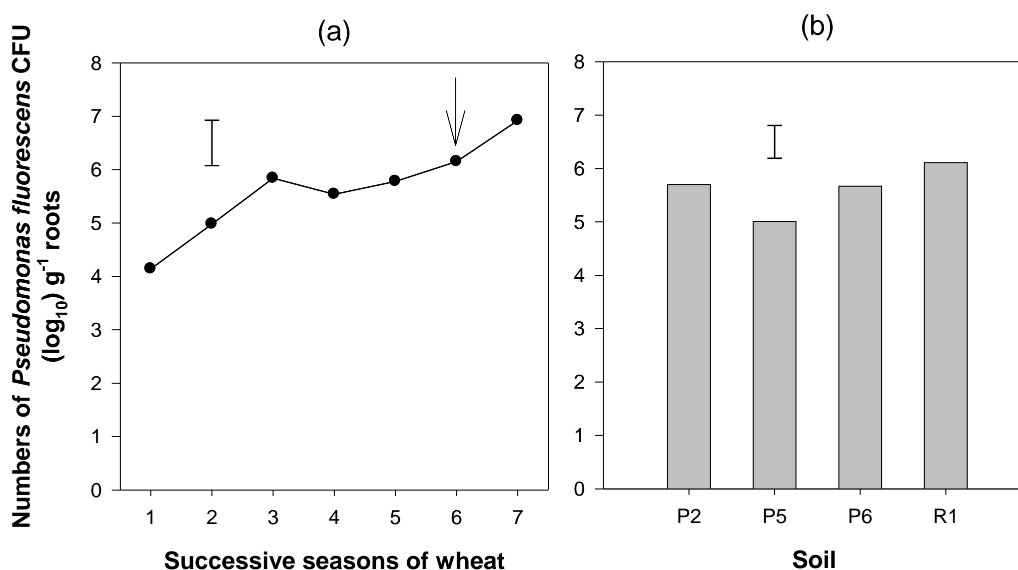

Figure 7.5 Mean numbers of *Pseudomonas fluorescens* CFU in the roots of wheat plants grown in (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111).

7.4.4 Soil pH

There was a significant effect of successive seasons of wheat crops on soil pH (P<0.001), with the mean pH progressively increasing over seasons 1-4 (from 5.4 to 6.1), being constant for season 5 (Figure 7.6a). After season 6, the mean pH dropped to 5.6, which was similar to that after season 2, and subsequently increased again to be similar to that after seasons 4 and 5 (pH 6.1). The soil pH also varied significantly with soil origins (P<0.001), with soils P2 and R1 (pH 6 and 6.2, respectively) having significantly higher pH than P5

and P6, which were similar (pH 5.5 and 5.7, respectively) (Figure 7.6b). There was a significant interaction with successive wheat seasons and soil origins (P<0.001) (Figure 7.7). Over the seven successive seasons, all the four soils had similar trends in pH shift, with gradual increases for seasons 1-4, a decrease after season 6 and then an increase after season 7. In general, soil P5 had the lowest pH throughout the seven successive seasons of wheat crops, followed by soils P6, P2 and R1.

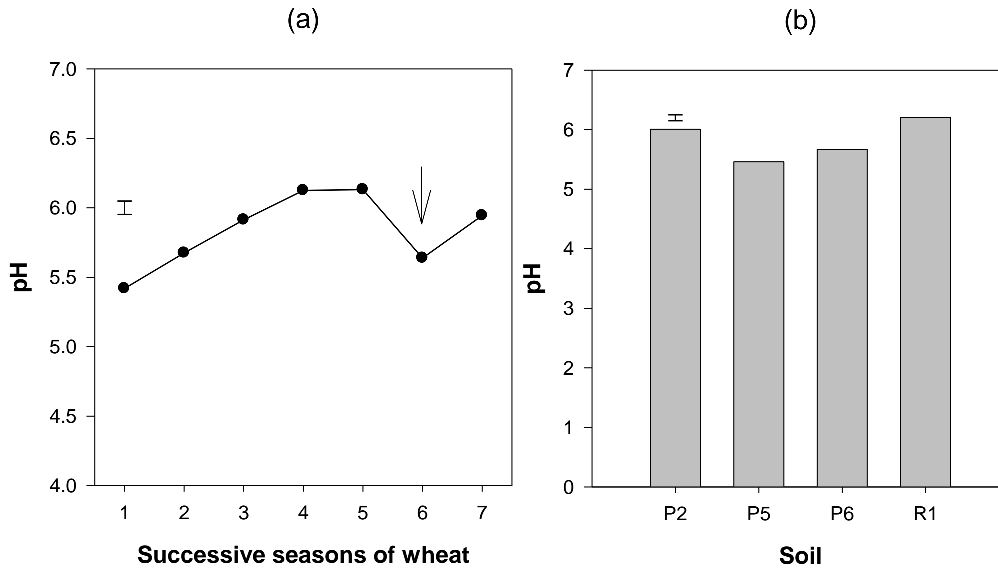

Figure 7.6 Mean pH of (a) soils grown with successive wheat crops and (b) the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences (LSD) at the 5% level (df = 111).

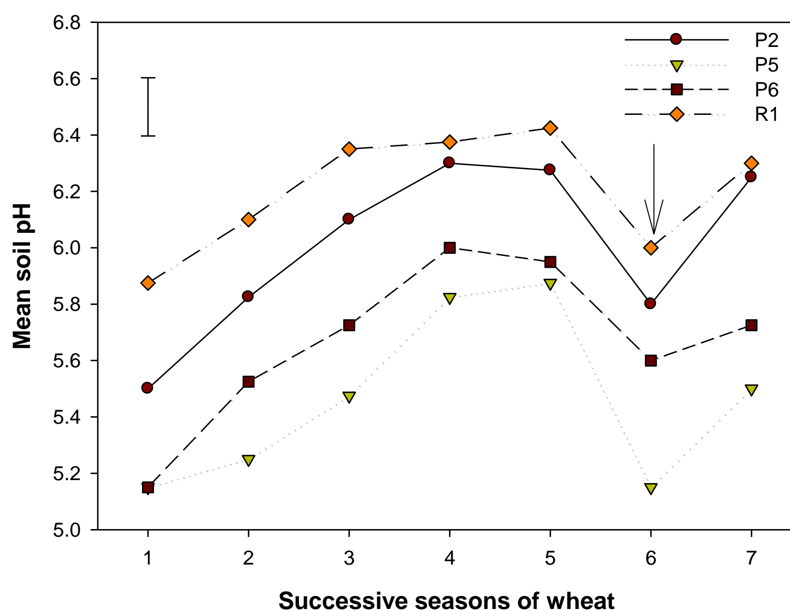

Figure 7.7 Mean pH of the four soils sown with successive seasons of wheat crops. Arrow indicates the time when the second nutrient solution was added into the soils. Error bar is the least significant difference (LSD) at the 5% level (df = 111).

7.4.5 Nutrient status in the soil

In the present study, nutrient solution was added to the bins of soils twice, first being at the time of setting up season 1, and the second being before season 6. Statistical analyses showed that the concentrations of Mg were significantly affected by soil origins ($P<0.001$), but not the successive seasons of wheat crops. The mean Mg concentration was highest in soil R1 (1.9 me 100 g^{-1} soil) followed by soil P6 (0.9 me 100 g^{-1} soil), whereas Mg concentrations in soils P2 and P5 were similarly low (0.6 me 100 g^{-1} soil) (Figure 7.8). There was no interaction effect between successive seasons of wheat crops and soil origins on the Mg concentrations.

The Ca concentrations were significantly affected by successive seasons of wheat and soil origins ($P = 0.003$ and <0.001, respectively). The effects of successive seasons of wheat alone had indicated that the highest mean Ca concentration (10.9 me 100 g^{-1} soil) was after season 7, which was significantly higher than all the other seasons except season 2 (Figure 7.9a). Season 6 had the significantly lowest mean Ca concentration (10.2 me 100 g^{-1} soil), whereas the Ca concentrations did not differ among seasons 1-5 (being 10.5-10.6 me 100

g^{-1} soil). Of the four soils tested, soil P6 had the highest Ca concentration (mean concentration = 11.8 me 100 g^{-1} soil), followed by soils P2, P5 and R1 (11.5, 9.9 and 8.9 me 100 g^{-1} soil, respectively) (Figure 7.9b). There was no interaction effect between successive seasons of wheat crops and soil origins on the Ca concentrations.

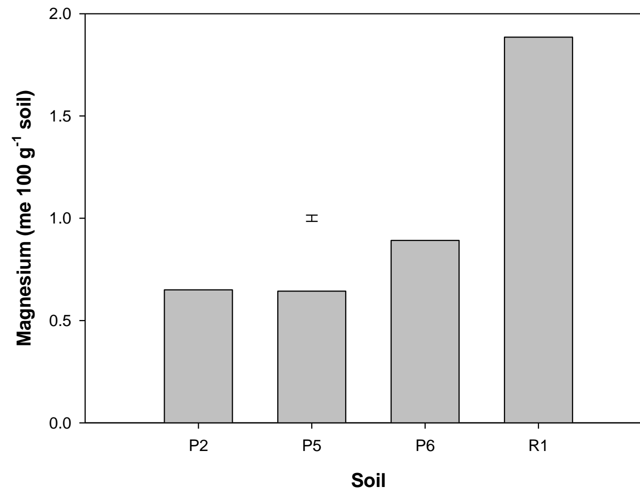

Figure 7.8 Mean Magnesium concentrations in the four soils tested over seven successive wheat seasons. Error bar is the least significant difference at the 5% level (df = 111).

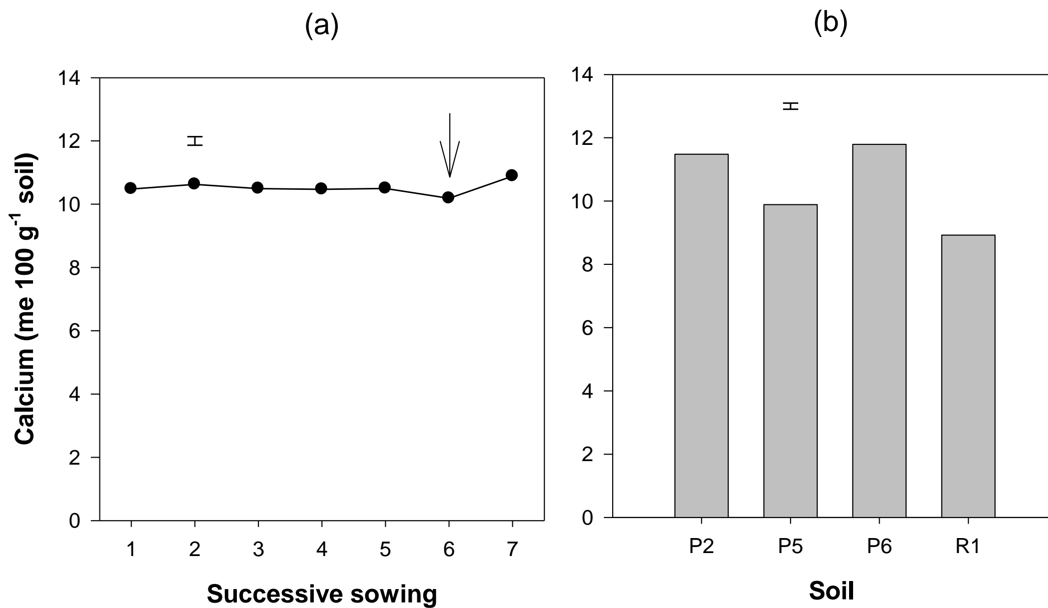

Figure 7.9 Mean calcium concentrations of (a) soils grown with successive seasons of wheat crops and (b) in the four soils tested over seven successive wheat seasons. Arrow indicates the time when the second nutrient solution was added into the soils. Error bars are the least significant differences at the 5% level (df = 111).

The concentrations of available N, Olsen P, K, and Na in the soils were significantly affected by the successive seasons of wheat crops and soil origins ($P<0.001$ for all nutrients for both effects). There were significant interaction effects between successive seasons of wheat crops and soil origins on the concentrations of available N, Na and K ($P = 0.024$, <0.001 and <0.001, respectively), and a marginal interaction effect on Olsen P ($P = 0.073$). As the mean concentrations of available N, Olsen P, K, and Na, showed similar trends to that of the interaction effects (Appendix 5, A7. 2 and A7. 3), only the results from the interaction effects are presented (Figures 7.10a-d). Since similar trends were also shown in the mean concentrations for Mg and Ca (Figures 7.8 and 7.9; Appendix 5, A7. 2 and A7. 3), the mean results from the non-significant interaction effects are also included (Figures 7.10e-f).

Both the available N and Olsen P concentrations shifted in very similar trends over the different numbers of successive sowings for nearly all the soils (Figures 7.10a-b). In general, the available N and Olsen P concentrations in the soils decreased gradually over seasons 1-5, increased after season 6 and decreased thereafter. An exception occurred in soil P2, for which the available N remained relatively uniform throughout the successive seasons of wheat crops (Figure 7.10a). In all soils, the K concentrations also decreased with successive seasons of wheat, but the Na concentrations increased steadily over the successive seasons of wheat crops (Figures 7.10c-d). For all the soils, the mean concentrations of Mg and Ca remained reasonably constant for the successive wheat crops grown (Figures 7.10e-f). Overall, soil R1 had the highest pH (Figure 7.7), available N, Olsen P, Na, K and Mg concentrations (Figure 7.10).

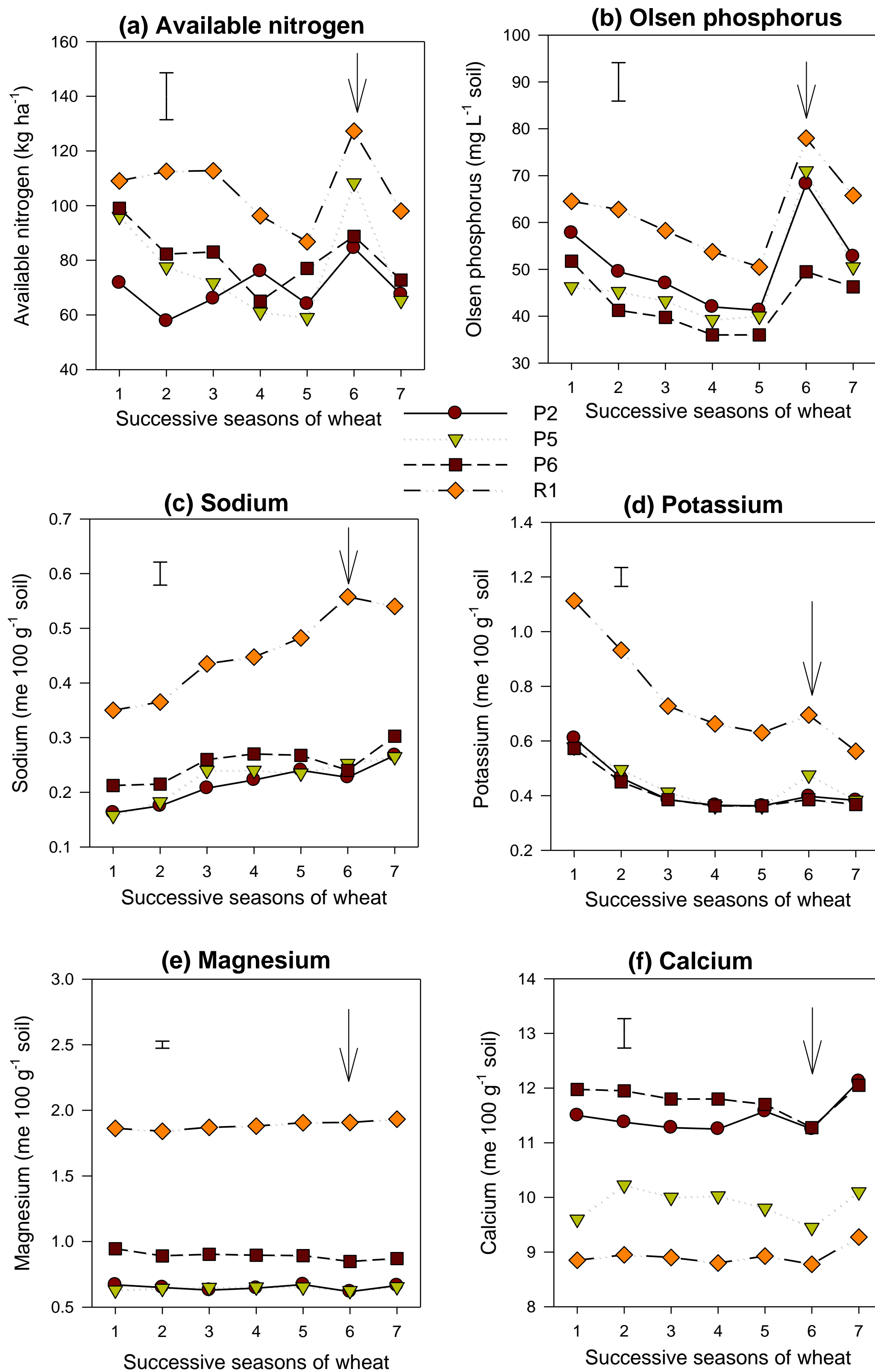

Figure 7.10 Mean concentrations of (a) available nitrogen, (b) Olsen phosphorus, (c) sodium, (d) potassium, (e) magnesium and (f) calcium in the four soils sown with successive seasons of wheat crops. Arrows indicate the time when the second nutrient solution was added into the soils. Error bars are the least significant differences at the 5% level (df = 111).

7.4.6 Correlations between the parameters

Initial analyses using the complete raw data set showed poor correlations between disease incidence and *Ggt* DNA concentrations, numbers of *P. fluorescens* CFU or pH, as well as between these four parameters and the individual nutrients. However, further analyses using the mean values of the individual parameters from the successive seasons of wheat crops showed high correlations between some of them (correlation coefficient, $r > 0.7$). Therefore, the results from analysing the means of successive seasons of wheat crops are presented in Table 7.3, and a matrix of scatter plots showing the relationships among these parameters are presented in Appendix 5, A7. 4. Overall, there were no significant correlations between the disease incidence and *Ggt* DNA concentrations ($r = 0.12$), and between these two variables and the numbers of *P. fluorescens* CFU ($r = 0.25$ and 0.34, respectively) (Table 7.3). However, there was a strong correlation between the concentrations of soil *Ggt* DNA and Olsen P ($r = 0.71$). The increasing numbers of *P. fluorescens* CFU was found to correlate strongly with the Na and K concentrations ($r =$ 0.96 and -0.87, respectively) (Appendix 5, A7. 4).

