Directed Evolution of Disease Suppressive Bacteria: The Role of Root Lesions on Take-all Diseased Wheat.

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Abstract

Take-all disease (caused by *Gaeumannomyces graminis var tritici*, Ggt) can be suppressed by soil microorganisms after continuous monoculture of wheat (take-all decline, TAD). Fluorescent pseudomonads have been implicated in this suppression. Two strategies for controlling take-all are the *in situ* development of disease suppressive soil, and/or the application of a biocontrol agent. However, TAD takes up to 10 years to develop after initially high levels of disease, and the performance of bacterial biocontrol agents has been inconsistent. It is not known what environmental factors select for disease antagonists. In this work the role of diseased root lesions in directing the evolution of a native pseudomonad community, and a model disease antagonist, *Pseudomonas corrugata* strain 2140 (Pc2140) for increased disease suppression was investigated.

This work shows that root lesions are a distinct niche, supporting increased populations of total aerobic bacteria (TAB), pseudomonads and Pc2140 (compared to non-lesioned sections of diseased roots and healthy roots). Lesions selected for fluorescent pseudomonads and pseudomonads which increase take-all severity. In contrast, lesions selected for non-pseudomonads which decrease take-all, and healthy roots selected for non-fluorescent pseudomonads which decrease take-all. It was concluded that non-fluorescent pseudomonads and non-pseudomonads were important in reducing take-all, but not fluorescent pseudomonads.

Pc2140 produced multiple variant phenotypes *in vitro* and on wheat roots which were altered in (1) their ability to inhibit pathogens *in vitro* and control take-all, and (2) GC-FAME and BIOLOG profiles to the extent that some variants were identified as different species. Different sets of phenotypes were produced *in vitro* and on roots. After 108 weeks culture of Pc2140 on root lesions and healthy wheat roots, variant colony types were generally slightly decreased in ability to reduce take-all, and reisolates with the wild type colony morphology were generally slightly increased in ability to reduce take-all compared to the ancestral Pc2140. This is the first report on the diversification of a pseudomonad biocontrol agent on roots, and has implications for the taxonomic identification and grouping of isolates based on phenotypic characteristics.

Declaration

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

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopied.

Signed Date 16/3/99

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Table of Contents

Abstract ... i

Declaration ... ii

Acknowledgments ... iii

Table of Contents ... iv

List of Tables ... x

List of Figures ... xii

Chapter 1. Introduction ... 1

1.1. Take-all disease on wheat ... 4

1.2. Take-all decline ... 5

1.2.1. Development of take-all decline ... 5

1.2.2. Take-all disease, a prerequisite for take-all decline ... 5

1.2.3. Microbial Suppression of take-all disease ... 6

1.2.4. Suppressive soils have more antagonists ... 6

1.2.5. Mechanisms of take-all decline ... 7

1.3. Biological suppression of soil-borne disease ... 8

1.3.1. Mechanisms of pathogen and disease suppression ... 8

1.3.2. Global regulation of antibiotic production ... 10

1.3.3. Root colonisation by PGPR ... 11

1.3.4. Using mixtures of microorganisms for disease control ... 11

1.4. Microbial Plasticity ... 12

1.4.1. Multiple new phenotypes in response to the same selection pressure ... 13

1.4.2. Cryptic genes ... 13

1.4.3. Microbial plasticity and disease suppression ... 14

1.4.4. Environmental selection for new phenotypes ... 15

1.4.5. The heterogeneity of the root-soil environment ... 16

1.5. Evolution of disease suppression ... 16

1.5.1. The origin of disease suppressive bacteria ... 17

1.5.2. Selection within and between bacterial populations ... 18

1.5.3. Diseased root lesions: a selection site for antagonists? ... 18

1.6. Research aims ... 19

Chapter 2. General Materials and Methods ... 20

2.1. Introduction ... 20

2.2. Materials and Methods 20
2.2.1. Source of microorganisms 20
2.2.1.1. *Pseudomonas corrugata* 2140 strains 20
2.2.1.2. Wheat pathogens and other microorganisms 21
2.2.2. Media 21
2.2.2.1. DB, Defined broth medium 21
2.2.2.2. LB, Luria Bertani medium 22
2.2.2.3. Minimal Medium 22
2.2.2.4. MNA, Modified Nutrient Agar 22
2.2.2.5. NB, Nutrient broth 22
2.2.2.6. NA, Nutrient agar 22
2.2.2.7. PDA/4 Potato Dextrose Agar, 1/4 strength 22
2.2.2.8. TB-T, Trypan Blue, Tetracycline medium 23
2.2.2.9. TSA, Tryptic Soy Agar 23
2.2.2.10. TSBA, Trypticase Soy Broth Agar 23
2.2.2.11. TZCA, Tetrazolium chloride agar 23
2.2.2.12. X-Gal MM, X-Gal Minimal Medium 23
2.2.3. Growth conditions of *in vitro* cultures 24
2.2.4. Purification and storage of bacterial cultures 24
2.2.5. Pathogen (Ggt) inoculum for pot microcosms 24
2.2.6. Soil 24
2.2.7. Wheat seeds 25
2.2.8. Wheat-soil pot microcosms 25
2.2.9. Bacterial inoculum for wheat-soil microcosms 25
2.2.10. Growth conditions of wheat-soil microcosms 26
2.2.11. Estimation of bacterial populations from wheat roots and soil. 26
2.2.12. Measurement of plant growth parameters 27
2.2.13. GC-FAME 27
2.2.14. *In vitro* inhibition of microorganisms 28
2.2.15. Statistical analysis 29