Table 7.3 A matrix showing the correlations between the parameters measured (disease incidence, *Ggt* DNA concentrations, or numbers of *P. fluorescens* CFU, pH and nutrient status). A negative correlation coefficient indicates that the two variables are inversely related. The highlighted coefficients are the ones that showed strong relationships (r > 0.7).

	% infected roots	*Ggt* DNA	*Pseudomonas*	pH	Available N	Ca	K	Mg	Na	Olsen P
% infected roots	1									
***Ggt* DNA**	0.126	1								
Pseudomonas	-0.254	0.338	1							
pH	0.351	-0.234	0.564	1						
Available N	-0.452	0.472	-0.271	-0.833	1					
Ca	-0.043	-0.314	0.260	0.246	-0.636	1				
K	-0.050	-0.091	-0.868	-0.875	0.549	-0.137	1			
Mg	-0.320	-0.534	0.119	0.224	-0.168	0.106	-0.151	1		
Na	-0.068	0.468	0.958	0.614	-0.258	0.116	-0.886	0.028	1	
Olsen P	-0.512	0.711	0.115	-0.705	0.875	-0.352	0.292	-0.337	0.102	1

7.5 Discussion

The current study was set up to attempt induction of suppressiveness in pots of soils naturally infested with *Ggt* by growing seasons of wheat, 4 wk each, successively in the same soils, as reported by Deacon (1997). Hence, all the four soils selected for the study had natural concentrations of *Ggt* inoculum sufficient to cause high severity or incidence of take-all (Table 7.1). However, results did not reflect those reported for TAD, which occurred after 4-6 successive wheat crops in the field (McSpadden Gardener & Weller 2001; Mazzola 2002; Weller *et al.* 2002), since there was no severe outbreak or sustained decreases in incidence of the disease and concentrations of *Ggt* inoculum (Figures 7.3 and 7.4). In fact, disease incidence and *Ggt* inoculum concentrations fluctuated throughout the whole course of the pot monoculture, indicating unsuccessful induction of suppressiveness. Although up to eight consecutive years of monoculture of barley crops has been reported for TAD to occur elsewhere (Hornby & Henden 1986), there was no guarantee that TAD would definitely develop when wheat is grown continuously. For instance, in this PhD research programme, one of the soils, which had been grown with wheat for 9 y, did not show to suppress take-all in the Chapters 5 and 6 studies.

Some studies have attempted to induce take-all suppression in pot assays by introducing *Ggt* inoculum into the soils (Gerlagh 1968; Zogg & Jäggi 1974; Wildermuth 1980). For instance, Wildermuth (1980) added an unknown quantity of either dead or living *Ggt* inoculum into fumigated soils and planted with wheat for 28 d, after which it was claimed that ‘slightly suppressive’ and ‘suppressive’ soils had been successfully induced. The mechanisms for the suppressiveness of these soils were not convincing mainly because there were likely to be few or no natural soil organisms/antagonists in the fumigated soils, which is generally considered a necessary component of suppressiveness. These artificially created ‘slightly suppressive’ and ‘suppressive’ soils were used by the author as controls in a pot trial to compare their suppressiveness (as severity of root infection on wheat plants) with that of other field soils cropped with various numbers of continuous wheat crops. In the study, a fumigated soil was supplemented with 1% (w/w) of each test soils (including the two induced suppressive soils) and 0.1% of *Ggt* (on ground oat), and wheat plants were grown in them for 28 d. The created suppressive soils provided a standard for determining suppression of the test soils.

Zogg and Jäggi (1974) tried to induce take-all suppression by adding 4 wk old *Ggt* mycelia (unspecified amount) into different soils at weekly intervals for 5, 9 and 18 times. For each interval, wheat plants were grown in a portion of the soils at 0, 1 or 3 d after being mixed with the *Ggt* inoculum. For the soils that were assayed immediately after being mixed with the *Ggt* inoculum, the disease severity decreased steadily (from 46-48% to 4-19%) across the increasing periods of adding *Ggt* inoculum. For the soils that were assayed either 1 or 3 d after being mixed with the *Ggt* inoculum, the disease severity varied initially (30-66%) for all three periods of adding the *Ggt* inoculum, and declined thereafter (to 7-8%). The occurrence of severe disease symptoms before the onset of decline was however, not reported. Zogg and Jäggi (1974) postulated that adding *Ggt* inoculum on repeated occasions had caused increasing populations of soil bacteria and actinomycetes, which were responsible for the suppressiveness. Moreover, they also found that up to 60% of the bacterial isolates showed some forms of antagonistic actions to *Ggt* on various media (e.g. 2% malt extract agar). Using a similar technique, Pope *et al.* (1974) conducted a pot assay using soils that had consecutive monthly crops of wheat seedlings and addition of *Ggt* inoculum between the successive wheat crops. They found that regardless of the numbers of successive wheat crops (i.e. third, sixth or thirteenth successive wheat), root infection increased with the second and third successive crop in the assay and thereafter decreased. These results indicated that with repeated additions of *Ggt* inoculum to the soil, TAD could be induced experimentally, and that the populations of bacteria and actinomycetes in the soils could increase with successive wheat cropping.

Ggt is a semi-obligate fungus, which survives saprophytically in soils as mycelium in host plant debris (Wilkinson *et al.* 1985), hence, the natural *Ggt* inocula in the soils in this study, which were initially high, could have been reduced by the repeated destructive harvesting of the wheat plants as seen during the first few wheat seasons (Figure 7.4). Soils P5, P6 and R1 had demonstrated decreases in *Ggt* DNA concentrations from the beginning to end of their last field season (by 312, 331 and 1487 pg g^{-1} soil, respectively) (Table 7.1), suggesting that the inocula in the soils were in decline. As expected, soil P2, which increased in *Ggt* DNA concentration from 533 to 678 pg g^{-1} soil during the 2004/05 field season (Table 7.1), had the highest mean percent infected roots and *Ggt* DNA concentration (Figures 7.3b and 7.4b) in the pot experiment. This may have been because it was less suppressive than the other three soils.

In this study, the poor correlation between disease incidence and *Ggt* DNA concentrations (Table 7.3) was mainly associated with the highest *Ggt* DNA concentration occurring after season 6 instead of after season 5, when the disease incidence was highest (Figures 7.3 and 7.4). Both variables were however, strongly correlated from seasons 1-5 ($r = 0.88$), indicating that the extent of infection on the roots was related to the amount of natural *Ggt* inoculum in the soils until season 5. This may have indicated the beginning of suppression but further wheat seasons would have been needed to show it. However, the application of nutrient solutions to all the soils at season 6 was considered the most probable cause for the differences seen. This was supported by soil analyses, which showed increased available N and Olsen P concentrations at season 6, to concentrations (125 kg g^{-1} soil and 78 mg L^{-1} soil, respectively) more similar to those at season 1 (110 kg g^{-1} soil and 65 mg L^{-1} soil, respectively), when the nutrient solution was added. Other nutrients, including Na, K and Ca, were only slightly affected by the addition of nutrient solution at season 6.

In this study, the concentrations of K decreased with increasing successive sowing, and did not increase very much with the addition of nutrients at season 6. This suggests that the amount of K in the nutrient solution was not as high as the initial level in the soils. In fact, for soils P2, P5 and P6 since season 3, the nutrient had been deficient and below the recommended concentrations (0.5-0.8 me 100^{-1}g soil) for New Zealand cereal crops (R. J. Hill Laboratories Ltd 2002). Few references imply a separate role for K relative to take-all, but it has been suggested to reduce take-all when applied together with N and P (Huber 1981).

Overall, the available N and Olsen P concentrations in the soils decreased from seasons 1 to 5 (Figure 7.10) and showed an opposite trend to that of disease incidence (Figure 7.3), suggesting that these two elements might play a part in reducing disease incidence. In the field, increased application rates of N and P, especially at critical stages of plant growth, such as tillering and stem elongation, is known to promote new root growth, thereby compensating for any diseased roots and so improving the host's capacity to tolerate *Ggt* infection (Huber 1981; Hornby *et al.* 1998; Cook 2003). In the pot trial, Olsen P concentrations in all the soils were still within the range (20-30 mg L^{-1} soil, R. J. Hill Laboratories Ltd., Hamilton, New Zealand) recommended for growing wheat, but the available N levels were always below the recommended rate (150 kg ha^{-1}) for optimal growth (Hornby *et al.* 1998).

The application of nutrients at season 6 might have contributed to the high *Ggt* concentration in the soils (Figure 7.4), in spite of decreased disease incidence at that point, since N is believed to favour the saprophytic survival of the *Ggt* in the absence of host (Garrett 1938; Huber 1981). However, if the soil is well aerated, it might favour microbial activity and hasten the disappearance of *Ggt* by promoting more rapid consumption of the available food material by both the pathogen itself and by other associated microorganisms (Chambers & Flentje 1969).

At present, there is insufficient evidence to determine whether the general effects of N on suppressive organisms are affected by the forms of N in the fertilisers (Alabouvette *et al.* 2004). This is because almost all studies on the effects of N on disease suppression have been confounded by, or shown to be caused by, the effects of nitrate and ammonium ions on rhizosphere pH (Smiley & Cook 1973). The form of nutrients applied may also affect take-all incidence/severity. For example, Smiley & Cook (1973) reported that when N was applied in the ammonium form (i.e. as NH_4^+), the rhizosphere pH became more acidic (<5), inhibiting the growth of *Ggt* completely and resulting in reduced disease ratings (decreased from 5 to 1). Further work conducted by Smiley (1978a; 1978b) also showed that the populations of *P. fluorescens* capable of inhibiting the growth of *Ggt* on the wheat roots, were 10-67% higher in the soils fertilised with ammonium N than those fertilised with nitrate N. These problems however, should have been minimised in this study as N was applied as ammonium nitrate (NH_4NO_3).

Although the soil pH differed significantly between the soils, they showed similar changes at each successive season (Figure 7.7) and remained within the acidic range of 4.8-6.1. It is evident that the declined pH at season 6 (Figure 7.6) was due to the addition of nutrient solution. It is known that Ca, Mg, Na and K, are most readily available in neutral (pH=7) or alkaline (pH>7) soils mainly because under acidic conditions, they are excluded from cation exchange sites by H^+ and Al^{3+} (McLaren & Cameron 1996). The effects of pH in the range 4.5-8.5 on the availability of nutrients (added as Hoagland's solution at normal to three times the normal strength) to wheat and the resulting take-all severity, were investigated by Reis and co-workers (1983) in pot experiments. They found that disease severity increased with increasing pH but not with the increasing concentrations of Ca or Mg, and suggested that pH above 6.5, was favourable to take-all. In the current study, pH did not increase above 6.5 and so was unlikely to have affected the results. The Ca and Mg concentrations were within the recommended range (5-12 and 0.8-3 me g^{-1} soil,

respectively) for New Zealand cereal (R. J. Hill Laboratories Ltd 2002), and remained relatively constant throughout the course of this study, indicating that these nutrients were absorbed at very low rates. The roles of other micronutrients such as manganese (Mn), can also affect take-all incidence/severity (Huber & McCay Buis 1993), but they were not assessed in this study.

In spite of the variation in disease incidence and *Ggt* DNA concentrations in this study (Figures 7.3 and 7.4), the mean numbers of *P. fluorescens* CFU increased steadily with successive seasons of wheat crops from 4.1 to 6.9 (log_{10}) CFU g^{-1} roots with adhered soil (i.e. equivalent to $10^{4.1}$ and $10^{6.9}$ CFU g^{-1} roots with adhered soil) (Figure 7.5). The presence of *Pseudomonas* spp. in the soil has been linked to the suppression of *Ggt* and the development of TAD. For instance, Raaijmakers *et al.* (1997) used molecular techniques to show that 2,4-diacetylphloroglucinol (DAPG)-producing strains of *Pseudomonas* spp. were present in only TAD soils, and when the fluorescent *Pseudomonas* spp. were eliminated, the suppressiveness was lost (Raaijmakers & Weller 1998). A series of pot experiments also revealed that the DAPG-producing *Pseudomonas* spp. were naturally present in TAD soils at rhizosphere populations of 10^5-10^6 CFU g^{-1} soil, which were sufficient to suppress take-all (Raaijmakers *et al.* 1999). This suggests that in the current study, the mean numbers of *P. fluorescens* CFU in the rhizosphere were sufficient to suppress take-all from season 2 onwards. However, it was not known whether the *P. fluorescens* isolated from the roots, belonged to the 2,4-DAPG-producing strains. The increased numbers of *P. fluorescens* colonies could also be a natural event, which had occurred with the successive seasons of wheat crops (Zogg & Jäggi 1974), since there was no correlation between the numbers of *P. fluorescens* CFU and the disease incidence or *Ggt* DNA concentrations (Table 7.3). However, in a previous study, which investigated the microbial populations using denaturing gradient gel electrophoresis, *Pseuodomonas* spp. were implicated to be associated with a New Zealand soil that was specific in its suppressiveness (Chapter 6).

The strong correlation between the numbers of *P. fluorescens* CFU and the concentrations of Na (Table 7.3), suggests that Na might have a positive effect on the bacterium. Using a tube assay, Ownley *et al.* (2003) investigated the efficacy of a phenazin-1carboxylic acid-producing *P. fluorescens* strain in controlling *Ggt* (applied as an oat inoculum), by treating the seeds with the bacterium and adding 5 mL of nutrients solution twice weekly. They found negative correlations between the concentrations of Na and the disease ratings on the

wheat roots (r = -0.64 to -0.68). Ownley *et al.* (2003) hypothesised that Na might have improved the biocontrol activity of the *P. fluorescens*. In the current study, the exact effect of Na on the numbers of *P. fluorescens* CFU was unknown, although many *Pseudomonas* spp. are known to be halophilic (i.e. able to tolerate saline conditions) (DasSarma & Massachusetts 2001). In addition, the strong correlation did not mean that there was a direct effect of Na on *P. fluorescens* CFU. More carefully designed experiments are required to verify or investigate the direct and indirect effects of Na on the survival of *P. fluorescens* under a range of soil conditions.

The results in this study indicated that the relationship between the disease incidence and *Ggt* DNA concentrations in the soils, might be partly dependent on some of the nutrients, particularly, the concentrations of available N and Olsen P. More experiments are required to verify the possible roles of the increased available N and Olsen P on disease incidence, root growth to offset the disease incidence, and the survival of soil *Ggt*. The increased numbers of *P. fluorescens* CFU with successive seasons of wheat crops might be a natural event independent of the *Ggt* DNA concentrations and disease incidence. However, more work is required to validate the tolerance of *P. fluorescens* to Na in the soil. This study failed to show development of TAD under controlled conditions probably because the additions of nutrients at seasons 1 and 6, and the repeated removal of roots or *Ggt* during destructive harvesting, caused conflicting effects. Other soil microflora, which could play a part in the development of TAD, might had been removed with the roots during harvest as well. The destructive harvesting of plants was essential for the successive monitoring of disease parameters. Future experiments aimed at inducing TAD will have to minimise the variations by adding small amounts of the nutrient solutions and *Ggt* inoculum into the soils before each new season, and to include an additional set of soils for disease/inoculum monitoring.

7.6 References

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Chapter 8
Concluding discussion

This study was undertaken to investigate the hypothesis that take-all decline (TAD) is a specific suppression caused by an individual or a selected group of microorganisms, which suppressed *Ggt* in the rhizosphere of the host. This chapter summarises the results of the many experiments undertaken in this research programme and comments on the possible mechanisms behind the soil suppressiveness. It also proposes some future experiments and some potential uses of these findings in the context of controlling take-all in New Zealand wheat cropping.

8.1 Prediction of take-all risk in subsequent wheat crop by quantification of *Ggt* DNA concentrations in soil

Major findings from using Ggt DNA concentrations in soils selected for this study were:

- In some test soils that had several years of continuous wheat cropping and take-all disease, the decreased *Ggt* DNA concentrations and disease incidence in the subsequent wheat crops, indicated presence of potential take-all suppressive soils.

- The numbers of successive wheat crops required before the onset of suppressiveness varied, possibly due to the differences in soil origins, soil *Ggt* DNA concentrations, the microbial status of the soils, and crop management factors, such as fertilisation.

The prediction of take-all risk by quantifying *Ggt* DNA concentrations in soils prior to sowing subsequent wheat crops, has been available commercially to Australian growers since 2001 (Ophel-Keller & McKay 2001), and New Zealand growers since 2008. In this study, the DNA quantification service was used to measure the amount of natural *Ggt* inoculum in the soils. However, there were poor or variable correlations between the soil *Ggt* DNA concentrations (raw data) and the subsequent disease levels in the host plants (Chapters 2 and 7). Cook (1985) also reported that a high concentration of *Ggt* inoculum does not always denote high incidence or severity of disease in a subsequent wheat crop. In this study, the mean values of *Ggt* DNA

concentrations and soil origins, compared over successive seasons of wheat, were positively correlated with each other. This suggests that other factors, such as the physical and chemical properties (e.g. nutrient status) of the soils, and environmental conditions might have an impact on the natural *Ggt* inoculum concentrations within the soils. Similar effects have been reported in the literature (Herdina & Roget 1999, 2000). These studies indicate that the differences in soil origins need to be minimised, by sampling soils from the same region and optimising experimental protocols and design, to achieve strong correlations between the *Ggt* DNA concentrations in the soil and the take-all incidence in the subsequent crop.

Some of the soils used in this study were from fields which had high *Ggt* DNA concentrations but low levels of take-all severity and were cropped with 3 and 4 y of wheat successively, so could be starting to express TAD (McSpadden Gardener & Weller 2001). Clearly, a soil undergoing TAD might be unsuitable for predicting take-all risks in the subsequent wheat crops by *Ggt* DNA quantification. However, the number of successive wheat crops before the onset of TAD has been reported to vary for different soils, generally ranging from 3 to 6 y (Weller *et al.* 2002). Alternatively, a soil grown with wheat for successive years may not even develop TAD. Factors such as variation of aggressive *Ggt* strains between soils, gradual reduction and replacement of the aggressive strains by the less aggressive strains (Shipton 1977; Lebreton *et al.* 2004), and reduction of inoculum over time due to the breakdown of viable *Ggt* infected propagules/host residue (in press:Bithell *et al.* 2009), might also affect the inoculum and disease correlation.

The DNA quantification tool is still useful for New Zealand growers to predict take-all risks during the early successive wheat crops. However, growers should be aware of the limitations and that soils showing significant reductions in *Ggt* DNA concentrations after a wheat crop, and declined disease severity in a subsequent crop, could be undergoing TAD.