Chapter 3. Selection by Root Lesions for Disease Antagonists Within a Native Bacterial Population 31

3.1. Introduction 31
3.1.1. Aims 33
3.2. Materials and Methods 33
3.2.1. Media for selective isolation of bacterial populations. 33
3.2.2. Ggt inoculum for *in vitro* inhibition assays 33

3.2.3. Estimation of bacterial populations 34

3.2.4. Recovery and scale up of bacterial populations for inoculation in microcosms..34

3.2.5. Native populations of Bacteria in Kapunda red-brown earth soil: initial cycle of wheat (C0) 34

3.2.6. Cycling of bacterial populations on successive generations of wheat roots and the effect on take-all disease and plant growth 35

3.2.7. *In vitro* inhibition and fluorescence by pseudomonads........................37

3.2.8. Take-all control by the total aerobic bacterial population from root lesions...38

3.2.9. Populations of bacteria in Avon soil 38

3.3. Results 39

3.3.1. Native populations of bacteria in Kapunda red-brown earth soil.............39

3.3.2. Pseudomonads cycled on healthy roots in Kapunda soil: effect on take-all and shoot growth...40

3.3.3. Pseudomonads cycled on lesioned and non-lesioned sections of diseased roots in Kapunda soil: effect on take-all and shoot growth 42

3.3.4. TAB cycled on root lesions in Kapunda soil: effect on take-all and shoot growth..42

3.3.5. Kapunda soil cycled with successive generations of diseased wheat: effect of take-all and shoot growth..45

3.3.6. Bacterial populations after cycling on wheat roots..............................45

3.3.7. Inhibition and fluorescence in pseudomonads after the third cycle of wheat in Kapunda soil..49

3.3.8. Bacterial populations on wheat roots grown in Avon soil......................52

3.4. Discussion 53

3.5. Conclusions...56

Chapter 4. *In vitro* Diversification of *Pseudomonas corrugata* strain 2140 ....**57**

4.1. Introduction 57

4.1.1. Aims..58

4.2. Materials and Methods 59

4.2.1. Production of variant phenotypes from Pc2140R and Pc2140RL.........59

4.2.2. Confirmation that variant isolates are derived from Pc2140..................59

4.2.3. Comparison of variant colony types by GC-FAME profiles....................59

4.2.4. Comparison of similar colony types by GC-FAME profiles 60

4.2.5. Production of further variants from variant colony types.......................60

4.2.6. Appearance of variants on common laboratory media....60
4.2.7. Production of variants from other *Pseudomonas* isolates....61
4.3. Results....61
4.3.1. Production of variant phenotypes from Pc2140R and Pc2140RL....61
4.3.2. Confirmation that variant isolates are derived from Pc2140....64
4.3.3. Comparison of variant colony types by GC-FAME profiles....64
4.3.4. Comparison of similar colony types by GC-FAME profiles....64
4.3.5. Production of further variants from variant colony types....66
4.3.6. Production of variants on common laboratory media....67
4.3.7. Production of variants from other *Pseudomonas* isolates....68
4.4. Discussion....69
4.5. Conclusions....72

Chapter 5. Production and Selection for Spontaneous Phenotype Variants of *Pseudomonas corrugata* strain 2140R on Root Lesions and Healthy Roots of Wheat....73

5.1. Introduction....73
5.1.1. Aims....74
5.2. Materials and Methods....75
5.2.1. Colonisation of take-all diseased and healthy wheat roots by Pc2140R....75
5.2.2. Production of variant colony types from Pc2140R in wheat-soil microcosms....75
5.2.3. Control of take-all by mixed Pc2140R populations after three cycles of wheat....77
5.2.4. Control of take-all by Pc2140R wild type and variant colony types from one microcosm after three cycles of wheat....77
5.2.5. Control of take-all by mixed Pc2140R populations after eight cycles of wheat....77
5.2.6. Control of take-all by individual Pc2140R reisolates recovered after eight cycles of wheat....78
5.2.7. Comparison of Pc2140R wild type and variant colony types from pot +3 after five cycles of wheat by GC-FAME and inhibition profiles....78
5.2.8. Comparison by *in vitro* inhibition of Pc2140R wild type and variant colony types from eight populations after six cycles of wheat....79
5.2.9. GC-FAME and *in vitro* inhibition profiles of Pc2140R colony types reisolated from roots after eight cycles of wheat....79
5.3. Results....79