8.2 Efficacy of the pot assays used for screening of take-all suppressive soils

The Major finding from the development studies was:

- Identification of the controlled conditions, at 16°C, 80% RH with alternate 12 h light/dark conditions, and watering the plants twice weekly to FC, that were suitable for differentiating soils with different levels of suppressiveness in pot assays used in this PhD research programme.

Assessment of take-all suppressiveness in the field can be difficult mainly due to the presence of many varying factors, such as climate, physico-chemical properties and the uneven concentrations of *Ggt* inoculum between soils of different origins (Rovira & Wildermuth 1981). The present study therefore began by developing a reliable pot assay to investigate the potential of take-all suppressiveness in the New Zealand wheat soils, as determined by comparing the *Ggt* DNA concentrations and the subsequent disease incidence in individual soils (Chapter 2). The results did reveal which soils had potential take-all suppressiveness but also led to further studies which aimed to improve the methodology (Chapters 3 and 4) by choosing the most appropriate *Ggt* inoculum level, ensuring consistency in treatment of the designated soils, and selecting the environmental conditions most suitable for disease development. The identified conditions were shown to permit plant growth and to demonstrate different levels of suppressiveness in different soils (Chapter 5, Experiments 1 and 2). A drawback of this study was that a TAD soil was not included as a positive control in the pot assays, but no such soil had been identified in New Zealand at the time. It is proposed that for future screening of suppressive soils, a known suppressive soil and a fallow soil be included in the pot assay as positive and negative controls, respectively.

8.3 New Zealand TAD soils and their possible mechanisms

Major findings from studies that investigated the occurrence of TAD in New Zealand wheat soils were:

- Identification of three suppressive soils, H1, H3 and M2, from the 12 potential New Zealand wheat soils screened for TAD.

- The transferability trial indicated that the TAD mechanisms were specific for soils H1 and H3, and general for soil M2.
- High levels of biological/microbial involvement were present in soils that were specific in suppression.

The existence of soils with the natural ability to suppress take-all has prompted researchers to investigate the mechanisms by which the suppressive effects are mediated (Baker & Cook 1974; Chin-A-Woeng *et al.* 2003). Past studies on suppression were not able to separate inoculum from disease, but this problem was overcome in this study by using real-time PCR to quantify the concentrations of *Ggt* DNA in the soils and relate them to the subsequent disease levels. Experiments 1 and 2 in Chapter 5 were designed to determine the suppressive properties of 12 New Zealand wheat soils that seemed likely to experience TAD, and to investigate the possible mechanisms associated with the suppressiveness. The results showed that the wheat soils, H1, H3 (3 y of consecutive wheat) and M2 (4 y of consecutive wheat), were suppressive to take-all, and also experienced *Ggt* DNA reductions (>1000 pg g^{-1} soil) (Chapter 2). This indicated potential TAD in the early successive wheat crops, with a suppressive effect labelled by Hornby (1983) as 'pathogen suppression', which occurs when the pathogen grows saprophytically in the debris. However, in the current experiments, *Ggt* DNA concentrations were analysed from bulk soils and so the suppressive effect could not be distinguished from 'disease suppression', which occurs when *Ggt* survives parasitically within the host. Past research (Simon *et al.* 1987) has indicated that pathogen and disease suppressions did not occur independently. To determine whether simultaneous suppression of *Ggt* could occur saprophytically in the soil and parasitically in the host, the DNA concentrations of the pathogen inhabiting the rhizosphere of the host must be measured as well.

From Experiments 1 and 2 (Chapter 5), the suppressiveness of soils H1 and H3 was shown to be due to a biotic factor or associated with microbial nature since autoclaving these soils caused consistent increases in disease severity. For the autoclaved M2 soil, increases in the disease severity and levels of biological involvement were inconsistent. In Experiment 3, addition of a small amount (1% w/w) of soil H1 or H3 to a γ-irradiated soil inoculated with *Ggt* (0.1% w/w), changed the γ-irradiated soil into a suppressive one (Experiment 3, Chapter 5). However, this

transference of suppressiveness did not occur for soil M2. According to Cook & Rovira (1976), one of the most important characteristics which distinguishes specific and general suppression is the transferability of the suppressive factors from a specific suppressive soil into a natural, or sterilised soil which has no microorganisms present. The results from this study collectively suggest that the mechanisms of the TAD suppressiveness could be 'specific' for soils H1 and H3, and 'general' for soil M2. In future, incorporation of a specific suppressive soil into a conducive soil may be an alternative way of controlling take-all in the field by accelerating the development of TAD in the conducive soil. Soil P7, which was found to be conducive in Experiments 1 and 2, also showed some suppression to *Ggt* after a small amount of it was transferred into the γ-irradiated base soil. The results of these studies demonstrate the complexities of the interactions among the soil chemical, physical and biological properties, and raised questions about the robustness of its suppressiveness. This soil was initially categorised as one that could be either conducive or specific in suppressiveness, but was later indicated to be conducive due to the similarity in its DGGE banding patterns with those from the non-suppressive soil control (H15, ryegrass soil) in the Chapter 6 study.

8.4 Microorganisms associated with the suppressiveness of TAD

Major findings from the PCR-DGGE analyses of roots and rhizosphere soils were:

- The microbial DGGE fingerprints were similar for soils with similar suppressiveness, thus able to differentiate the soils into their suppressive types.
- The presence of some specific microorganisms, not the microbial diversities, in the rhizosphere and roots of plants in the soils, was responsible for the differentiation of the soils.
- In general, *Streptomyces bingchengensis*, *Terrabacter* sp., *Nocardioides* sp., *Fusarium lateritium*, *Microdochium bolleyi* and an unidentified fungus, were associated with the suppressive soils, but *Pseudomonas putida*, *P. fluorescens*, *Nocardioides oleivorans*, *Gibberella zeae*, and *Penicillium allii* were unique in the specific suppressiveness.

The study conducted in Chapter 6 used PCR-DGGE to characterise and compare the microbial communities within the rhizosphere of plants grown in soils that had demonstrated conduciveness and suppressiveness (both specific and general) to take-all. Rhizosphere competence is viewed as an ecologically desirable attribute for microorganisms, when screening for those with potential growth promotion or control activities against root-infecting pathogens (Whipps 1997). In this study, total DNA was extracted from the roots and rhizosphere soil of wheat plants, which had grown in the suppressive soils in a pot assay (Experiment 2, Chapter 5).

Overall, the PCR-DGGE analyses supported the results of pot assays. The test soils were clustered into conducive and suppressive types, also into specific or general suppression, with similar microbial DGGE fingerprints for soils with similar suppressiveness. However, the diversity of microorganisms in the rhizosphere/roots of plants grown in the individual soils (indicated by the number of bands), were not responsible for this clustering, rather it was the presence of distinguishing bands that was associated with the soil suppressiveness. Further excision, re-amplification, cloning and sequencing of the distinguishing bands that differentiated the soils, showed that some actinomycetes (*Streptomyces bingchengensis*, *Terrabacter* sp. and *Nocardioides* sp.), ascomycetes (*Fusarium lateritium* and *Microdochium bolleyi*) and an unidentified fungus, were associated with the suppressive soils (both specific and general). Others, such as the proteobacteria (*Pseudomonas putida* and *P. fluorescens*), an actinomycete (*Nocardioides oleivorans*), ascomycete (*Gibberella zeae*), and basidiomycete (*Penicillium allii*) were unique in the specific suppressiveness. This suggests that some microorganisms were common in all take-all suppressive soils, but a selected group of distinguishing microorganisms might be responsible for or associated with specific suppressiveness. Since nearly all of these distinguishing microorganisms in the suppressive soils have been reported to show antagonistic activities against *Ggt* or other soilborne pathogens, they are likely to be associated with the suppressiveness. However, due to the many limitations that come with DGGE, the results have to be interpreted with caution. More work is required to validate the effects of the distinguishing microorganisms, either individually or interactively, against *Ggt* in plate assays and/or pot experiments before confident conclusions can be drawn. The absence of distinguishing microorganisms in the

general suppressive soil agrees with Cook (2003) and Weller *et al.* (2002), who stated that general suppression is not related to any one microorganism.

Knowledge of the microorganisms responsible for take-all suppressiveness, could allow development of better strategies for control of the disease in the future, for example by introducing them into the soil. Genetic markers could also be developed to enumerate the distinguishing microorganisms in the suppressive soil, providing growers with decision-making tools to be used before planting wheat in the same field.

8.5 Fluorescent *Pseudomonas* spp. as potential biological control agents of *Ggt*

Major findings on fluorescent Pseudomonas from the TAD induction trial were:

- The numbers of *Pseudomonas fluorescens* CFU in the rhizosphere of wheat plants in pots of soils naturally infested with *Ggt* increased over successive wheat seasons.

- The increases in the numbers of *Pseudomonas fluorescens* CFU did not correlate with either disease incidence or *Ggt* DNA concentrations.

The involvement of fluorescent pseudomonads in the suppressiveness of TAD has been widely reported (Cook & Rovira 1976; Simon & Sivasithamparam 1989; Raaijmakers *et al.* 1997; Raaijmakers & Weller 1998; Raaijmakers *et al.* 1999; McSpadden Gardener *et al.* 2000; McSpadden Gardener & Weller 2001; Mazzola 2002). Although *Pseudomonas putida* and *P. fluorescens* were found in the PCR-DGGE study (Chapter 6), to be associated with the specific suppressiveness of the New Zealand TAD soils, other microorganisms were also unique to the suppressiveness. An attempt to induce TAD suppressiveness showed that the numbers of *P. fluorescens* CFU in the rhizosphere of plants grown in pots of soils naturally infested with *Ggt*, did increase with successive wheat crops (Chapter 7). However, this was independent of the *Ggt* DNA concentrations and disease incidence, suggesting that the increase in the numbers of *P. fluorescens* CFU might be a natural event regardless of *Ggt* inoculum concentrations in the soil. In addition, the numbers of *P. fluorescens* CFU was also found to correlate positively with the Na

concentrations in the soil. However, more research is required to validate this effect since strong correlation did not mean that there was a direct effect of Na on *P. fluorescens* CFU. If the types of antibiotics, metabolites or siderophores, which are believed to play a part in inhibiting *Ggt* (Scher & Baker 1982; Thomashow & Weller 1988; Hornby *et al.* 1998; Delaney *et al.* 2001), are produced by the *P. putida* and *P. fluorescens* identified in this study, this may allow their antagonistic activities against *Ggt* to be determined.

8.6 Soil nutrition and TAD induction

The major finding on soil nutrition from the TAD induction trial was:

- The decreased disease incidence at season 6 coincided with the high concentrations of available N and Olsen P, which were added prior to setting up the season 6 trial.

An attempt to induce TAD experimentally was unsuccessful in the present study (Chapter 7), since results did not reflect the common symptoms reported for TAD, which were reported to occur after 4-6 successive wheat crops in the field (McSpadden Gardener & Weller 2001; Mazzola 2002; Weller *et al.* 2002). This indicates that other factors (e.g. variable concentrations of *Ggt* DNA in the soils and the additions of nutrients mid way through the experiment), might have influenced the induction process. Although the influences of nutrition on TAD was not the main focus in this study, some of the nutrients measured, particularly the higher concentrations of available N and Olsen P, seemed to be associated with lower disease incidence and high *Ggt* DNA concentrations in the soils. This may have been due to reported effects (Huber 1981; Hornby *et al.* 1998; Cook 2003) of high concentrations of available N and Olsen P in the soils reducing the disease incidence by promoting root growth. The same increases in N and P were reported to improve the survival and so increase the concentration of *Ggt* in soils (Garrett 1938, 1940; Chambers & Flentje 1969; Huber 1981). More experiments are required to investigate the possible roles of available N and Olsen P on disease incidence, root growth and the survival of *Ggt* and on distinguishing microorganisms in the rhizosphere/roots during consecutive wheat cropping. However, these effects of N and P on the development of TAD indicate that New Zealand growers practising monoculture of wheat for successive seasons should

consider applying sufficient nutrients to the soils annually to encourage plant vigour and also potential development of TAD.

For the current research, financial constraints allowed seasonal analysis of nutrient concentrations in the soils of only the basic soil nutrients, not other micronutrients which may affect disease incidence. For instance, it is well established that the available manganese (Mn) in the soil is inversely related to take-all incidence or severity (Thompson & Huber 2007). Therefore, future work investigating the effects of soil nutrients on TAD should include both the major and micronutrients.

8.7 The modified hypothesis

The initial hypothesis proposed for this study was that TAD was a specific suppression caused by individual or selected groups of microorganisms. Some soils had specific suppression with distinguishing microorganisms unique to these soils (Section 8.4), thus agreeing with the initial hypothesis. According to Cook *et al.* (1986), *Ggt* development in suppressive soils is limited by suppression of the pathogen (i.e. parasitic stage) after initial infection of roots. Since the distinguishing microorganisms were determined from the total DNA extracted from the roots, this indicated likely antagonistic effects on the parasitic and endophytic *Ggt*. However, the results also showed that at least one TAD soil was associated with general suppression, the microorganisms within the rhizosphere and roots of plants acting to suppress the pathogen during its pre-penetration and penetration stages (i.e. resting phase and saprophytic stage) (Cook *et al.* 1986). Clearly, the initial hypothesis should have been expanded into the following:

> Take all decline is due to either specific suppression, which is associated with an individual or a selected group of microorganisms that suppress *Ggt* in the rhizosphere and roots of the host, or due to general suppression, which is not associated with any distinguishing microorganisms.

8.8 Recommendations for future research

This study has proposed the possible mechanisms behind TAD and identified the microorganisms associated with the suppression, but has also provided further key questions on the factors affecting the natural suppression of take-all. Further research

in some areas has to be carried out to give a more complete understanding of the mechanisms and microbial interactions in TAD. These are listed below:

1. *Improvement to the prediction of take-all risks in subsequent wheat crops using Ggt DNA concentrations*

    a) Using soils of similar origins to establish better correlation between the *Ggt* DNA concentrations in bulk soil and the subsequent disease severity/incidence of plants in pot assays or in the field, so that take-all risk in subsequent crops can be predicted accurately.

2. *Investigations into the possibility of soils with general suppressiveness developing specific suppression over time*

    b) Simultaneous measurements of the *Ggt* DNA concentration should be made in the soil and within the roots of host plants grown in the same soil over successive seasons of wheat crops. This will show whether suppression can target the *Ggt* growing saprophytically in the soil and parasitically in the host and whether these change over time.

    c) Use of PCR-DGGE for continued characterisation of the microbial populations within the rhizosphere/roots of plants grown in the general suppressive soils over successive seasons of wheat in inoculated pot assays. This will show whether microbial populations change over successive wheat seasons.

3. *Investigations into soil nutrient effects on take-all levels*

    d) Pot and field experiments to investigate the effects of nutrients (in particular N and P) in promoting new roots to offset diseased roots of plants infected with *Ggt* over time. This could be achieved by measuring cumulative new roots (by total weight) relative to the infected roots over time.

    e) Pot and field experiments to investigate whether optimum N and P concentrations in consecutive wheat crops increased the rate of initial take-all development and subsequently TAD development. This could be achieved by

monitoring field *Ggt* DNA concentrations and disease incidence, followed by pot trials as detailed in this thesis.

4. *Continued improvement in protocols for pot assays to investigate the mechanisms of soil suppressiveness*

    f) Further pot assays to confirm the mechanisms of suppression (i.e. specific and general forms of suppressiveness) proposed in this thesis should integrate other known/reported characteristics, to reduce ambiguity in defining suppression types (Rovira & Cook 1981). These include:

        - steam treating the soils at a range of temperatures around 60°C since specific suppression is known to be eliminated at this temperature, whereas general suppression remains functional.

        - fumigating the soils to determine the persistence of the suppressiveness, since specific suppression is known to be eliminated by fumigation, whilst general suppression is reduced but not eliminated by this treatment.

5. *Investigations into the mechanisms used by the distinguishing microorganisms to antagonise Ggt and the prospects of using them as biocontrol control agents*

    - Field studies and pot assays to investigate the efficacy and ecological fitness of the distinguishing microorganisms as biocontrol agents against *Ggt* under controlled and natural conditions.

    - Field trials to encourage or accelerate the development of TAD in conducive soil by introducing the distinguishing microorganisms or a portion of a specific suppressive soil.

    - *In vitro* studies using plate assays to identify the antagonistic mechanisms used by the distinguishing microorganisms individually and as a group against *Ggt*, and against one another.

- Identification of the types of antibiotics, metabolites or siderophores, produced by the *P. putida* and *P. fluorescens* from the specific suppressive soils, and investigation of their activities against *Ggt*.

6. *Development of molecular markers*

- Molecular studies to design and develop genetic probes or markers for analysing the rhizosphere soil and roots of wheat plants grown in a soil. Detection of the distinguishing microorganisms could provide a tool to determine the suppressive types (specific suppressiveness and conduciveness) of the soil.

8.9 Concluding remarks

This study has enhanced our understanding of the microbial interaction within the rhizosphere/roots of plants grown in soils with differing suppressiveness to take-all, and has also identified some key factors, such as soil physico-chemical properties and nutrients, which may affect the development of TAD. It has verified the initial hypothesis proposed for this thesis by identifying specific suppressive soils in New Zealand as well as finding soils with general suppression. This study has, therefore, provided valuable information that will improve the prospects of integrating suppressive soils into conventional cropping systems, and acted as a foundation for further research into the possibility of using the distinguishing microorganisms as natural control agents against *Ggt*.