5.3.1. Colonisation of take-all diseased and healthy wheat roots by Pc2140R 79
5.3.2. Production of variant colony types from Pc2140R in wheat-soil microcosms 80
5.3.3. Control of take-all by mixed Pc2140R populations after three cycles of wheat 84
5.3.4. Control of take-all by Pc2140R wild type and variant colony types from one microcosm after three cycles of wheat 85
5.3.5. Control of take-all by mixed Pc2140R populations after eight cycles of wheat 86
5.3.6. Control of take-all by individual Pc2140R reisolates recovered after eight cycles of wheat 86
5.3.7. Comparison of Pc2140R wild type and variant colony types from pot +3 after five cycles of wheat by GC-FAME and inhibition profiles 89
5.3.8. Comparison by *in vitro* inhibition of Pc2140R wild type and variant colony types from eight populations after six cycles of wheat 90
5.3.9. GC-FAME and *in vitro* inhibition profiles of Pc2140R colony types reisolated from roots after eight cycles of wheat 92
5.4. Discussion 92
5.5. Conclusions 95

Chapter 6. Phenotypic Characterisation, Identification and Hierarchical Clustering of a Range of *Pseudomonas corrugata* strain 2140 Variants 96

6.1. Introduction 96
6.1.1. Aims 97
6.2. Materials and Methods 97
6.2.1. Source of Pc2140 variant phenotypes 97
6.2.2. Phenotypic characterisation of Pc2140R, Pc2140RL and variant phenotypes 98
6.2.2.1. GC-FAME 98
6.2.2.2. BIOLOG 99
6.2.2.3. *In vitro* inhibition 99
6.2.3. Hierarchical clustering of Pc2140R, Pc2140RL and variant phenotypes 100
6.2.4. Comparison of cluster analysis using Procrustes comparison 100
6.3. Results 101
6.3.1. Phenotypic characterisation of Pc2140R, Pc2140RL and variant phenotypes 101

6.3.2. Taxonomic identification of Pc2140 and variants by GC-FAME and BIOLOG 104

6.3.3. Hierarchical clustering of Pc2140R Pc2140RL and variant phenotypes 108

6.3.4. Comparison of cluster analysis using Procrustes comparison 108

6.4. Discussion 109

6.5. Conclusions 112

Chapter 7. Molecular Confirmation that Variant Phenotypes of *Pseudomonas corrugata* 2140R are Derived from the Original Parent Strain 113

7.1. Introduction 113

7.2. Materials and Methods 114

7.2.1. PFGE 114

7.2.1.1. DNA extraction 114

7.2.1.2. Restriction digests 114

7.2.1.3. Electrophoresis conditions 115

7.2.2. ERIC-PCR 115

7.2.2.1. Preparation of cells 116

7.2.2.2. Preparation of PCR mix 116

7.2.2.3. PCR conditions 116

7.2.2.4. Electrophoresis of ERIC-PCR products 116

7.3. Results 117

7.4. Discussion 117

Chapter 8. General Discussion 121

8.1. Directed selection by root lesions 122

8.2. The implications of targeting the fluorescent pseudomonad group in studies on take-all decline 124

8.3. Selection for variant phenotypes 125

8.4. Phenotype conversion and microbial inoculants 127

8.5. Phenotype plasticity: implications for studies on bacterial populations 128

8.6. Conclusions 129

Appendix: GENSTAT 5 commands 131

References 134

Publications Arising From This Thesis 152

List of Tables

Table 1.1. *Pseudomonas* strains isolated as potential biological disease control agents. 3

Table 1.2. Secondary metabolites implicated in disease or pathogen suppression....9

Table 1.3. Regulatory genes controlling production of metabolites involved in disease suppression, or pathogenesis, by pseudomonads. 10

Table 2.1. Description of Pc2140 colony morphology types on TZCA after 48 h at 25°C....20

Table 2.2. Source of wheat pathogens and other soil microorganisms used in *in vitro* inhibition assays and pathogen inoculum for pot microcosms....21

Table 2.3. *In vitro* inhibition of soil microorganisms by Pc2140 isolates and description of inhibition rating....29

Table 3.1. Populations of bacteria in Kapunda bulk soil....40

Table 3.2. Inhibition and fluorescence characteristics of pseudomonads in Kapunda soil....41

Table 3.3. Percentage of pseudomonads to TAB in C1, C2, and C3 on root lesions and non-lesioned sections of diseased roots....48

Table 3.4. Percentage of TB-T isolates compared to TAB and pseudomonads on root lesions and non-lesioned sections of diseased roots after one cycle (C1) on wheat roots....48

Table 3.5. Ggt-inhibition and fluorescence by pseudomonad isolates on root lesions and non-lesioned roots after three cycles on wheat roots in Kapunda soil. 51

Table 3.6. Populations of TAB, pseudomonads and percentage of pseudomonads on root lesions and non-lesioned roots grown in Avon soil....53

Table 3.7. Ggt-inhibition and fluorescence in pseudomonads isolated from roots and bulk soil from wheat-Avon soil microcosms. 53

Table 4.1. Number of *P. corrugata* 2140RL cultures (maximum 4) containing TR colony types after growth on different media for 16 and 31 days. 68

Table 4.2. Production of variants from a range of soil and rhizosphere bacteria. 69

Table 5.1. Inhibition of 7 microorganisms by the ancestral Pc2140R, and wild type (OP) and variant (RC) colony types reisolated from pot +3 after 5 cycles (65 wk) on diseased wheat roots....89

Table 5.2. Inhibition of soil microorganisms by Pc2140R reisolates with the wild type (OP) and variant (RC) colony types reisolated from different pot microcosms after 6 cycles (83 wk) on wheat roots....90

Table 6.1. Source of variant colony types derived from Pc2140R and Pc2140RL....98

Table 6.2. Fatty acid, BIOLOG and inhibition profiles of Pc2140R, Pc2140RL and their descendants. 102

Table 6.3. BIOLOG dye reduction after 24 h at 28°C for carbon sources that did not vary between Pc2140 and variant isolates. 104

Table 6.4. Taxonomic classification of Pc2140R, Pc2140RL and variant phenotypes by GC-FAME and BIOLOG taxonomic identification systems. 105

Table 6.5. Procrustes paired comparisons of GC-FAME, BIOLOG and inhibition profiles by PCA. 108

Table 7.1. PCR reaction mixes 1 and 2. 116

List of Figures

Fig. 1.1. Take-all disease interaction modified from Huber and McCay-Buis (1993). 4

Fig. 3.1. Cycling of native bacterial populations in wheat-Kapunda soil microcosms. 36

Fig. 3.2. Populations of total aerobic bacteria (TAB), pseudomonads and percentage of pseudomonads to TAB on wheat roots grown in Kapunda soil. 39

Fig. 3.3. Association between fluorescence and inhibition of Ggt among pseudomonads isolated from various locations on wheat roots and soil. 41

Fig. 3.4. Shoot dry weight (DW, mg) of wheat inoculated with bacterial populations after one (C1), two (C2) and three (C3) cycles of wheat in Kapunda soil. 43

Fig. 3.5. Percentage of root lesions on wheat roots inoculated with bacterial populations after one (C1), two (C2) and three (C3) cycles of wheat in Kapunda soil 44

Fig. 3.6. Shoot and root dry weight (A) and percent root lesion (B) of wheat after inoculation with TAB cycled on root lesions, and the pseudomonad and non-pseudomonad sub-populations. 45

Fig. 3.7. Populations of TAB (A), pseudomonads (B) and percentage of pseudomonads (C) on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after one cycle of wheat (C1). 46

Fig. 3.8. Population size of TAB and pseudomonads on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after two cycles of wheat (C2) 47

Fig. 3.9. Population size of TAB and pseudomonads on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after three cycles of wheat (C3) 47

Fig. 3.10. Population size of TB-T isolates on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after one cycles of wheat (C1). 48

Fig. 3.11. Inhibition score of pseudomonad isolates from healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after three cycles of wheat (C3) 50

Fig. 3.12. Number of pseudomonad isolates which fluoresce on KB medium after three cycles of wheat 50

Fig. 3.13. Percentage of pseudomonad isolates with each of the four character combinations of fluorescence (F) and Ggt-inhibition (I) after 3 cycles of wheat (C3). 51

Fig. 4.1. Mixed populations of Pc2140R and variant colony types on TZCA from three replicate cultures of Pc2140R after 25 days in static A-DB medium. 62

Fig. 4.2. Number of variant colony types in each of 25 replicate cultures of Pc2140RL after 11, 18 and 25 days growth in static A-DB medium. 63

Fig. 4.3. Percentage of TR (A) and FNT (B) colony types in each of 25 replicate cultures of Pc2140RL after 11, 18 and 25 days growth in static A-DB medium. 63

Fig. 4.4. Comparison of five different variant colony types by GC-FAME profiles. 65

Fig. 4.5. Comparison by GC-FAME profiles of variant isolates with the same colony morphology. 65

Fig. 4.6. Evolution of three variant colony types derived from Pc2140RL as represented by PCA of GC-FAME profiles. 67

Fig. 5.1. Root colonisation by Pc2140R on healthy wheat roots, and on root lesions and non-lesioned sections of diseased roots. 80

Fig. 5.2. Proportion of different colony types in Pc2140R populations cultured on healthy roots (A) and diseased root lesions (B) of wheat after two (C2, 24 wk), five (C5, 65 wk) and eight (C8, 108 wk) cycles of wheat. 81