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Appendices

Appendix 1 Growth stages of cereals

A1. 1 Decimal codes for growth stages of cereals (Tottman 1987)

0 Germination

00 Dry Seed
01 Start of imbibition
02 ---
03 Imbibition complete
04 ---
05 Radicle emerged from seed
06 -
07 Coleoptile emerged from seed
08 ---
09 Leaf just at coleoptile tip

1 Seedling Growth

10 First leaf through coleoptile
11 First leaf unfolded
12 2 leaf unfolded
13 3 leaf unfolded
14 4 leaf unfolded
15 5 leaf unfolded
16 6 leaf unfolded
17 7 leaf unfolded
18 8 leaf unfolded
19 9 or more leaves unfolded

2 Tillering

20 Main shoot only
21 Main shoot and 1 tiller
22 Main shoot and 2 tillers
23 Main shoot and 3 tillers
24 Main shoot and 4 tillers
25 Main shoot and 5 tillers
26 Main shoot and 6 tillers
27 Main shoot and 7 tillers
28 Main shoot and 8 tillers
29 Main shoot and 9 or more tillers

3 Stem Elongation

30 Ear at 1 cm (psuedostem erect)
31 First node detectable
32 2nd node detectable
33 3rd node detectable
34 4th node detectable
35 5th node detectable
36 6th node detectable
37 Flag leaf just visible
38 ---
39 Flag leaf ligule just visible

4 Booting

40 ---
41 Flag leaf sheath extending
42 ---
43 Boots just visibly swollen
44 ---
45 Boots swollen
46 ---
47 Flag leaf sheath opening
48 ---
49 First awns visible

5 Inflorescence (ear/panicle) emergence

50 ---
51 First spikelet of inflorescence just visible
52 ---
53 1/4 of inflorescence emerged
54 ---
55 1/2 of inflorescence emerged
56 ---
57 3/4 of inflorescence emerged
58 ---
59 Emergence of inflorescence completed

6 Anthesis (flowering)

60 ---
61 Beginning of anthesis
62 ---
63 ---
64 ---
65 Anthesis half way
66 ---
67 ---
68 ---
69 Anthesis completed

7 Milk Development

70 ---
71 Caryopsis (kernel) water ripe
72 ---
73 Early milk
74 ---
75 Medium milk
76 ---
77 Late milk
78 ---
79 ---

8 Dough Development

80 ---
81 ---
82 ---
83 Early dough
84 ---
85 Soft dough
86 ---
87 Hard dough
88 ---
89 ---

9 Ripening

90 ---
91 Caryopsis hard (difficult to divide)
92 Caryopsis hard (not dented by thumbnail)
93 Caryopsis loosening in daytime
94 Over-ripe, straw dead and collapsing
95 Seed dormant
96 Viable seed giving 50% germination
97 Seed not dormant
98 Secondary dormancy induced
99 Secondary dormancy lost

Appendix 2 Soil types of tested soils and descriptions of the methods used for basic soil and mineral N analyses

a) Soil types of tested soils (Kear *et al.* 1967)

Field Codes	Soil types
B3	Kaweku
B6	Kaweku
B8	Kaweku
C1	Waimakariri silt loam
C3	Waimakariri silt loam + Templeton silt loam
C6	Wakana silt loam
C12	Hatfield silt loam + Barrhill fine sandy loam
G2	Ashwick stony silt loam
H1	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase
H2	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase
H3	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase
H4	Templeton silt loam + Waitohi silt loam + Claremont silt loam easy rolling phase
H5	Waitohi silt loam + Claremont silt loam
H6	Claremont silt loam easy rolling phase
H7	Claremont silt loam easy rolling phase + Waitohi silt loam
H8	Claremont silt loam easy rolling phase + Timaru silt loam
H9	Timaru silt loam
H10	Timaru silt loam
H11	Timaru silt loam
H12	Timaru silt loam
H13	Claremont silt loam easy rolling phase
H14	Waitohi silt loam + Claremont silt loam easy rolling phase
H15	Claremont silt loam easy rolling phase.
I6	Waitohi silt loam
L9	Timaru silt loam + Claremont silt loam easy rolling phase
M2	Waitohi silt loam
M3	Waitohi silt loam
M10	Waitohi silt loam
M11	Waitohi silt loam

b) Methods used in the basic soil and mineral N analyses (R. J. Hill Laboratories Ltd., 2002).

Analyte	Method
Soil preparation (dry)	Air dried at 35°C overnight (residual moisture typically 4%).
Available nitrogen	Anaerobic incubation followed by extraction using 2M KCL followed by Berthelot colorimetry. Calculation based on 15 cm depth sample.
Anaerobically mineralisable N	As for available N but reported as $\mu g\ g^{-1.}$
pH	1:2 (v/v) soil: water slurry followed by potentiometric determination of pH.
Phosphorus	Olsen extraction followed by Molybdenum Blue colorimetry.
Potassium, calcium, magnsium and sodium	1 M neutral ammonium acetate extraction followed by ICP-OES.
CEC	Summation of extractable Cations (K, Ca, Mg, Na) and extractable acidity.
Base saturation	Calculated from Extractable Cations and Cation Exchange Capacity.
Volume weight	The weight/volume ratio of dried, ground soil.

Appendix 3 General methods

A3. 1 Preparation of *Ggt* inoculum and inoculation of soils in pot experiments

A sand/maizemeal mixture or culture medium was prepared by mixing 500 g sand (2 mm), 15 g ground maize meal and 65 mL of water in a clear autoclavable bag (36 x 48 cm, capable of withstanding temperatures up to 150°C (Raylab NZ Ltd., Auckland, New Zealand). A 10 cm long, 5 cm diameter aluminium tube was secured to the opening of the bag using rubber bands and was sealed by plugging with cotton wool, and covered with foil. The medium was then thoroughly shaken and autoclaved at 121°C for 1 h. Once the sterilised medium was cooled, 40 circular potato dextrose agar (PDA) discs (8 mm diameter) from the actively growing edges of an 8 d old *Ggt* culture were added to the bag. The inoculated culture media was then shaken by hand before incubating at 23°C for 3 wk and shaken weekly. Prior to use, the five different *Ggt* isolates grown in separate bags of sand/maizemeal media were pooled and mixed thoroughly in one of the bags. Soil inoculation was carried out by mixing the required amount of *Ggt* inoculum with the soil to give 300 g of soil mixture per 400 mL pot.

A3. 2 Pathogenicity test of *Ggt* isolates

The pathogenicity of the *Ggt* isolates used in the experiments was tested by inoculating 7 d old wheat and oat seedlings (five of each), which were pre-germinated on the same moistened buff germination paper rolls (Anchor Paper Ltd, Minnesota, USA). Inoculation was done by placing a circular potato dextrose agar (PDA) disc (8 mm diameter) from the actively growing edges of an 8 d old *Ggt* culture (grown at 23°C), 2 mm above the tip of the root radical. There were four replicates for each isolate treatment. The paper rolls were then placed in separate plastic bags, sealed and incubated at 25°C for a further 10 d. Results obtained after 10 d showed that 100% of wheat seedlings inoculated with the five *Ggt* isolates had lesions on their roots and penetrated hyphae present within the root cortices. Symptoms of root infection was not found on any of the control oat seedlings.

A3. 3 Pre-germination of seeds

All the wheat seedlings used in the experiments were pre-germinated on three moistened buff germination papers (Anchor papers Ltd., Minnesota, USA), rolled up, and then sealed in plastic bags and incubated at 25°C for 3 d before planting.

A3. 4 Calculations for nutrient solutions used in pot experiment

(a) Ammonium nitrate (NH_4NO_3) required to give N = 150 µg g^{-1} soil

For 300 g of soil pot^{-1},

$$\begin{aligned}\text{Required N rate} &= 150 \times 300 \\ &= 45000\,\mu g \\ &= 0.045\,g\end{aligned}$$

For 0.045 g N,

$$\begin{aligned}\text{Required } NH_4NO_3 \text{ rate} &= 0.045 \times \left[\frac{\text{Total mol. mass of } NH_4NO_3\ (80.04)}{\text{Total atomic mass of N (28)}}\right] \\ &= 0.0129\,g\end{aligned}$$

(b) Potassium phosphate (KH_2PO_4) required to give P = 50 µg g^{-1} soil

For 300 g of soil pot^{-1},

$$\begin{aligned}\text{Required P rate} &= 50 \times 300 \\ &= 15000\,\mu g \\ &= 0.015\,g\end{aligned}$$

For 0.015 g P,

$$\begin{aligned}\text{Required } KH_2PO_4 \text{ rate} &= 0.015 \times \left[\frac{\text{Total mol. mass of } KH_2PO_4\ (136.09)}{\text{Total atomic mass of P (30.97)}}\right] \\ &= 0.066\,g\end{aligned}$$

(c) Potassium sulphate (K_2SO_4) required to give S = 20 µg g^{-1} soil

For 300 g of soil pot^{-1},

$$\begin{aligned}\text{Required S rate} &= 20 \times 300 \\ &= 6000\,\mu g \\ &= 0.006\,g\end{aligned}$$

For 0.006 g S,

$$\begin{aligned}\text{Required } K_2SO_4 \text{ rate} &= 0.006 \times \left[\frac{\text{Total mol. mass of } K_2SO_4\ (174.25)}{\text{Total atomic mass of S (32.06)}}\right] \\ &= 0.033\,g\end{aligned}$$

(d) KH_2PO_4 and K_2SO_4 required to give K = 100 µg g^{-1} soil

300 g of soil pot^{-1},

$$\begin{aligned}\text{Required K rate} &= 100 \times 300 \\ &= 30000\,\mu g \\ &= 0.03\,g\end{aligned}$$

$$\text{K present in (b) 0.066 g of } KH_2PO_4 = \frac{[0.66 \times \text{Total atomic mass of K (39.1)}]}{\text{Total mol. mass of } KH_2PO_4 \text{ (136.09)}}$$
$$= 0.019 \text{ g}$$

$$\text{K present in (c) 0.033 g of } K_2SO_4 = \frac{[0.33 \times \text{Total atomic mass of K (78.2)}]}{\text{Total mol. mass of } K_2SO_4 \text{ (174.25)}}$$
$$= 0.015 \text{ g}$$

$$\text{Total K obtained from } KH_2PO_4 \text{ and } K_2SO_4 = 0.019\text{g} + 0.015\text{g}$$
$$= 0.034\text{g}$$

The amount of K at 0.034 g, has exceeded the amount required to get 100 µg g^{-1} soil, hence, was sufficient for the plants.

The calculated amounts of chemicals in (a), (b) and (c) were required to prepare 20 mL of nutrient solutions, but these were prepared in greater volumes depending on the number of pots in each trial.

A3. 5 Moisture contents of test soils at field capacity

Prior to conducting the pot assays, the original moisture contents (MC) and the MC at field capacity (FC) of the soils were determined for watering purposes. The original MC were determined by weighing three replicates of each soil sample (about 5 g each) prior to drying in the oven at 105°C for 24 h. The oven-dried soils were then weighed again.

Original MC (%) was calculated with the formula:

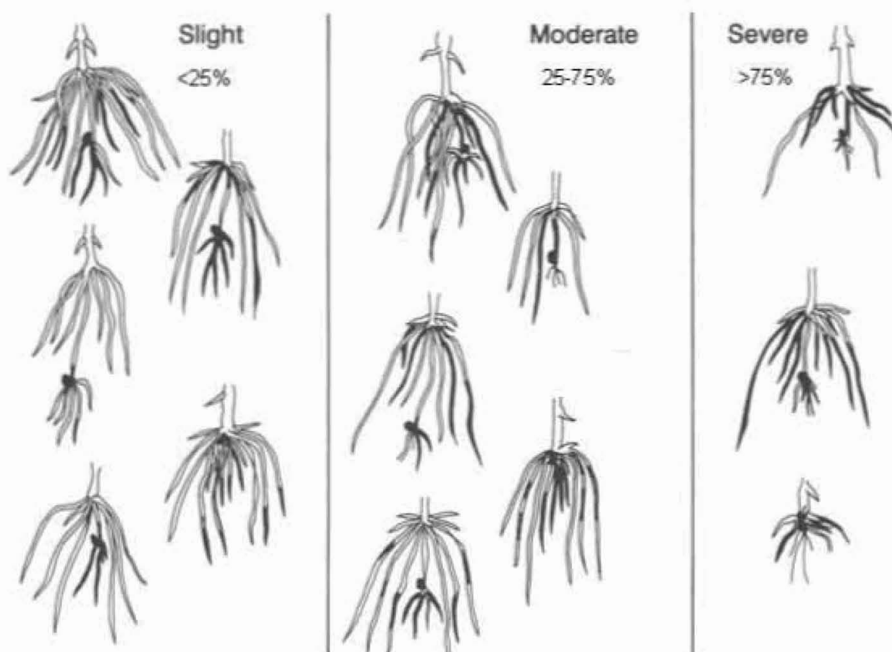
$$\text{MC (\% w/w)} = \left[\frac{\text{Wet weight (g) - Dry weight (g)}}{\text{Dry weight (g)}}\right] \times 100\%$$

MC of all the soils at FC were determined by using the tension tables based on the principles described by (Klute 1986). Three replicates of each soil sample (about 5 g each) were placed onto circular (90mm diameter) Whatman® glass micro-fibre papers (Whatman, International Ltd., USA) separately, and left on the tension tables, which were made up of silica flour and sand, and maintained at a suction pressure of -5 kPa by adjusting the height of an attached water bottle next to apparatus to 50 cm below the tension table. This pressure of -5kPa equates to the minimum amount of water retained by the soils at FC (McLaren & Cameron 1996). After 3 d, the soils were weighed, dried at 105°C for 24 h and weighed again. Wet and dry weights obtained were used to calculate the MC of soils at FC using the same formula used for calculating the original MC. The amount of water (weight by weight) required by each soil to reach FC was then calculated with the formula:

$$\text{Reqired water to reach FC} = \left(\frac{\text{\% MC at FC - \% original MC}}{100} \right) \times \text{Total amount of soil in each pot (g)}$$

All the data were then analysed with one-way ANOVA. Results obtained are presented in Appendix 5, A5. 1. The weights of nutrient solutions (20 mL), pots and soils were all taken into considerations when applying water to FC.

A3. 6 Infection keys used in Chapter 5 (Hornby *et al.* 1998).



Appendix 4 Media, gels and solutions

A4. 1 Potato dextrose agar (PDA)

39 g	Potato dextrose agar (Difco Laboratories, Detroit, USA)
1000 mL	Reverse osmosis water

The agar was mixed thoroughly with the water and heated with frequent agitation in a microwave (1000 Watts) for about 1 min to completely dissolve the powder. The agar solution was then autoclaved at 121°C and 15 psi for 15 min.

A4. 2 Nutrient Agar

23 g	Nutrient Agar (Difco Laboratories, Detroit, USA)
1000 mL	Reverse osmosis water

The agar was mixed thoroughtly with the water and heated with frequent agitation in a microwave (1000 Watts) for about 1 min to completely dissolve the powder. The agar solution was then autoclaved at 121°C and 15 psi for 15 min.

A4. 3 Modified King's B agar

15 g	Bateriological Agar (Difco Laboratories, Detroit, USA)
20 g	Peptone (Difco Laboratories, Detroit, USA)
1000 mL	Reverse osmosis water
1.5 g	K_2HPO_4 (MERCK, Merck KGaA, Germany)
1.5 g	$MgSO_4.7H_2O$ (BDH Laboratory Supplies, England)
10 mL	Glycerol (BDH, VWR International Ltd, England)
1 mL	Trimethoprim (20 mg L^{-1}) (Sigma Chemical Co., USA)

The agar and peptone were mixed thoroughly with the water and heated with frequent agitation in a microwave (1000 Watts) for about 1 min to completely dissolve the powders. The rest of the ingredients excluding antibiotic were then added to the agar solution and the pH adjusted to 7.2. The medium was then autoclaved at 121°C at 15 psi for 15 min. The molten agar was cooled to approximately 50°C and the antibiotic was added aseptically. Antibiotic stock was prepared by dissolving 0.5 g of Trimethoprim in 25 mL of Dimethyl sulphoxide (DMSO).

A4. 4 Potato dextrose broth (PDB)

24 g	Potato dextrose broth (Difco laboratories, Detroit, USA)
1000 mL	Reverse osmosis water

The PDB powder was added to the water and autoclaved at 121°C and 15 psi for 15 min.

A4. 5 1% (w/v) agarose gel

0.3 g	Agarose (Sigma, St. Louis, United States)
30 mL	0.5×TBE buffer (Tris Boric EDTA)

Dissolve and heat the mixture in the microwave on high power for 1min. Cool the molten agar to about 50°C and add 3 μL of ethidium bromide.

Appendix 5 Additional results and ANOVA tables of statistical analyses

Chapter 2

A5. 1 Means of original moisture content and moisture content at FC of soils

Soil Sample	Mean moisture content (%)	
	Original	FC
B3	24.77	33.16
B6	24.45	34.28
B8	27.86	35.73
C1	17.40	32.48
C3	16.95	36.63
C6	18.56	33.24
C12	13.60	31.95
G2	22.50	41.97
H1	21.38	35.85
H2	20.45	32.36
H3	20.40	35.84
H4	20.41	31.82
H5	20.09	36.22
H6	23.06	32.73
H7	20.71	34.22
H8	27.97	34.97
H9	21.49	33.18
H10	25.15	32.98
H11	23.42	30.90
H12	22.00	34.74
H13	14.16	35.67
H14	15.67	35.41
H15	18.51	34.69
I6	21.06	38.96
L9	21.41	33.44
M2	21.06	33.63
M3	22.23	35.41
M10	20.34	35.45
M11	18.02	32.19
Significance	$p<0.001$	$p<0.001$
LSD at 5%	11.61	1.89

A5. 2 Analysis of variance

A5.2 a ANOVA of moisture contents of soils

Original % MC of soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	7.703	3.8515	3.98	
Rep.Units.stratum					
Soils	28	1010.21	36.079	37.33	<.001
Residual	56	54.1299	0.9666		
Total	86	1072.04			

% MC at FC of soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Rep stratum	2	4.226	2.113	1.58	
Rep.Units.stratum					
Soils	28	437.378	15.621	11.7	<.001
Residual	56	74.734	1.335		
Total	86	516.337			

A5.2 b ANOVA of *Ggt* DNA concentrations of soils after crop harvest (quantified from soils collected for the pot experiment)

Ggt DNA concentrations for the 29 test soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3	0	0	0	
Block_Rep.Units. stratum					
Years of wheat cropping	4	8767899	2E+06	4.91	0.001
Residual	108	4.8E+07	446359		
Total	115	5.7E+07			

Ggt DNA concentrations for St Andrews soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3	0	0	0	
Block_Rep.Units. stratum					
Years of wheat cropping	5	1.2E+07	2E+06	5.87	<.001
Years of wheat.cropping.soils	10	7000856	700086	1.77	0.076
Residual	97	3.8E+07	395482		
Total	115	5.7E+07			

A5.2 c ANOVA of percent infected roots from the pot experiment

Initial analysis on transformed percent infected roots of the whole pot experiment (including both inoculated and uninoculated soils). Transformation was done with adjusted logit: LOG ((0.5 + percent infected roots) / (100.5 - percent infected roots)).