Fig. 5.3. Changes in the Pc2140R population cultured on diseased root lesions in pot +1 after two (A; C2, 24 wk), five (B; C5, 65 wk) and eight (C; C8, 108 wk) cycles of wheat. 82

Fig. 5.4. Changes in the Pc2140R population cultured on diseased root lesions in pot +3 after two cycles (A, 24 wk), five cycles (B, 65 wk) and eight cycles (C, 108 wk) of wheat. 83

Fig. 5.5. Shoot dry weight (DW) of wheat (A) and average percent root lesions (B) on wheat roots of take-all infected wheat inoculated with the ancestral Pc2140R and mixed Pc2140R populations from the third cycle (C3, 39 wk) of wheat. 84

Fig. 5.6. Shoot dry weight (DW) of wheat (A) and average percent root lesions (B) on wheat roots inoculated with wild type and variant colony types from pot +3 after three cycles of wheat (C3, 39 wk). 85

Fig. 5.7. Shoot dry weight (DW) of wheat plants inoculated with individual Pc2140R reisolates with the wild type (OP) and variant (RC and OPS) colony types recovered after eight cycles (C8, 108 wk) of wheat. 87

Fig. 5.8. Percentage of root length occupied by lesions on wheat seedlings inoculated with individual Pc2140R reisolates with the wild type (OP) and variant (RC and OPS) colony types recovered after eight cycles (C8, 108 wk) of wheat. 88

Fig. 5.9. Comparison of GC-FAME profiles of Pc2140R reisolates from pot +3 after five cycles of wheat by principal component analyses (PCA). 89

Fig. 5.10. Inhibition of wheat pathogens (A) and cluster analyses of GC-FAME profiles (B) by Pc2140R re-isolates with the wild type and variant colony morphologies recovered from a healthy (pot -3) and a diseased (pot +3) microcosm after eight cycles of wheat (C8, 108 weeks). 91

Fig. 6.1. Comparison of Pc2140R and 3 variant phenotypes (TR4.Ri, FNT8.Ri and PPr6.Ri) to Pseudomonas spp. in the MIDI GC-FAME MIS Sherlock TSBA aerobe library version 3.8. 106

Fig. 6.2. Hierarchical cluster analysis of Pc2140R, Pc2140RL and descendent variants by GC-FAME (A), BIOLOG (B) and *in vitro* inhibition (C) profiles. 107

Fig. 7.1. Macrorestriction fragment profiles of Pc2140R and variant colony types after digestion with *Xba*1 and *Spe*1 restriction enzymes and separation by CHEF-PFGE. 118

Fig. 7.2. ERIC-PCR profiles of Pc2140R and variant colony types. 119

Fig. 8.1. Development of a disease suppressive microbial community by: (A) selection between existing populations..122

Fig. 8.2. Comparisons of bacterial populations between: (A) non-suppressive and suppressive soil..123

Fig 8.3. Changes in the pseudomonad population, and the effect on take-all disease, by bacterial populations cultured on successive generations of wheat roots in Kapunda soil. 124

Fig. 8.4. Summary of the diversification of Pc2140 on agar media, in static ammonium defined broth (A-DA) and on wheat roots. 126

Chapter 1. Introduction

During the last decade, in Australia and overseas, there has been an increasing emphasis on "sustainable" agricultural practices, recognising the need to prevent degradation of our agricultural lands. In the future the aim must surely be not just to sustain our agricultural soils, but to improve them. Much of Australia's agricultural land is on geologically old soils, with soil organisms adapted to perennial dry sclerophyll vegetation. These organisms would not be considered as the optimum biota for agricultural crops. Nonetheless, many Australian soils have been very productive and Australia is one of six countries which is a net exporter of agricultural produce. One major problem facing Australian farmers, in particular cereal growers, are soil-borne root diseases which cause millions of dollars loss in crop yields every year. The yield loss of wheat to take-all disease, caused by the fungal pathogen *Gaeumannomyces graminis* (Sacc.) von Arx and Oliver var. *tritici* Walker (Ggt), has been estimated at $A200 million per year in Australia (CSIRO Division of Soils 1991). There are currently no commercially available resistant cultivars (Huber and McCay-Buis, 1993; Rengel *et al.*, 1993), or chemical (Huber and McCay-Buis, 1993) or biological controls (Wong, 1994) available to control take-all disease. Current control of take-all is by crop rotation with legumes (Rothrock and Cunfer, 1991; DeBoer *et al.*, 1993), and by stubble management (Murray *et al.*, 1991), but take-all is increased in drier areas (<350 mm rainfall) using direct drill seeding (Rovira and Ridge, 1983; Roget, 1988). Two other strategies for the control of take-all and other soil-borne diseases are (1) the *in situ* development of disease suppressive soils; and (2) the introduction of a disease antagonist or biocontrol agent.