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		121.427	40.476	40.59	
Block_Rep.Units. stratum						
Inoc	1		613.736	613.74	615.43	<.001
Years of wheat cropping	4		12.943	3.2357	3.24	0.014
Inoc.Years of wheat cropping	4		11.8377	2.9594	2.97	0.021
Years of wheat cropping.Soils	24		30.9975	1.2916	1.3	0.174
Inoc.Years of wheat cropping.Soils	24		29.5033	1.2293	1.23	0.221
Residual	168	-3	167.537	0.9972		
Total	228	-3	982.687			

Analysis on transformed percent infected roots of the 29 test soils without added *Ggt* inoculum. Transformation was done with adjusted logit: LOG ((0.5 + percent infected roots) / (100.5 - percent infected roots)).

Source of variation	df	(m.v).	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		146.163	48.721	34.88	
Block_Rep.Units. stratum						
Years of wheat cropping	4		22.366	5.591	4	0.005
Years of wheat cropping.Soils	24		55.983	2.333	1.67	0.046
Residual	83	-1	115.922	1.397		
Total	114	-1	340.128			

Analysis on transformed percent infected roots of the 15 St Andrews soils without added *Ggt* inoculum. Transformation was done with adjusted logit: LOG ((0.5 + percent infected roots) / (100.5 - percent infected roots)).

Source of variation	df(m.v.)	(m.V.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		146.163	48.721	34.88	
Block_Rep.Units. stratum						
Set	1		7.552	7.552	5.41	0.022
Set.not St Andrews	13		22.63	1.741	1.25	0.263
Set.St Andrews years of wheat cropping	4		19.289	4.822	3.45	0.012
Set.St Andrews years of wheat .St Andrews soils	10		28.877	2.888	2.07	0.036
Residual	83	-1	115.922	1.397		
Total	114	-1	340.128			

A5.2 d ANOVA of the root and shoot dry and fresh weights from the pot experiment

Shoot fresh weight of the 29 test soils without added *Ggt*

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		45.997	15.332	106.09	
Block_Rep.Units.stratum						
Years of wheat cropping	4		4.4485	1.1121	7.7	<.001
Years of wheat cropping.Soils	24		14.574	0.6073	4.2	<.001
Residual	83	-1	11.995	0.1445		
Total	114	-1	77.006			

Shoot dry weight of the 29 test soils without added *Ggt*

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		0.2515	0.0838	16.78	
Block_Rep.Units.stratum						
Years of wheat cropping	4		0.1417	0.0354	7.09	<.001
Years of wheat cropping.Soils	24		0.4574	0.0191	3.82	<.001
Residual	83	-1	0.4145	0.005		
Total	114	-1	1.2604			

Root fresh weight of the 29 test soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		23.274	7.7581	16.93	
Block_Rep.Units.stratum						
Years of wheat cropping	4		1.4489	0.3622	0.79	0.535
Years of wheat cropping.soils	24		22.697	0.9457	2.06	0.008
Residual	83	-1	38.04	0.4583		
Total	114	-1	85.347			

Root dry weight of the 29 test soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		0.5848	0.1949	31.45	
Block_Rep.Units. stratum						
Years of wheat cropping	4		0.0044	0.0011	0.18	0.949
Years of wheat cropping.Soils	24		0.2603	0.0108	1.75	0.033
Residual	83	-1	0.5144	0.0062		
Total	114	-1	1.354			

Root dry weight of the 15 Andrews soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		0.5848	0.1949	31.45	
Block_Rep.Units. stratum						
Set	1		0.0002	0.0002	0.04	0.846
Set.not St Andrews	13		0.1736	0.0134	2.16	0.019
Set.St Andrews years of wheat cropping	4		0.0345	0.0086	1.39	0.243
Set.St Andrews years of wheat cropping.St Andrews soils	10		0.0563	0.0056	0.91	0.529
Residual	83	-1	0.5144	0.0062		
Total	114	-1	1.354			

Root fresh weight of the 15 St Andrews soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		23.274	7.7581	16.93	
Block_Rep.Units. stratum						
Set	1		0.6141	0.6141	1.34	0.25
Set.not St Andrews	13		11.565	0.8896	1.94	0.037
Set.St Andrews years of wheat cropping	4		5.5626	1.3907	3.03	0.022
Set.St Andrews years of wheat cropping.St Andrews soils	10		6.4039	0.6404	1.4	0.196
Residual	83	-1	38.04	0.4583		
Total	114	-1	85.347			

Shoot dry weight of the 15 St Andrews soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		0.2515	0.0838	16.78	
Block_Rep.Units. stratum						
Set	1		0.0163	0.0163	3.26	0.074
Set.not St Andrews	13		0.378	0.0291	5.82	<.001
Set.St Andrews years of wheat cropping	4		0.1359	0.034	6.8	<.001
Set.St Andrews years of wheat cropping.St Andrews soils	10		0.0689	0.0069	1.38	0.204
Residual	83	-1	0.4145	0.005		
Total	114	-1	1.2604			

Shoot fresh weight of the 15 St Andrews soils without added *Ggt* inoculum

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block_Rep stratum	3		45.997	15.332	106.09	
Block_Rep.Units. stratum						
Set	1		0.4236	0.4236	2.93	0.091
Set.not St Andrews	13		11.835	0.9104	6.3	<.001
Set.St Andres years of wheat cropping	4		4.8662	1.2166	8.42	<.001
Set.St Andrews years of wheat cropping.St Andrews soils	10		1.8978	0.1898	1.31	0.237
Residual	83	-1	11.995	0.1445		
Total	114	-1	77.006			

Chapter 3

A5. 3 Analysis of Variance

A5.3 a ANOVA of percent infected roots from the pot experiment

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	274.96	91.65	1.42	
Block.Units.stratum					
% inoc	4	40132.35	10033.09	155.46	<.001
Soil	2	832.74	416.37	6.45	0.004
% inoc.Soil	8	1363.37	170.42	2.64	0.019
Residual	42	2710.64	64.54		
Total	59	45314.07			

A5.3 b ANOVA of root and shoot dry and fresh weights

Root dry weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	0.043167	0.014389	3.47	
Block.Units.stratum					
% inoc	4	0.035667	0.008917	2.15	0.092
Soil	2	0.044333	0.022167	5.34	0.009
% inoc.Soil	8	0.032333	0.004042	0.97	0.469
Residual	42	0.174333	0.004151		
Total	59	0.329833			

Shoot dry weight

Source of variation	df	(m.v.)	s.s.	m.s.	v.r.	F pr.
Block stratum	3		0.049603	0.016534	3.71	
Block.Units.stratum						
% inoc	4		0.109159	0.02729	6.13	<.001
Soil	2		0.162738	0.081369	18.26	<.001
% inoc.Sol	8		0.06389	0.007986	1.79	0.107
Residual	41	-1	0.182663	0.004455		
Total	58	-1	0.559322			

Root fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	9.57	3.19	8.75	
Block.Units.stratum					
% inoc	4	6.231	1.5577	4.27	0.005
soil	2	5.7	2.85	7.81	0.001
% inoc.Soil	8	2.025	0.2531	0.69	0.695
Residual	42	15.32	0.3648		
Total	59	38.846			

Shoot fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	2.7613	0.9204	3.03	
Block.Units.stratum					
% inoc	4	1.3883	0.3471	1.14	0.35
Soil	2	5.356	2.678	8.81	<.001
% inoc.Soil	8	3.4507	0.4313	1.42	0.217
Residual	42	12.7637	0.3039		
Total	59	25.72			

A5.3 c ANOVA of water uptake

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	1130.5	376.8	1.06	
Block..Units.stratum					
% inoc	4	13080.7	3270.2	9.22	<.001
Soil	2	69704.9	34852.5	98.29	<.001
% inoc.Soil	8	2912.9	364.1	1.03	0.431
Residual	42	14892.7	354.6		
Total	59	101721.7			

Chapter 4

A5. 4 Analysis of Variance

A5.4 a ANOVA of percent infected roots

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	630.02	315.01	5.56	0.027
Residual	9	509.57	56.62	0.69	
Chamber.Block.Plots stratum					
Soils	2	1904.24	952.12	11.61	<.001
Water	1	4388.33	4388.33	53.5	<.001
Chamber.Soils	4	315.17	78.79	0.96	0.438
Chamber.Water	2	242.07	121.04	1.48	0.239
Soils.Water	2	312	156	1.9	0.161
Chamber.Soils.Water	4	731.38	182.85	2.23	0.081
Residual	45	3691.12	82.02		
Total	71	12723.9			

A5.4 b ANOVA of root and shoot dry and fresh weights

Root dry weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	1.72	0.86	254.47	<.001
Residual	9	0.030417	0.00338	0.97	
Chamber.Block.Plots stratum					
Soils	2	0.1875	0.09375	26.86	<.001
Water	1	0.233472	0.233472	66.88	<.001
Chamber.Soils	4	0.085	0.02125	6.09	<.001
Chamber.Water	2	0.187778	0.093889	26.9	<.001
Soils.Water	2	0.030278	0.015139	4.34	0.019
Chamber.Soils.Water	4	0.027222	0.006806	1.95	0.119
Residual	45	0.157083	0.003491		
Total	71	2.65875			

Shoot dry weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	2.491944	1.245972	154.67	<.001
Residual	9	0.0725	0.008056	1.88	
Chamber.Block.Plots stratum					
Soils	2	0.567778	0.283889	66.36	<.001
Water	1	0.245	0.245	57.27	<.001
Chamber.Soils	4	0.117222	0.029306	6.85	<.001
Chamber.Water	2	0.0975	0.04875	11.4	<.001
Soils.Water	2	0.13	0.065	15.19	<.001
Chamber.Soils.Water	4	0.05	0.0125	2.92	0.031
Residual	45	0.1925	0.004278		
Total	71	3.964444			

Root fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	212.909	106.454	86.99	<.001
Residual	9	11.014	1.224	1.13	
Chamber.Block.Plots stratum					
Soils	2	30.501	15.251	14.07	<.001
Water	1	3.645	3.645	3.36	0.073
Chamber.Soils	4	21.307	5.327	4.91	0.002
Chamber.Water	2	15.656	7.828	7.22	0.002
Soils.Water	2	2.303	1.152	1.06	0.354
Chamber.Soils.Water	4	4.273	1.068	0.99	0.425
Residual	45	48.791	1.084		
Total	71	350.399			

Shoot fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	17.52528	8.76264	62.88	<.001
Residual	9	1.25417	0.13935	1.48	
Chamber.Block.Plots stratum					
Soils	2	27.90861	13.95431	148.6	<.001
Water	1	0.88889	0.88889	9.47	0.004
Chamber.Soils	4	4.94639	1.2366	13.17	<.001
Chamber.Water	2	0.70194	0.35097	3.74	0.032
Soil.Water	2	6.59528	3.29764	35.12	<.001
Chamber.Soils.Water	4	0.62639	0.1566	1.67	0.174
Residual	45	4.22583	0.09391		
Total	71	64.67278			

A5.4 c ANOVA of soil sieving

Percent infected roots

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	630.02	315.01	5.56	0.027
Residual	9	509.57	56.62	0.69	
Chamber.Block.Plots stratum					
Sieving	1	447.73	447.73	5.46	0.024
Water	1	4388.33	4388.33	53.5	<.001
Chamber.Sieving	2	0.44	0.22	0	0.997
Sieving.Soils	1	1456.51	1456.51	17.76	<.001
Chamber.Water	2	242.07	121.04	1.48	0.239
Sieving.Water	1	74.79	74.79	0.91	0.345
Chamber.Sieving.Soils	2	314.73	157.37	1.92	0.159
Chamber.Sieving.Water	2	455.08	227.54	2.77	0.073
Sieving.Soils.Water	1	237.21	237.21	2.89	0.096
Chamber.Sieving.Soils.Water	2	276.31	138.15	1.68	0.197
Residual	45	3691.12	82.02		
Total	71	12723.9			

Root dry weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	1.72	0.86	254.47	<.001
Residual	9	0.030417	0.00338	0.97	
Chamber.Block.Plots stratum					
Sieving	1	0.140625	0.140625	40.29	<.001
Water	1	0.233472	0.233472	66.88	<.001
Chamber.Sieving	2	0.06125	0.030625	8.77	<.001
Sieving.Soils	1	0.046875	0.046875	13.43	<.001
Chamber.Water	2	0.187778	0.093889	26.9	<.001
Sieving.Water	1	0.025069	0.025069	7.18	0.01
Chamber.Sieving.Soils	2	0.02375	0.011875	3.4	0.042
Chamber.Sieving.Water	2	0.021806	0.010903	3.12	0.054
Sieving.Soils.Water	1	0.005208	0.005208	1.49	0.228
Chamber.Sieving.Soils.Water	2	0.005417	0.002708	0.78	0.466
Residual	45	0.157083	0.003491		
Total	71	2.65875			

Shoot dry weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	2.491944	1.245972	154.67	<.001
Residual	9	0.0725	0.008056	1.88	
Chamber.Block.Plots stratum					
Sieving	1	0.380278	0.380278	88.9	<.001
Water	1	0.245	0.245	57.27	<.001
Chamber.Sieving	2	0.045972	0.022986	5.37	0.008
Sieving.Soils	1	0.1875	0.1875	43.83	<.001
Chamber.Water	2	0.0975	0.04875	11.4	<.001
Sieving.Water	1	0.1225	0.1225	28.64	<.001
Chamber.Sieving.Soils	2	0.07125	0.035625	8.33	<.001
Chamber.Sieving.Water	2	0.04875	0.024375	5.7	0.006
Sieving.Soils.Water	1	0.0075	0.0075	1.75	0.192
Chamber.Sieving.Soils.Water	2	0.00125	0.000625	0.15	0.864
Residual	45	0.1925	0.004278		
Total	71	3.964444			

Root fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	212.909	106.454	86.99	<.001
Residual	9	11.014	1.224	1.13	
Chamber.Block.Plots stratum					
Sieving	1	6.418	6.418	5.92	0.019
Water	1	3.645	3.645	3.36	0.073
Chamber.Sieving	2	2.407	1.203	1.11	0.338
Sieving.Soils	1	24.083	24.083	22.21	<.001
Chamber.Water	2	15.656	7.828	7.22	0.002
Sieving.Water	1	0.902	0.902	0.83	0.366
Chamber.Sieving.Soils	2	18.9	9.45	8.72	<.001
Chamber.Sieving.Water	2	0.823	0.411	0.38	0.686
Sieving.Soils.Water	1	1.401	1.401	1.29	0.262
Chamber.Sieving.Soils.Water	2	3.45	1.725	1.59	0.215
Residual	45	48.791	1.084		
Total	71	350.399			

Shoot fresh weight

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	17.52528	8.76264	62.88	<.001
Residual	9	1.25417	0.13935	1.48	
Chamber.Block.Plots stratum					
Sieving	1	19.06778	19.06778	203.05	<.001
Water	1	0.88889	0.88889	9.47	0.004
Chamber.Sieving	2	0.38097	0.19049	2.03	0.143
Sieving.Soils	1	8.84083	8.84083	94.14	<.001
Chamber.Water	2	0.70194	0.35097	3.74	0.032
Sieving.Water	1	6.58778	6.58778	70.15	<.001
Chamber.Sieving.Soils	2	4.56542	2.28271	24.31	<.001
Chamber.Sieving.Water	2	0.54764	0.27382	2.92	0.064
Sieving.Soils.Water	1	0.0075	0.0075	0.08	0.779
Chamber.Sieving.Soils.Water	2	0.07875	0.03938	0.42	0.66
Residual	45	4.22583	0.09391		
Total	71	64.67278			

A5.4 d ANOVA on repeated measurement of water use

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Chamber.Block stratum					
Chamber	2	16937.29	8468.643	85.92	<.001
Residual	9	887.047	98.561	0.83	
Chamber.Block.Plots stratum					
Soil_type	2	7694.078	3847.039	32.48	<.001
Chamber.Soil_type	4	2473.69	618.423	5.22	0.006
Residual	18	2132.072	118.448	15.25	
Chamber.Block.Plots.Time stratum					
df correction factor 0.3685					
Time	8	41462.84	5182.854	667.4	<.001
Chamber.Time	16	3462.401	216.4	27.87	<.001
Time.Soil_type	16	2637.588	164.849	21.23	<.001
Chamber.Time.Soil_type	32	786.643	24.583	3.17	0.001
Residual	216	1677.408	7.766		
Total	323	80151.05			

(df are multiplied by the correction factors before calculating F probabilities)

Chapter 5

A5. 5 Field management practice and crop yield information of the four wheat soils provided by the growers

Soil / season	Residue management after crop	Pre-sowing cultivation	[1]Weed Problems	Glyphosate application before sowing	Fertiliser application before crop sowing	Seed treatment before sowing	Irrigation during growing season	Yield (t ha^{-1})
H1								
1999/00	-	Min-tillage	-	-	-	-	-	-
2000/01	-	Min-tillage	-	-	-	-	-	-
2001/02	-	Plough, min-tillage	-	-	-	-	-	-
2002/03	Burned	Min-tillage	-	-	-	-	-	-
2003/04	-	Min-tillage	C	Yes	-	No	No	-
2004/05	-	-	-	-	-	-	-	-
2005/06	-	-	-	-	-	-	-	-
H3								
1999/00	-	-	-	-	-	-	-	-
2000/01	-	-	-	-	-	-	-	-
2001/02	-	Plough	-	-	-	-	-	-
2002/03	Burned	Min-tillage	-	-	-	-	-	-
2003/04	-	Min-tillage	RB and FP	Yes	10 % potash super (400 kg ha^{-1}) and N (180 kg ha^{-1})	No	No	4.4
2004/05	-	-	-	-	-	-	-	-
2005/06	-	-	-	-	-	-	-	-
M2								
1999/00	-	Plough	-	-	-	-	-	-
2000/01	Incorporated	Plough	-	Yes	-	-	-	-
2001/02	Burned	Min-tillage	-	Yes	-	-	-	-
2002/03	Burned	Min-tillage	-	Yes	-	-	-	-
2003/04	-	Min-tillage	MW, WW, F, C and WO	Yes	Sulphur super + copper (1000 kg ha^{-1}), urea (130 kg ha^{-1}) and Potassium choloride (75 kg ha^{-1})	No	Yes	7.8
2004/05	-	-	-	-	-	-	-	-
2005/06	-	-	-	-	-	-	-	-
P7								
1999/00	-	-	-	-	-	-	-	-
2000/01	Burned	Min-tillage	-	Yes	-	-	-	-
2001/02	Burned	Min-tillage	-	Yes	-	-	-	-
2002/03	Burned	Min-tillage	-	Yes	-	-	-	-
2003/04	Burned	Min-tillage	-	Yes	-	-	-	-
2004/05	Baled	plough	CW, B and RG	Yes	Sulphur super (600 kg ha^{-1}) and urea (520 kg ha^{-1})	Raxil® (containing active ingredient 25 g L^{-1} tebuconazole) and Gaucho® (containing active ingredient 600 g L^{-1} imidacloprid)	Yes	12.24
2005/06	Burned	Min-tillage	CW, B and RG	Yes	Sulphur super (500kg ha^{-1}) and urea (550 kg ha^{-1})	Raxil® (containing active ingredient 25 g L^{-1} tebuconazole)	Yes	9.64

[1] Weed proplems

C	Couch
RB	Ripgut brome
FP	field pansy
MW	Mayweed
WW	Wireweed
F	Fumitory
C	Cleaves
WO	Wild oats
CW	Chickweed
B	Brome
RG	Ryegrass

A5. 6 Analysis of Variance

A5.6 a Experiment 1-ANOVA on TAR

Effect of sterilisation on inoculated soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	1903	634.3	0.83	
Block.Plot_no stratum					
Soil	12	20412.2	1701	2.23	0.014
Inoc	1	66354.2	66354.2	87.15	<.001
Soil.Inoc	12	16342.6	1361.9	1.79	0.058
Inoc.Ster	1	41733.4	41733.4	54.81	<.001
Soil.Inoc.Ster	12	21322.1	1776.8	2.33	0.01
Residual	114	86794.9	761.4		
Total	155	254862.4			

Effect of inoculation on non-sterilised soil

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	1903	634.3	0.83	
Block.Plot_no stratum					
Soil	12	20412.2	1701	2.23	0.014
Ster	1	93461.5	93461.5	122.76	<.001
Soil.Ster	12	27724.8	2310.4	3.03	<.001
Ster.Inoc	1	14626.1	14626.1	19.21	<.001
Soil.Ster.Inoc	12	9939.9	828.3	1.09	0.377
Residual	114	86794.9	761.4		
Total	155	254862.4			

A5.6 b Experiment 2-ANOVA on TAR

Effect of sterilisation on inoculated soils

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	101.9	34	0.1	
Block.PlotNo stratum					
Soil	4	22342.6	5585.6	17.19	<.001
Inoc	1	43446.8	43446.8	133.7	<.001
Soil.Inoc	4	11692.1	2923	9	<.001
Inoc.Ster	1	16000	16000	49.24	<.001
Soil.Inoc.Ster	4	4694.4	1173.6	3.61	0.013
Residual	42	13648.1	325		
Total	59	111925.9			

Effect of inoculation on non-sterilised soil

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	101.9	34	0.1	
Block.PlotNo stratum					
Soil	4	22342.6	5585.6	17.19	<.001
Ster	1	45695	45695	140.62	<.001
Soil.Ster	4	2803.2	700.8	2.16	0.091
Ster.Inoc	1	13751.7	13751.7	42.32	<.001
Soil.Ster.Inoc	4	13583.3	3395.8	10.45	<.001
Residual	42	13648.1	325		
Total	59	111925.9			

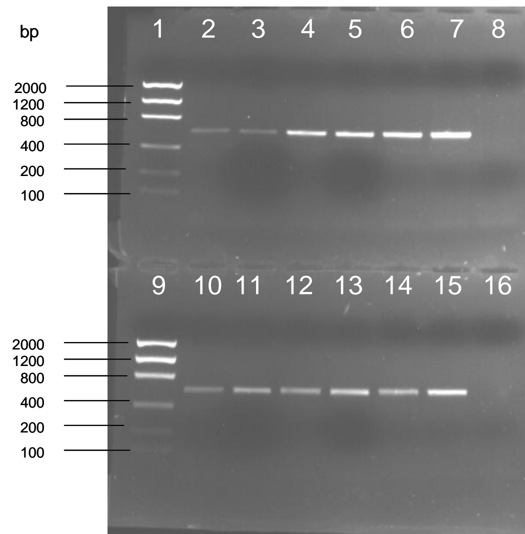
A5.6 c Experiment 3-ANOVA on TAR

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Block stratum	3	171.9	57.3	0.14	
Block.*Units* stratum					
Soil_sample	4	7875	1968.8	4.73	0.005
inoc	1	15015.6	15015.6	36.09	<.001
Soil_sample.inoc	4	6312.5	1578.1	3.79	0.014
Residual	27	11234.4	416.1		
Total	39	40609.4			

Chapter 6

A6. 1 Amplified products of the ITS rDNA fragments from the 12 *Ggt* isolates and their nucleotide sequences

A6.1 a Amplified products of the ITS rDNA fragments from the 12 *Ggt* isolates on 1% agarose gel. Lanes 1 and 9 are the high molar mass ladders, while lanes 8 and 16 are the negative controls. Lanes 2-7 represent *Ggt* isolates A3SL4, H9T3R1/1.2, BIOMILL, H9T3R3, BIOMILLSC3 and H11T3R1/3, respectively. Lanes 10-15 are BIO3, WF99/3, BIO4B, BIO4A, MAL1/8 and DSMZ12044, respectively.



A6.1 b A3SL4

GTCGCG*GGT*ATTCCTACCTGATCCGA*GGT*CAACCTAAGAAGTTTAGGG*G*
*GT*TTAGCGGCTGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTA
CCGCGAGTTACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGG
GGGCCCCGACCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTT
GAGT*GGT*GAAATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCG
GGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC
ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAG
AGATCCGTTGTTGAAAGTTTTAATTATTT*GGT*TTTGTACTCAGAGATACA
CTAAAATTCAG*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGG
CCATCGTCCGCCGAAGCAACAGTAAA*GGT*ATGTTCACAGGG*GGT*TGGA
GTTTTTCAACTCTGTAATGATCCCTCCGCT*GGT*TCACCAACG

A6.1 c H9T3R1/1.2

*GGT*ATTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGC
GGCTGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGA
GTTACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCC
CGACCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GG*
*T*GAAATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTA
CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCC

GTTGTTGAAAGTTTTAATTATTT*GGT*TTTGTACTCAGAGATACACTAAAA
TTCAG*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGCGCCAT
CGTCCGCCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTT
CAACTCTGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACC

A6.1 d BIOMILL

TTCCTACCTGATCCGA*GGT*CAACCTAAGAAGTTTAGGG*GGT*TTAGCGGC
TGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTT
ACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGA
CCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GA
AATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAATTATTT*GGT*TTGTACTCAGAGATACACTAAAATTCA
G*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGCCATCGTCCG
CCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTC
TGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACACCTTTT
TACTTCCA

A6.1 e H9T3R3

TTCCTACCTGATCCGA*GGT*CAACCTAAGAAGTTTAGGG*GGT*TTAGCGGC
TGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTT
ACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGA
CCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GA
AATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAATTATTT*GGT*TTATACTCAGAGATACACTAAAATTCA
G*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGGCCATCGTCC
GCCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACT
CTGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACgACTTT
TACTTCCA

A6.1 f BIOMILLSC3

TTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGCGGCT
GGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTTA
CTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGAC
CGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GAA
ATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATGT
GCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTAATTATTT*GGT*TTGTACTCAGAGATACACTAAAATTCAG
*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGCCATCGTCCGC
CGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTCT
GTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACACCTTTTA
CTTCCA

A6.1 g H11T3R1/3

*GGT*ATTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGC
GGCTGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGA
GTTACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCC
CGACCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GG*
*T*GAAATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTA
CTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCC
GTTGTTGAAAGTTTTAATTATTT*GGT*TTATACTCAGAGATACACTAAAA
TTCAG*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGGCCATC
GTCCGCCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTC
AACTCTGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGATACA
ACC

A6.1 h BIO3

TTCCTACCTGATCCGA*GGT*CACCTAAGGAGTTTAGGG*GGT*TTAGCGGCT
GGAGCCCGCCAGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTTA
CTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGAC
CGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GAA
ATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATGT
GCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTAT
CGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGT
TGAAAGTTTTAATTATTT*GGT*TTGTACTCAGAGATACACTAAAATTCAG
*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGCCATCGTCCGC
CGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTCT
GTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGATACGACTTTTT
TACTTCC

A6.1 i WF99/3

ATTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGCGGC
TGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTT
ACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGA
CCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GA
AATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAATTATTT*GGT*TTGTACTCAGAGATACACTAAAATTCA
G*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGCCATCGTCCG
CCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTC
TGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACGACCTTT
TACTTCCA

A6.1 j BIO4B

TGATCCGA*GGT*CAACCTAAGAAGTTTAGGG*GGT*TTAGCGGCTGGAGCCC
GCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTTACTGCGTTC
AG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGACCGCCGGGC
GGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GAAATGACGCTC
GGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATGTGCGTTCAAA
GATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCG
CTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT
TAATTATTT*GGT*TTTATACTCAGAGATACACTAAAATTCAG*GGT*TTGTAA
CCTCCGGCGGCGTCCGGCCCCCGGGGGGGGCCATCGTCCGCCGAAGCA
ACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTCTGTAATGA
TCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACGACTTTTACTTCCA

A6.1 k BIO4A

TTTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGCGGC
TGGAGCCCGCCGGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTT
ACTGCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGA
CCGCCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GA
AATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAATTATTT*GGT*TTTATACTCAGAGATACACTAAAATTCA
G*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGGCCATCGTCC
GCCGAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACT
CTGTAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTACGACTTT
TACTTC

A6.1 l MAL 1/8

TCCTACCTGATCCGA*GGT*CACCTAAGGAGTTTAGGG*GGT*TTAGCGGCTG
GAGCCCGCCAGGAAGCCTCCGAACGAAGCGCGTTTTACCGCGAGTTACT
GCGTTCAG*GGT*CCTGGCGAGACCGCCGATGTTCTTGGGGGCCCCGACCG
CCGGGCGGCCG*GGT*GCCCCAACACCAAGCTGGGCTTGAGT*GGT*GAAAT
GACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATGTGC
GTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCG
CATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTG
AAAGTTTTAATTATTT*GGT*TTTGTACTCAGAGATACACTAAAATTCAG*G*
*GT*TTGTAACCTCCGGCGGCGTCCGGCCCCCGGGGGGGGCCATCGTCCGCC
GAAGCAACAGTAAA*GGT*ATGTTCACAGG*GGT*TGGAGTTTTTCAACTCTG
TAATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTAGTTACACCTTTTAC
TTCA

A6.1 m DSMZ12044

TTCCTACCTGATCCGA*GGT*CACCTAAGAAGTTTAGGG*GGT*TTAGCGGCT
GGAGCCCGCCGGGAAAGCCTCCGAGCGAAGCGCGTTTTACCGCGAGTT
ACTGCGCTCAG*GGT*CCTAGCGAGACCGCCGATGTTCTTGGGGGCCCCGA
CCGCCGGGCGGCCG*GGT*GCCCCAACGCCAAGCTGGGCTTGAGT*GGT*GA
AATGACGCTCGGACAGGCATGCCCGCCGGAATACCGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA
TCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTG
TTGAAAGTTTTAATTATTT*GGT*TTCGTACTCAGGAGATACACTAAAATTC
AG*GGT*TTGTAACCTCCGGCGGCGTCCGGCCCGGGGGCCATCGTCCGCCG
AAGCAACAGTAAA*GGT*A*GGT*TCACAGG*GGT*TGGAGTTTTTCAACTCTGT
AATGATCCCTCCGCT*GGT*TCACCAACGGAGACCTTGTTAC

A6. 2 Summary of number of replicates where a band was present for the five soils. DBM refere to the distance of band migration (mm) along the gel.

Bacterial 16S

Band No.	DBM	H1	H15	H3	M2	P7
1	10.5	4	4	4	4	4
2	16.0	4	3	4	4	4
3	19.1	4	4	4	4	4
4	20.9	0	0	2	0	0
5	22.3	1	0	2	0	0
6	24.5	0	1	0	0	1
7	25.9	0	1	0	0	1
8	26.8	0	0	1	0	0
9	31.5	4	4	4	4	4
10	37.2	4	4	4	4	4
11	40.0	0	2	4	0	2
12	43.6	0	0	4	0	0
13	45.1	1	0	4	0	0
14	46.3	0	0	2	1	0
15	47.1	0	0	4	0	2
16	48.7	0	4	4	0	4
17	51.3	0	4	4	4	4
18	52.5	0	0	1	0	0
19	53.9	4	0	4	0	1
20	55.3	0	4	4	4	4
21	56.7	0	0	2	0	0
22	57.8	0	0	1	0	0
23	60.6	4	4	4	4	4
24	62.6	0	4	4	4	4
25	64.9	1	4	4	4	4
26	66.7	4	4	4	4	4
27	68.3	4	4	4	4	4
28	69.3	4	4	4	4	4
29	70.7	1	3	4	4	4
30	72.3	4	4	4	4	4
31	73.0	0	4	4	4	4
32	74.5	4	4	4	4	4
33	76.2	4	0	4	4	0
34	77.1	0	4	4	4	4
35	79.4	4	4	4	4	4
36	80.8	4	0	4	4	0
37	82.6	4	0	4	4	0
38	84.4	4	4	4	4	3
39	86.1	4	4	4	0	4
40	87.9	4	4	4	4	4

Fungal ITS

Band No.	DBM	H1	H15	H3	M2	P7
1	7.9	2	4	4	0	1
2	9.7	0	3	4	0	1
3	11.0	4	4	4	0	2
4	12.7	4	4	4	4	0
5	14.7	0	3	4	0	2
6	15.8	0	0	0	4	0
7	17.0	4	4	4	4	0
8	18.2	4	1	4	4	3
9	19.5	4	1	4	4	0
10	20.6	4	1	4	0	4
11	22.8	4	4	4	0	4
12	24.7	4	1	4	4	0
13	26.2	4	4	4	0	4
14	28.2	4	4	4	0	1
15	30.0	4	4	4	4	4
16	31.0	4	1	4	4	3
17	38.1	4	4	4	4	4
18	46.1	0	2	2	0	0
19	47.6	0	1	3	0	0
20	49.8	4	4	4	4	0
21	50.9	2	4	4	0	4
22	52.2	1	0	1	0	0
23	53.6	4	4	4	4	1
24	55.3	4	3	4	0	4
25	56.7	4	0	4	0	0
26	58.2	4	4	4	4	4
27	60.3	4	4	4	0	4
28	62.0	4	4	4	0	4
29	63.1	4	0	4	4	4
30	64.3	4	4	4	4	4
31	65.9	4	4	4	4	4
32	67.6	4	0	4	4	4
33	69.5	1	0	4	1	1
34	70.7	1	0	4	1	0
35	72.4	0	4	0	3	4
36	74.3	4	4	4	4	4
37	76.2	1	4	1	0	1
38	78.2	1	0	0	0	0
39	79.6	0	0	0	0	1
40	83.9	0	1	1	0	0
41	86.5	0	1	1	0	0
42	90.4	0	1	0	1	0
43	92.8	2	3	4	2	2
44	95.2	0	0	4	0	4

Actinomycetes

Band No.	DBM	H1	H15	H3	M2	P7
1	10.5	4	4	4	4	4
2	11.5	2	4	0	4	0
3	13.1	3	4	4	4	4
3a	18.7	1	0	0	0	1
4	20.5	4	4	4	4	4
5	22.8	4	4	4	4	4
5a	24.1	1	1	0	0	0
6	25.0	4	4	4	4	4
7	29.5	4	4	4	4	4
8	31.3	3	4	4	0	3
8a	33.3	1	4	0	0	0
9	36.8	4	4	0	4	4
10	38.4	0	4	0	0	4
11	39.9	4	4	0	0	4
12	41.8	4	4	4	4	4
13	46.5	4	4	4	4	4
14	50.6	4	4	4	4	4
15	51.8	4	4	4	4	4
16	53.5	4	4	4	4	4
17	55.5	4	4	0	4	4
18	57.7	2	4	0	4	3
19	58.9	4	4	0	4	3
20	59.8	0	1	0	0	2
21	61.2	4	4	0	4	4
22	63.0	0	4	0	0	3
23	64.0	4	4	4	4	4
24	65.3	1	4	0	4	4
25	66.5	4	4	4	4	4
26	68.2	0	4	0	0	4
27	69.5	4	4	4	4	4
28	70.8	4	4	4	4	4
29	71.4	0	4	0	0	4
30	72.5	0	4	0	0	4
31	73.4	4	0	0	4	4
32	74.1	4	0	0	4	4
33	76.6	4	0	0	4	0
34	80.0	0	0	0	1	0
35	80.8	1	0	0	2	0
36	83.3	1	0	0	1	0
37	85.1	3	0	0	2	0

Ascomycetes

Band No.	DBM	H1	H15	H3	M2	P7
1	9.4	0	0	0	4	0
2	11.5	4	4	0	4	0
3	14.1	4	4	4	0	0
4	15.0	4	4	4	0	1
5	16.4	4	4	4	0	4
6	17.1	4	4	4	4	4
7	18.4	0	4	4	4	4
8	19.0	0	4	4	0	4
9	19.7	4	4	4	4	4
10	23.9	4	4	4	0	4
11	26.5	4	4	4	0	4
12	29.1	0	1	1	4	4
13	35.0	4	4	4	4	4
14	56.7	4	4	4	4	4
15	60.1	4	4	4	4	4
16	62.1	4	4	0	4	4
17	63.5	4	0	4	0	0
18	65.8	4	4	4	4	4
19	68.2	4	4	4	0	4
20	69.7	4	4	4	4	4
21	71.1	4	0	4	4	0
22	72.8	4	4	4	4	4
23	74.8	4	4	4	4	4
24	78.6	4	4	0	4	4
25	80.1	4	4	4	4	4
26	82.1	0	4	0	4	4
27	83.8	4	4	4	0	4
28	86.3	4	4	4	4	4

A6. 3 Similarity among the soils derived from the cluster analyses using the Jaccard method on bands separated on DGGE gels. Numbers next to the soil codes on each axis are the replicate numbers of the rhizosphere samples loaded onto the gel.