Soils have been described which have been found to suppress plant disease in the presence of the pathogen. Huber and Schneider (1982) list examples of soils suppressive to 14 plant pathogens. These soils have been described as historically suppressive, and are naturally suppressive to a particular disease. Suppression can also be induced by a particular cropping regime (Lockwood, 1988), and soils have become suppressive to take-all disease (take-all decline, TAD; Gerlagh, 1968; Shipton, 1972; Brown, 1981) and Rhizoctonia bare patch disease of wheat (Rhizoctonia decline; Roget, 1995). Take-all suppression is based on microbiological interactions, and suppression can be transferred to non-suppressive soils (Shipton *et al.*, 1973), as is suppression to Rhizoctonia bare patch (Wiseman *et al.*, 1996). The development of suppressive soils *in situ* would be advantageous, but suppression of disease during TAD and Rhizoctonia decline occurs over a lengthy period (up to 10 years) with a greatly elevated level of disease in the first few years (Werker *et al.*, 1991; Roget, 1995). This results in economically unacceptable losses in crop yields. It is not known what factors select for the suppressive microflora found in suppressive soils. If we know the factors which select for a disease

suppressive microflora, and also the niches within the root-soil environment where selection for these organisms occurs, then these factors could be manipulated to direct the evolution of a disease suppressive microflora. The initial high levels of disease and the time till suppression develops could then be reduced.

Disease suppressive soils also provide a source of potential biocontrol agents. The introduction of plant growth promoting rhizobacteria (PGPR) or biocontrol agents that reduce disease would seem an attractive proposition, with the potential for long term disease control if the introduced organisms become established in the soil. There are numerous examples of bacteria, fungi and actinomycetes that have been isolated, and which can increase plant growth, and/or, reduce disease in many host plant-pathogen interactions. Only a few examples are currently available commercially, including formulations containing *Agrobacterium* (Kerr and Htay, 1974; Ryder and Jones, 1990), *Streptomyces* sp. (Mohammadi and Lahdenperä, 1994) and *Bacillus subtilis* (Backman *et al.*, 1994). Many examples of potential disease control agents can be found in recent books on biological control organisms (Cook and Baker, 1983; Hornby, 1990; Tjamos *et al.*, 1992), and the list is ever increasing. Pseudomonads appear to be important in the natural suppression of a number of diseases on a range of important crop species, and many have been isolated which can suppress disease. Some of these pseudomonads are listed in Table 1.1. The use of biological control agents, or PGPR, is of increasing importance considering the implications of further restrictions in the use of chemical fungicides (Gullino *et al.*, 1994).

A major problem that prevents many plant growth promoting rhizobacteria (PGPR) from becoming commercially available to farmers is in achieving consistent results in increasing plant growth and reducing disease in field trials by application of a PGPR (Capper and Campbell, 1986; Weller, 1988; Wong, 1994). One factor which could contribute to inconsistent performance is the production of variant phenotypes which do not control disease. Bacteria are plastic and spontaneously produce new phenotypes (Terzaghi and O'Hara, 1990; Rainey *et al.*, 1994). This is of importance in pseudomonad PGPR isolates because the common variant phenotypes produced during stationary phase culture in the laboratory do not control disease (Laville *et al.*, 1992; Gaffney *et al.*, 1994). That is, *in vitro* conditions direct the evolution of PGPR towards a population which does not control disease. These new variant phenotypes can be difficult to detect on most culture media, however the consequences of selecting a non-functioning mutant for scale up is failure of the PGPR inoculum to control disease. It is not known to what extent variant phenotypes occur in the root-soil environment or the impact phenotype change has on disease control and long term establishment of bacteria in the field. Conditions in the root-soil environment which select for a disease suppressive microflora could also select for new variant phenotypes with an increased ability to suppress disease.

If we are to direct the evolution of bacteria or bacterial populations we need to understand how bacteria change, and how environmental pressures influence the direction of change within

bacterial populations. The aim of this thesis was to investigate the role of diseased root lesions produced after infection of Ggt on wheat roots in directing the evolution of (1) a single pseudomonad isolate (Pc2140) and (2) a native pseudomonad population for increased disease suppression. Pc2140 was chosen as a model pseudomonad biocontrol agent against take-all disease as its distinctive corrugated colony morphology on an agar medium containing triphenyl tetrazolium chloride (TZCA; Kelman, 1954) allows easy detection of variant phenotypes by virtue of their altered colony morphology on this medium. This allows for investigation into the evolution of bacteria in the laboratory and the effect of phenotype change on the phenotypic characterisation of a range of new variant phenotypes with a known ancestor.

Table 1.1. *Pseudomonas* strains isolated as potential biological disease control agents.