A6.3a On all the 152 DGGE bands of the four microbial groups

		H1 1	H1 2	H1 3	H1 4	H15 1	H15 2	H15 3	H15 4	H3 1	H3 2	H3 3	H3 4	M2 1	M2 2	M2 3	M2 4	P7 1	P7 2	P7 3	P7 4
H1	1	1																			
	2	0.88	1																		
	3	0.88	0.93	1																	
	4	0.84	0.87	0.89	1																
H15	1	0.62	0.63	0.65	0.65	1															
	2	0.61	0.62	0.64	0.66	0.93	1														
	3	0.60	0.61	0.64	0.66	0.92	0.93	1													
	4	0.61	0.61	0.63	0.64	0.85	0.88	0.87	1												
H3	1	0.69	0.68	0.67	0.66	0.63	0.63	0.62	0.65	1											
	2	0.69	0.69	0.67	0.66	0.63	0.65	0.62	0.66	0.95	1										
	3	0.69	0.68	0.68	0.67	0.63	0.64	0.63	0.63	0.92	0.92	1									
	4	0.68	0.67	0.67	0.66	0.63	0.65	0.63	0.66	0.93	0.93	0.94	1								
M2	1	0.62	0.62	0.64	0.68	0.57	0.57	0.57	0.58	0.55	0.56	0.56	0.54	1							
	2	0.61	0.62	0.63	0.67	0.57	0.58	0.57	0.59	0.53	0.54	0.53	0.52	0.94	1						
	3	0.61	0.63	0.65	0.68	0.58	0.58	0.59	0.59	0.55	0.56	0.55	0.54	0.94	0.92	1					
	4	0.61	0.63	0.63	0.66	0.59	0.60	0.58	0.60	0.56	0.56	0.55	0.55	0.93	0.94	0.97	1				
P7	1	0.58	0.57	0.60	0.63	0.74	0.75	0.75	0.71	0.57	0.58	0.57	0.56	0.61	0.60	0.62	0.62	1			
	2	0.60	0.60	0.63	0.63	0.77	0.78	0.80	0.76	0.64	0.65	0.64	0.63	0.59	0.58	0.60	0.60	0.91	1		
	3	0.61	0.60	0.61	0.62	0.75	0.77	0.75	0.74	0.63	0.64	0.63	0.62	0.59	0.59	0.60	0.62	0.89	0.87	1	
	4	0.62	0.59	0.62	0.62	0.78	0.76	0.75	0.74	0.63	0.65	0.64	0.64	0.59	0.58	0.59	0.60	0.84	0.87	0.85	1

A6.3b On all the bands from the Bacterial DGGE analysis

		H1 1	H1 2	H1 3	H1 4	H15 1	H15 2	H15 3	H15 4	H3 1	H3 2	H3 3	H3 4	M2 1	M2 2	M2 3	M2 4	P7 1	P7 2	P7 3	P7 4
H1	1	1																			
	2	0.95	1																		
	3	1	0.95	1																	
	4	0.86	0.83	0.86	1																
H15	1	0.54	0.52	0.54	0.53	1															
	2	0.56	0.54	0.56	0.61	0.88	1														
	3	0.50	0.48	0.50	0.55	0.81	0.92	1													
	4	0.54	0.52	0.54	0.59	0.85	0.96	0.96	1												
H3	1	0.56	0.59	0.56	0.60	0.61	0.68	0.68	0.71	1											
	2	0.59	0.63	0.59	0.64	0.65	0.72	0.72	0.75	0.94	1										
	3	0.54	0.57	0.54	0.63	0.59	0.66	0.66	0.69	0.82	0.86	1									
	4	0.56	0.59	0.56	0.65	0.61	0.68	0.68	0.71	0.89	0.89	0.92	1								
M2	1	0.65	0.63	0.65	0.70	0.71	0.81	0.74	0.78	0.71	0.75	0.69	0.71	1							
	2	0.65	0.63	0.65	0.70	0.71	0.81	0.74	0.78	0.71	0.75	0.69	0.71	1	1						
	3	0.65	0.63	0.65	0.70	0.71	0.81	0.74	0.78	0.71	0.75	0.69	0.71	1	1	1					
	4	0.63	0.61	0.63	0.68	0.69	0.78	0.71	0.75	0.74	0.73	0.67	0.74	0.96	0.96	0.96	1				
P7	1	0.52	0.50	0.52	0.57	0.84	0.96	0.88	0.92	0.65	0.69	0.63	0.65	0.77	0.77	0.77	0.74	1			
	2	0.52	0.50	0.52	0.57	0.81	0.92	0.92	0.96	0.74	0.78	0.71	0.74	0.75	0.75	0.75	0.72	0.88	1		
	3	0.56	0.54	0.56	0.61	0.88	1	0.92	0.96	0.68	0.72	0.66	0.68	0.81	0.81	0.81	0.78	0.96	0.92	1	
	4	0.52	0.50	0.52	0.56	0.86	0.82	0.82	0.86	0.72	0.76	0.70	0.72	0.68	0.68	0.68	0.66	0.79	0.89	0.82	1

A6.3c On all the bands from the fungal DGGE analysis

		H1 1	H1 2	H1 3	H1 4	H15 1	H15 2	H15 3	H15 4	H3 1	H3 2	H3 3	H3 4	M2 1	M2 2	M2 3	M2 4	P7 1	P7 2	P7 3	P7 4
H1	1	1																			
	2	0.81	1																		
	3	0.78	0.90	1																	
	4	0.87	0.86	0.89	1																
H15	1	0.57	0.61	0.63	0.59	1															
	2	0.53	0.56	0.58	0.55	0.85	1														
	3	0.53	0.56	0.63	0.60	0.88	0.81	1													
	4	0.56	0.59	0.61	0.58	0.70	0.70	0.66	1												
H3	1	0.81	0.75	0.72	0.74	0.62	0.58	0.54	0.65	1											
	2	0.78	0.78	0.70	0.72	0.61	0.61	0.53	0.63	0.92	1										
	3	0.85	0.79	0.76	0.79	0.66	0.61	0.57	0.60	0.94	0.92	1									
	4	0.76	0.71	0.73	0.70	0.63	0.63	0.55	0.70	0.89	0.92	0.89	1								
M2	1	0.56	0.50	0.52	0.53	0.36	0.32	0.35	0.38	0.49	0.47	0.51	0.46	1							
	2	0.52	0.55	0.52	0.53	0.41	0.36	0.35	0.46	0.45	0.44	0.47	0.42	0.82	1						
	3	0.52	0.55	0.57	0.59	0.35	0.31	0.34	0.37	0.44	0.43	0.47	0.42	0.85	0.85	1					
	4	0.53	0.57	0.53	0.55	0.42	0.38	0.37	0.43	0.46	0.45	0.49	0.44	0.86	0.95	0.89	1				
P7	1	0.50	0.48	0.50	0.57	0.48	0.43	0.48	0.36	0.47	0.46	0.50	0.45	0.36	0.36	0.35	0.37	1			
	2	0.53	0.56	0.58	0.60	0.57	0.52	0.57	0.43	0.58	0.57	0.62	0.55	0.35	0.35	0.34	0.37	0.82	1		
	3	0.55	0.53	0.50	0.57	0.53	0.53	0.48	0.49	0.56	0.54	0.59	0.53	0.32	0.37	0.31	0.38	0.77	0.72	1	
	4	0.60	0.54	0.56	0.58	0.59	0.55	0.50	0.50	0.61	0.59	0.64	0.62	0.39	0.39	0.34	0.41	0.69	0.71	0.74	1

A6.3d On all the bands from the actinomycete DGGE analysis

		H1 1	H1 2	H1 3	H1 4	H15 1	H15 2	H15 3	H15 4	H3 1	H3 2	H3 3	H3 4	M2 1	M2 2	M2 3	M2 4	P7 1	P7 2	P7 3	P7 4
H1	1	1																			
	2	0.83	1																		
	3	0.86	0.89	1																	
	4	0.73	0.80	0.83	1																
H15	1	0.57	0.62	0.65	0.69	1															
	2	0.57	0.62	0.65	0.69	1	1														
	3	0.57	0.62	0.65	0.69	1	1	1													
	4	0.58	0.58	0.61	0.65	0.94	0.94	0.94	1												
H3	1	0.57	0.58	0.62	0.50	0.53	0.53	0.53	0.50	1											
	2	0.57	0.58	0.62	0.50	0.53	0.53	0.53	0.50	1	1										
	3	0.57	0.58	0.62	0.50	0.53	0.53	0.53	0.50	1	1	1									
	4	0.57	0.58	0.62	0.50	0.53	0.53	0.53	0.50	1	1	1	1								
M2	1	0.72	0.79	0.83	0.93	0.63	0.63	0.63	0.59	0.54	0.54	0.54	0.54	1							
	2	0.73	0.74	0.77	0.87	0.59	0.59	0.59	0.56	0.50	0.50	0.50	0.50	0.93	1						
	3	0.71	0.79	0.82	0.86	0.67	0.67	0.67	0.63	0.58	0.58	0.58	0.58	0.93	0.86	1					
	4	0.71	0.79	0.82	0.86	0.67	0.67	0.67	0.63	0.58	0.58	0.58	0.58	0.93	0.86	1	1				
P7	1	0.61	0.62	0.70	0.69	0.82	0.82	0.82	0.82	0.48	0.48	0.48	0.48	0.68	0.64	0.72	0.72	1			
	2	0.64	0.65	0.73	0.67	0.85	0.85	0.85	0.85	0.52	0.52	0.52	0.52	0.66	0.62	0.70	0.70	0.97	1		
	3	0.67	0.68	0.71	0.65	0.78	0.78	0.78	0.74	0.59	0.59	0.59	0.59	0.64	0.60	0.68	0.68	0.84	0.87	1	
	4	0.69	0.65	0.73	0.67	0.85	0.85	0.85	0.80	0.52	0.52	0.52	0.52	0.66	0.62	0.70	0.70	0.91	0.94	0.87	1

A6.3e On all the bands from the ascomycete DGGE analysis

		H1 1	H1 2	H1 3	H1 4	H15 1	H15 2	H15 3	H15 4	H3 1	H3 2	H3 3	H3 4	M2 1	M2 2	M2 3	M2 4	P7 1	P7 2	P7 3	P7 4
H1	1	1																			
	2	1	1																		
	3	1	1	1																	
	4	1	1	1	1																
H15	1	0.81	0.81	0.81	0.81	1															
	2	0.81	0.81	0.81	0.81	1	1														
	3	0.81	0.81	0.81	0.81	1	1	1													
	4	0.78	0.78	0.78	0.78	0.96	0.96	0.96	1												
H3	1	0.80	0.80	0.80	0.80	0.77	0.77	0.77	0.74	1											
	2	0.77	0.77	0.77	0.77	0.74	0.74	0.74	0.78	0.96	1										
	3	0.80	0.80	0.80	0.80	0.77	0.77	0.77	0.74	1	0.96	1									
	4	0.80	0.80	0.80	0.80	0.77	0.77	0.77	0.74	1	0.96	1	1								
M2	1	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.63	0.46	0.50	0.46	0.46	1							
	2	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.63	0.46	0.50	0.46	0.46	1	1						
	3	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.63	0.46	0.50	0.46	0.46	1	1	1					
	4	0.56	0.56	0.56	0.56	0.59	0.59	0.59	0.63	0.46	0.50	0.46	0.46	1	1	1	1				
P7	1	0.67	0.67	0.67	0.67	0.84	0.84	0.84	0.88	0.69	0.73	0.69	0.69	0.64	0.64	0.64	0.64	1			
	2	0.70	0.70	0.70	0.70	0.88	0.88	0.88	0.92	0.73	0.77	0.73	0.73	0.62	0.62	0.62	0.62	0.96	1		
	3	0.67	0.67	0.67	0.67	0.84	0.84	0.84	0.88	0.69	0.73	0.69	0.69	0.64	0.64	0.64	0.64	1	0.96	1	
	4	0.67	0.67	0.67	0.67	0.84	0.84	0.84	0.88	0.69	0.73	0.69	0.69	0.64	0.64	0.64	0.64	1	0.96	1	1

A6. 4 Nucleotide sequences of the distinguishing bands generated on DGGE specific for the four microbial groups

A6.4 1 Bacterial band 12

GTACCGCGACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGA
GTTGCAGACTGCGATCCGGACTACGATCG*GGT*TTGTGGGATTAGCTCCACCTCG
CGGCTTGGCAACCCTCTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCG
TAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCC*GGT*TTGTCACCGGA
GCGCTCTAGGGCACT

A6.4 2 Bacterial band 13

GACATTCTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAG
ACTGCGATCCGGACTACGATC*GGT*TTATGGGATTAGCTCCACCTCGCGGCTTG
GCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGC
CATGATGACTTGACGTCATCCCCACCTTCCTCC*GGT*TTGTCACCGGCAGTCTCC
TTAGAGTGCCCACCATAACGTGCT*GGT*AACTAAGGACAAG*GGT*TGCGCTCGTT
ACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCAC
CTGTCTCAATGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGT
CAAGGCCT*GGT*AA*GGT*TCTTCGCGTT

A6.4 3 Bacterial band 14

GATTCCGCGTCACTGCTGATTCTCGCGATTCCTGCCATTCCCACTTCCGGAAGC
CAAGTTGCAAACGCGATCCGGACTACGATC*GGT*TTTTGGGATTAGCTCCACCT
CGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGC
CGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCC*GGT*TTGTCACCG
GCAGTCTCCTTAGAAGTGCCCACCATTACGTGCT*GGT*AACTAAGGACAAG*GGT*
TGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGC
CATGCAGCACCTGTCTCAATGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTT
CATTGGATGTCAAGGCCT*GGT*AA*GGT*TCTTCGCGTTCCCCCCGGGCCCCCGCCC
CCCCC

A6.4 4 Bacterial band 19

ATCTGCGATTACTAGCGATTCCGACTTCATGG*GGT*CGAGTTGCAGACCCCAATC
CGAACTGAGACAGGCTTTTTGAGATTCGCTCCGCCTCACGGGATCGCAGCCCA
TTGTACCGTGCCATTGTAGCACGTGTGAAGCCCTGGCATAAGGGGCAATGATG
ACTTGACGTCATCCCCACCTTCCTCCGATTTGCCCCCGGC*GGT*CTCCTGTGAGT
CCCCACCATTACGTGCTGATAACTGACAACAAG*GGT*TGCGCTCGTTGCGGGAC
TTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGTA

A6.4 5 Bacterial band 33

CAAGGCTTGACATACACCGGAAACATCCAGAGATG*GGT*GCCCCCTTGT*GGT*C*G*
*GT*GTACA*GGTGGT*GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG*GGT*TAA
GTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGG*GGT*G
ATGGGGACTCACAGGAGACTGCCGG*GGT*CAACTCGGAGGAA*GGT*GGGGACGA
CGTCAAGTCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCC*G*
*GT*ACAAAG*GGT*TGCGATACTGTGA*GGT*GGAGCTAATCCCAAAAAGCC*GGT*CTC
AGTTCGGATTGG*GGT*CTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAAT
CGCAGATCAGCATTGCTGC*GGT*GAATACGTTCCCGGGCCTTGTACACACCGA

A6.4 6 Bacterial band 36

CGGG*GGT*TGCTTGTTCTGCGATTACTAGCGACTCCGACTTCAGGG*GGT*CAAGTT
GCAAACTCCAAtCCGAACTGAGACG*GGT*TTTTTGGGATTCGCTCCCCCTTCGC*G*
*GT*TTCGCACCCCTTTGTACCGGCCATTGTAGCATGTGTGAACCCCAAGACATAA
GGGGCATGATGATTTGACGTCCTCCCCACCTTCCTCCGAGTTGACCCCGGCAGT
CTCCTATAGAGTCCCCACCATTACGTGCTGGCAACAAAGAACGAG*GGT*TGCGC
TCGTTGC*GGT*ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGC
ACCACCTGTACACCTCC

A6.4 7 Bacterial band 37

CGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGAGTCGAGTTGCAGA
CTCCAATCCGAACTGAGACCGGCTTTTCTGGGATTCGCTCCACCTCGC*GGT*ATC
GCAGCCCTTTGTACCGGCCATTGTAGCATGTGTGAAGCCCTGGACATAAGGGG
CATGATGACTTGACGTCATCCCCACCTTCCTCCGATTTGACCCCGGCAGTCTCC
TAAGAGTCCCCACCATTACGTGCTGGCAACATAGAAACGAG*GGT*TGCGCTCTT
GGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCATGCACCAC
CTGTGAAACGGCCCCAAAGG

A6.4 8 Fungal band 6

TTAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAG*GGT*G
TCTTCGGCGGGCGCGGGCCCGGGGGCGTGAGCCCCCCGGCGGCCTGTAAAGGC
GGGCCCGCCGAAGCAACAA*GGT*AAAATAAACACG*GGT*GGGA*GGT*TGGACCCA
GAGGGCCCTCACTC*GGT*AATGATCCTTCCGCA*GGT*TCACCTACGGAAACCTTGT
TACGACTTTTACTTCCTCTAAATGACC

A6.4 9 Fungal band 9

AAGTCGTAACAA*GGT*CTCCGTT*GGT*GAACCAGCGGAGGGATCATTACCGAGTT
TACAACTCCCAAACCCCTGTGAACATACCTTAATGTTGCCTCGGCGGATCAGCC
CGCGCCCCGTAAAACGGGACGGCCCGCCAGAGGACCCAAACTCTAATGTTTCT
TATTGTAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGA
TCTCTT*GGT*TCTGGCATCGATGAAGAACGCAGCA

A6.4 10 Fungal band 12

GATGAAAGTTGTATTTTGAATTGTTTATTCATCAATATTTTTCCGATCAAAGAG
TATATAAAATAAA*GGT*TGATGTTG*GGT*CGATCTCCATGAAGAAGATCGACTGA
CATTGCACACAA*GGT*GGATATGGATTTAAAAAGTGCCATAAAACACTTATTAT
GAATGATCCTTCCGCA*GGT*TCACCTACCGAAACCTAGTTTCGACTTTTACTTCC
TCTAAATGACCTATCACGTGCCCCACTTCCGCAAAAC

A6.4 11 Fungal band 25

AAGTCGTAACAA*GGT*CTCCGTT*GGT*GAACCAGCGGAGGGATCATTACCGAGTT
TACAACTCCCAAACCCCTGTGAACATACCTTATGTTGCCTCGGCGGATCAGCCC
GCGCCCCGTAAAAAGGGACGGCCCGCCGCAGGAACCCTAAACTCTGTTTTTAG
TGGAACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGATCT
CTT*GGT*TCTGGCATCGATGAAGAACGCAGCA

A6.4 12 Fungal band 33

TTTAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAG*GGT*
GTCTTCGGCGGGCGCGGGCCCGGGGGCGTGAGCCCCCCGGCGGCCTGTAAAGG
CGGGCCCGCCGAAGCAACAA*GGT*AAAATAAACACG*GGT*GGGA*GGT*TGGACCC
AGAGGGCCCTCACTC*GGT*AATGATCCTTCCGCA*GGT*TCACCTACGGAAACCTTG
TTACGACTTTTACTTCCTCTAAATGACCAAGCCCGT