Bacteria	Disease/Pathogen	Crop	Reference
P. corrugata 2140	take-all	wheat	Ryder and Rovira, 1993
P. fluorescens 2-79	take-all	wheat	Weller and Cook, 1983
P. putida K11	take-all	wheat	Vrany *et al.*, 1981
P. aureofaciens Q2-87	take-all	wheat	Harrison *et al*, 1993
P. fluorescens BL915	*Rhizoctonia solani*	cotton, variety of seedlings	Gaffney *et al.*, 1994
P. fluorescens CHAO	*Thielaviopsis basicola*	tobacco	Keel *et al*, 1992
P. fluorescens Pf-5	*Rhizoctonia solani* *Pythium ultimum*	cotton	Howell and Stipanovic, 1979; Howell and Stipanovic, 1980
P. fluorescens Hv37aR2	*Pythium ultimum*	cotton	Howie and Suslow, 1991
P. fluorescens *P. putida*	Increased yield	potato	Burr *et al.*, 1978
P. fluorescens R111	Increased yield	lettuce onion	Germida and de Freitas, 1994.
P. fluorescens M45 and MC07	*Rhizoctonia solani*, *Pythium ultimum*	cucumber	Chang and Ju, 1994.
P. corrugata 13 *P. fluorescens* bv C *P. fluorescens* bv E	*Pythium aphanidermatum*	cucumber	Zhou and Paulitz, 1993 Rankin and Paulitz, 1994
P. putida	*Fusarium*	Flax	Scher and Baker, 1982
P. putida	growth promotion	canola	Lifshitz *et al.*, 1987
Pseudomonas spp.	*Pseudomonas. solanacearum*	tomato	Peixoto *et al.*, 1994
P. putida 34-13 *P. fluorescens* G8-4 *P. aureofaciens* 25-33, 28-9, 36-5	*Colletotrichum orbiculare*	cucumber	Kloepper *et al.*, 1991
[a]*P. fluorescens*	*Pythium*, *Rhizoctonia*	cotton, other seedlings	Harris, 1994
[b]*P. fluorescens* C12 *Pseudomonas.* sp. A2	*Pseudomonas tolaasii*	mushrooms	Nair and Fahy, 1976 Lynch, 1992

[a]commercial isolate withdrawn from market due to short shelf life
[b]commercially available

1.1. Take-all disease on wheat

Take-all disease results from the interaction between the plant (wheat), pathogen (Ggt) and the environment. Huber (1981) and Huber and McCay-Buis (1993) have reviewed the factors that affect take-all, and have provided a comprehensive summary of the multiple factors involved, and some of the inter-relationships between these factors. Often disease symptoms are not evident even in the presence of a virulent pathogen. In Huber and McCay-Buis's review (1993) the disease interaction was represented by a Venn diagram, with take-all as the area where the plant, pathogen and environmental sets intersect. This work introduces a fourth major factor, disease suppressive soil bacteria.

As environmental conditions affect all three sets of organisms, plant, fungus and bacteria, the take-all interaction might be better represented as the intersection of the three biotic components, all of which are in the soil environment domain, as shown in Figure 1.1. The environmental conditions present at the root surface act on all three parts of the biota simultaneously, while each of the biotic components acts on the micro-environment and directly and indirectly on the other biota. The biotic components are also dependent on their evolutionary history, and Cook *et al.* (1995) suggest that plants with the ability to stimulate and respond to disease suppressive microorganisms have been selected for by the pressures imposed by soil pathogens. Here we are concerned with how a suppressive soil community develops, or evolves.

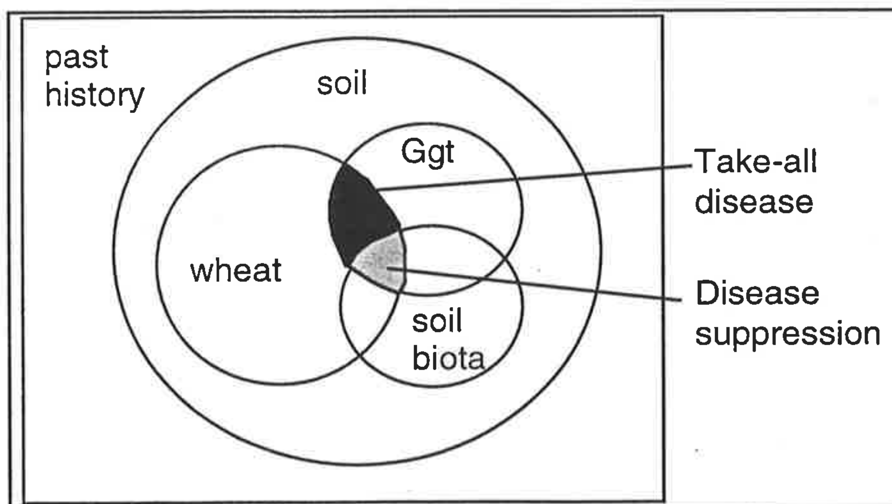


Fig. 1.1. Take-all disease interaction modified from Huber and McCay-Buis (1993).