A6.4 13 Fungal band 34

AGTTTTAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAG
*GGT*GTCTTCGGCGGGCGCGGGCCCGGGGGCGTGAGCCCCCCGGCGGCCAGTAA
AGGCGGGCCCGCCGAAGCAACAA*GGT*AAAATAAACACG*GGT*GGGA*GGT*TGGA
CCCAAAGGGCCCTCACTC*GGT*AATGATCCTTCCGCA*GGT*TCACCTACGGAAAC
CTTGTTACGACTTTTACTTCCTCTAAATGACCAAGC

A6.4 14 Fungal band 35

CAGAACCAAGAGATCCGTTGTTGAAAGTTGTAATAATTACATTGTGTACTGAC
GCTGATTGCAATTACAAAAAAA*GGT*TTAT*GGT*TG*GGT*CCT*GGT*GGCGGGCGAA
CCCGCCCAGGAAACAAGAAGTGCGCAAAAGACATG*GGT*GAATAATTCAGACA
AGCTGGAGCCCTCACCGAGATGA*GGT*CCCAACCTGCTTTCATATTGTGTAATGA
TCCCTCCGCA*GGT*TCACCTACGGAGACCTTGTTACGACTTTTACTTCCTCTAAA
TGACCAAGA

A6.4 15 Actinomycete band 10

TCAGCTTGTT*GGT*GA*GGT*GAAAGCTCACCAAGGCTTTGACG*GGT*AACCGGCCT
GAGAGGGC*GGT*C*GGT*CACATTGGGACTGAGATACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATTTTGCACAATGGGCGGAAGCCTGATGCANCGACNCC
NCGTGAGGGATGACGGCTTTCG*GGT*TGTAAACCTCTTTTATCCACCACGAAGG
CTCCGTATTCGCGG*GGT*TGAC*GGT*AGT*GGT*TGAATAAGCGCCGGCTAACTACGT
GCCAGCAGCCGCGGCCGA

A6.4 16 Actinomycete band 22

TCAGCTTGTT*GGT*GA*GGT*AGTGGCTCACCAAGGCGACGACG*GGT*AGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCG*GGT*TGTAAACCTCTTTCAGCAGGGAAGAAG
CGAAAGTGAC*GGT*ACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGC
CGCGGCCGA

A6.4 17 Actinomycete band 24

TCAGCTTGTTT*GGT*GG*GGT*AATGGCCTACCAAGGCGACGACG*GGT*AGCCGGCC
TGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACG
CCGCGTGAGGGATGACGGCCTTCG*GGT*TGTAAACCTCTTTCAGCAGGGAAGAA
GCGAAAGTGAC*GGT*ACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAG
CCGCGGCCGA

A6.4 18 Actinomycete band 26

TCAGCTTGTT*GGT*GG*GGT*AATGGCCTACCAAGGCGACGACG*GGT*AGCCGGCCT
GAGAG*GGT*GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCG*GGT*TGTAAACCTCTTTCAGCAGGGACGAAG
CGCAAGTGAC*GGT*ACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGCCGA

A6.4 19 Actinomycete band 29

TCAGCTTGTT*GGT*GG*GGT*AATGGCCTACCAAGGCGTCGACG*GGT*AGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCGCAATGGACGGAAGTCTGACGCAGCAACGC
CGCGTGAGGGATGACGGCCTTCG*GGT*TGTAAACCTCTTTCAGCAGGGACGAAG
CGAGAGTGAC*GGT*ACCTGCAGAAGAAGGACCGGCCAACTACGTGCCAGCAGC
CGCGGCCGA

A6.4 20 Actinomycete band 30

TCAGCTTGTT*GGT*GG*GGT*AATGGCCTACCAAGGCGACGACG*GGT*AGCCGGCCT
GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGC
CGCGTGAGGGATGACGGCCTTCG*GGT*TGTAAACCTCTTTCAGCAGGGACGAAG
CGCAAGTGAC*GGT*ACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGCCGA

A6.4 21 Ascomycete band 1

AAATCCCTGCTGCGTTCTTCATCGATGCAGAACGCAGCGAAATGCGATACGTA
ATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCC
CT*GGT*ATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGG
CTTGTGTGTTGGGCCCCGTCCTCCGATTTCCGGGGGACGGGCCCGAAAGGCAG
CGGCGGCACCGCGTCC*GGT*CCTCAAGCGTATGGGGCTTTGTCACCCGCTCTGTA
GGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTTATCCA*GGT*TGACCTCGGA
TCA*GGT*AGGGATACCCGCTGAATTTAAGCATATCAATAAGCGGAGGAAAAGA
AACCAACAGGGATTGCCTTAGTAACGA

A6.4 22 Ascomycete band 17

GTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCG*GGT*ATTCCTACCTGA
TCCGA*GGT*CAACATTCAGAAGTTGG*GGT*TTAACGGCGTGGCCGCGACGATTAC
CAGTAACGATATGTAAATTACTACGCTATGGAAGCTCGACGTGACCGCCAATG
TATTTGGGGAGTGCAGCAGGACTGCAGCTCCCAACACCAAGCTGGGCTTGAG*G*
*GT*TGAAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATG
TGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTTGCTGCGTTCTTCATCGATGCA

A6.4 23 Ascomycete band 21

GTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCG*GGT*ATTCCTACCTGA
TCCGA*GGT*CAACCATTAAAAAAGTGCCCCCCGAGAG*GGT*GC*GGT*TTATGGCTG
TCGTCTGGCCGGCTTGCAGAAGCGAGATAAAAAATTACTACGCTCAGAGCACG
AACAGACTCCGCCACT*GGT*TTTGAGGAGCTGCGTATTAGGCAGTCTCCCAACA
CTAAGCTAGGCTTAAG*GGT*TGAAATGACGCTCGAACAGGCATGCCCACTAGAA
TACTAATGGGCGCAATGTCCGTTCAAAGATTCGATGATTCACTGAATTCTGCAA
TTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCA

A6.4 24 Ascomycete band 26

GTTTCTTTTCCTCCGCTTATTGATATGCTTAAGTTCAGCG*GGT*ATCCCTACCTGA
TCCGA*GGT*CAACCTGGATAAAAATTTG*GGT*TGATCGGCAAGCGCCGGCCGGGC
CTACAGAGCG*GGT*GACAAAGCCCCATACGCTCGAGGACTGGACGC*GGT*GCCGC
CGCTGCCTTTCGGGCCCGTCCCCCGGAATCGGAGGACGGGGCCCAACACACAA
GCCGTGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACC
AGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCA
CATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCA

Chapter 7

A7. 1 Analysis of variance

A7.1 a Percent infected roots

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk stratum	3	239.74	79.91	1.28	
Blk.Plot stratum					
soil	3	1927.52	642.51	10.32	0.003
Residual	9	560.31	62.26	1.06	
Blk.Plot.*Units* stratum					
Sowing	6	5840.4	973.4	16.56	<.001
soil.Sowing	18	1012.57	56.25	0.96	0.516
Residual	72	4231.54	58.77		
Total	111	13812.08			

A7.1 b *Ggt* DNA concentrations

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	5733	1911	0.30	
Blk_1.Plot_1 stratum					
soil_1	3	140305	46768	7.28	0.009
Residual	9	57827	6425	1.46	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.4276					
Sowing	6	701255	116876	26.61	<.001
Sowing.soil_1	18	127022	7057	1.61	0.166
Residual	72	316260	4392		
Total	111	1348401	58.77		

A7.1 c Population of *Pseudomonas fluorescens* (log 10)

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	11.230	3.743	3.62	
Blk_1.Plot_1 stratum					
soil_1	3	17.594	5.865	5.68	0.018
Residual	9	9.298	1.033	0.90	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.5309					
Sowing	6	74.547	12.425	10.79	<.001
Sowing.soil_1	18	16.818	0.934	0.81	0.615
Residual	72	82.915	1.152		
Total	111	212.402			

A7.1 d pH

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	0.10884	0.03628	1.29	
Blk_1.Plot_1 stratum					
soil_1	3	9.33812	3.11271	110.75	<.001
Residual	9	0.25295	0.02811	2.13	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.3622					
Sowing	6	6.84107	1.14018	86.35	<.001
Sowing.soil_1	18	0.6025	0.03347	2.53	0.042
Residual	72	0.95071	0.0132		
Total	111	18.0942			

A7.1 e Available N

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	558.2	186.1	2.96	
Blk_1.Plot_1 stratum					
soil_1	3	20997.8	6999.3	111.18	<.001
Residual	9	566.6	63	0.45	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.6874					
Sowing	6	11787.4	1964.6	13.96	<.001
Sowing.soil_1	18	5603.5	311.3	2.21	0.024
Residual	72	10134.2	140.8		
Total	111	49647.7			

A7.1 f Olsen P

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	78.36	26.12	0.89	
Blk_1.Plot_1 stratum					
soil_1	3	5433.71	1811.24	62.03	<.001
Residual	9	262.79	29.2	1.23	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.3828					
Sowing	6	7006.87	1167.81	49.06	<.001
Sowing.soil_1	18	918.41	51.02	2.14	0.073
Residual	72	1713.86	23.8		
Total	111	15414			

A7.1 g Potassium

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	0.014603	0.004868	4.18	
Blk_1.Plot_1 stratum					
soil_1	3	2.374653	0.791551	680.27	<.001
Residual	9	0.010472	0.001164	0.62	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.4157					
Sowing	6	1.114605	0.185768	98.35	<.001
Sowing.soil_1	18	0.273166	0.015176	8.03	<.001
Residual	72	0.136	0.001889		
Total	111	3.923499			

A7.1 h Magnesium

Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	0.00691	0.002303	0.89	
Blk_1.Plot_1 stratum					
soil_1	3	29.220567	9.740189	3749.52	<.001
Residual	9	0.023379	0.002598	2	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.3815					
Sowing	6	0.014736	0.002456	1.89	0.165
Sowing.soil_1	18	0.044514	0.002473	1.91	0.108
Residual	72	0.093436	0.001298		
Total	111	29.403542			

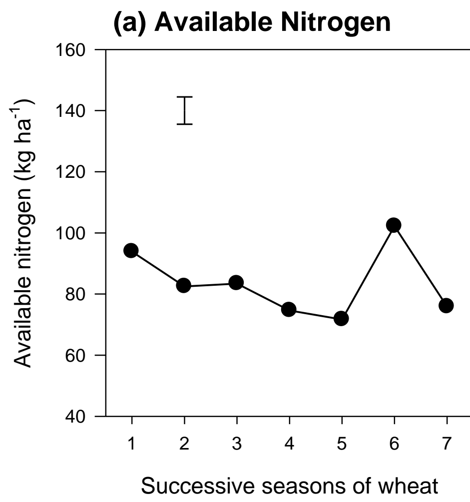
A7.1 i Calcium

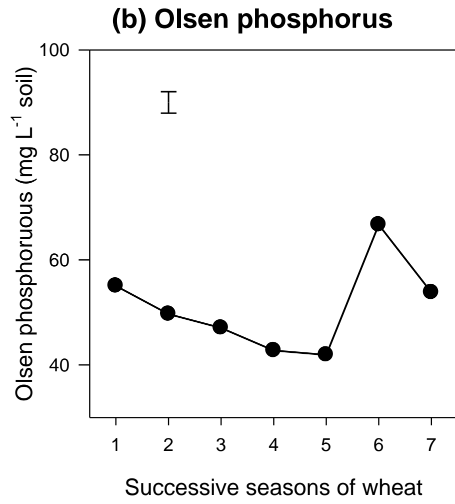
Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	0.9974	0.3325	3.21	
Blk_1.Plot_1 stratum					
soil_1	3	153.5903	51.1968	494.27	<.001
Residual	9	0.9322	0.1036	0.92	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.4498					
Sowing	6	4.1896	0.6983	6.2	0.003
Sowing.soil_1	18	2.3104	0.1284	1.14	0.364
Residual	72	8.1029	0.1125		
Total	111	170.1228			

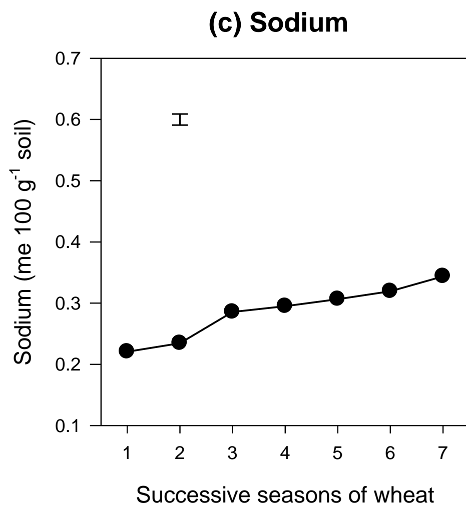
A7.1 j Sodium

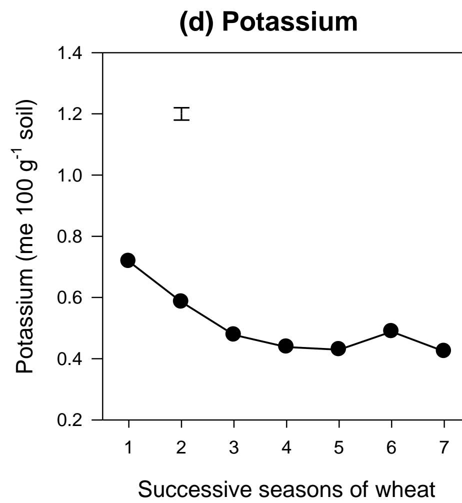
Source of variation	df	s.s.	m.s.	v.r.	F pr.
Blk_1 stratum	3	0.0072714	0.002424	1.29	
Blk_1.Plot_1 stratum					
soil_1	3	1.0689857	0.356329	189.6	<.001
Residual	9	0.0169143	0.001879	3.79	
Blk_1.Plot_1.Sowing stratum					
df correction factor 0.4814					
Sowing	6	0.1900464	0.031674	63.95	<.001
Sowing.soil_1	18	0.0562893	0.003127	6.31	<.001
Residual	72	0.0356643	0.000495		
Total	111	1.3751714			

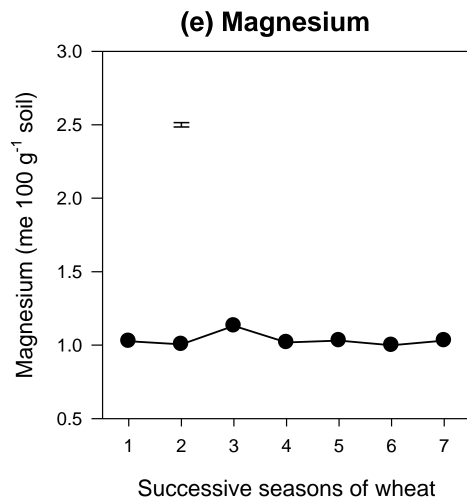
A7. 2 Mean concentrations of the various nutrients in soils grown with successive seasons of wheat crops.



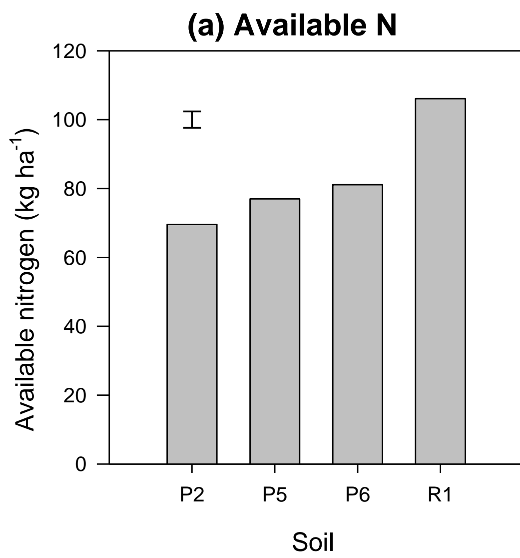


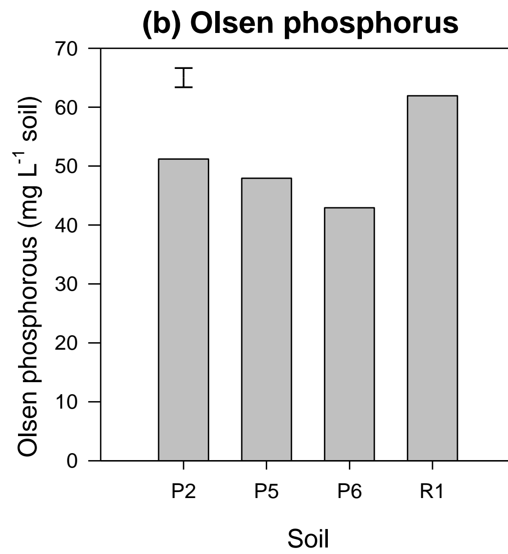


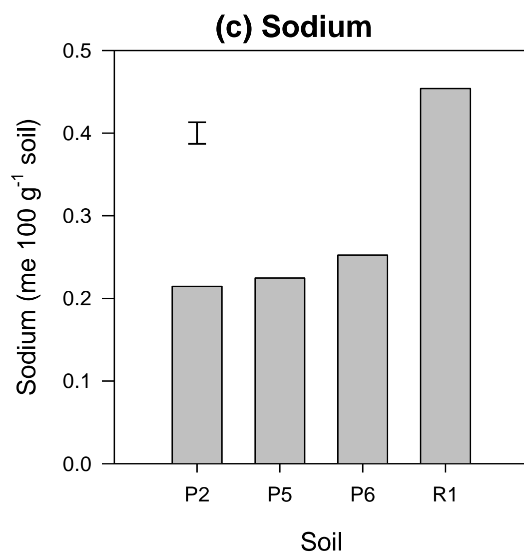


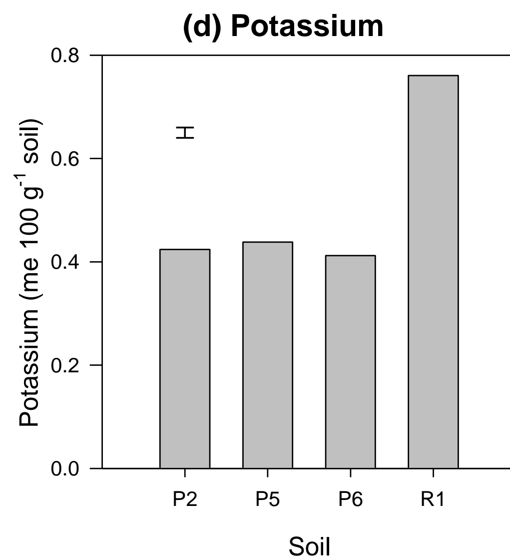


A7. 3 Mean concentrations of the various nutrients in the four tested soils.









A7. 4 A matrix of scatter plots showing the relationships between all the parameters measured.