The pathogen, in this case Ggt, is the causal agent of disease, not the disease itself. Disease results from the interaction of all components, and could be likened to the concept of dynamic equilibrium used in chemistry. A given set of conditions would produce a given level of disease, dependent on the combined influence of all components involved in the disease interaction. A change in one or more components shifts the equilibrium position to favour either the disease or the plant.

1.2. Take-all decline

Under continuous wheat monoculture, many soils become suppressive to take-all (Gerlagh, 1968; Shipton, 1972; Brown, 1981). This phenomenon, called take-all decline (TAD), is due to soil microorganisms, and the biological factors responsible for take-all decline can be transferred from one soil to another to induce suppression of take-all. Soils suppressive to take-all have provided a source of disease antagonists which might be used to control soil-borne diseases.

1.2.1. Development of take-all decline

In one example in the UK, take-all reached a peak in the third year of continuous wheat, and then declined steadily (Hornby, 1992), however, the rate of development can be variable (Shipton, 1975). Although TAD does not develop in all soils under continuous wheat cropping (Lucas *et al.*, 1989), TAD still develops in a wide range of soils, from clay to sandy loams (Gerlagh, 1968), from acid (Weller and Cook, 1983) to alkaline soils (Andrade *et al.*, 1994a) and in areas from low (Shipton *et al.*, 1973) to high (Shipton, 1972) annual rainfall, and under irrigation (Cook, 1981).

Hornby (1992) suggested that TAD is less frequent, weaker and ephemeral or absent in soils with low fertility and low inputs. In these soils, biological control agents and fertiliser treatments such as ammonium or manganese had a larger impact on disease compared to high input fertile soils where classical TAD develops, and disease is not easily influenced by biological control agents or fertiliser treatments. The addition of ammonium to soils can also stimulate take-all decline (MacNish; 1988; Sarniguet *et al*, 1992a).

Different crops used in rotation can influence the level of disease in subsequent wheat crops (Huber, 1981), and also disease suppression. Soybeans make soil conducive to take-all disease and negate the development of take-all decline (Cook, 1981; Rothrock and Cunfer, 1985). Cultivation practices also affect suppression, with take-all disease increased in direct drilled field plots compared to conventionally cultivated plots (Rovira *et al.*, 1990).

1.2.2. Take-all disease, a prerequisite for take-all decline

Shipton (1972) suggested that maximum disease development appeared to be a prerequisite for take-all decline to develop, and this still appears to be true. TAD does not develop in the absence of a virulent pathogen (Gerlagh, 1968), and there is little evidence that reduced pathogen virulence (Cook and Naiki, 1982) or reduced primary infection (Pope and Jackson, 1973; Wilkinson *et al.*, 1985; Cook *et al.*, 1986) is responsible for TAD. High levels of disease in the second to fourth year of continuous wheat before disease suppression occurs is a common feature of many studies on TAD (Hornby, 1992), and also occurs during Rhizoctonia decline (Roget, 1995). The initial high levels of disease, especially during direct drilling, is a major economic impediment to the *in situ* development of suppressive soil.

1.2.3. Microbial suppression of take-all disease

Gerlagh (1968) concluded that TAD was of microbial origin as suppression was lost after pasteurisation of the soil, and suppression was restored when pasteurised soil was inoculated with non-sterilised suppressive TAD soil. This result has been confirmed in many other studies, and the process of soil sterilisation and restoration of suppressiveness by inoculation with a small amount of non-sterilised suppressive soil, has become common to prove suppression of a particular disease is of microbial origin (Shipton *et al.*, 1973; Cook and Rovira, 1976).

Soils can also exhibit a general, non specific, suppression of soil-borne diseases, and the distinction between general and specific suppression is mainly based on sterilisation and restoration experiments. General suppression of disease survives 70°C moist heat, methyl bromide and chloropicrin, but not autoclaving, is not transferable and probably operates in the bulk soil (Cook and Rovira, 1976). Specific suppression to disease is eliminated by 60°C moist heat, methyl bromide, chloropicrin, is transferable, and operates in the rhizoplane and rhizosphere. The specific antagonism towards Ggt can be in both the saprophytic (Gerlagh, 1968; Zogg and Amiet, 1980; Kirk, 1984) and parasitic (Gerlagh, 1968; Wilkinson *et al.*, 1985; Cook *et al.*, 1986) stages of Ggt growth in the soil or plant respectively.

Pseudomonads have been implicated in take-all suppression (Cook and Rovira, 1976) and are amongst the most abundant Gram negative bacteria in the wheat rhizosphere (Kleeberger *et al.*, 1983). In light of this, a number of *Pseudomonas* spp. have been isolated from suppressive soils in the search for biological control agents which can be applied to Ggt infested soils to reduce take-all disease. Other soil organisms which can reduce take-all disease include *Bacillus* spp. (Capper and Campbell, 1986), avirulent *Gaeumannomyces graminis* (Wong and Southwell, 1980), *Trichoderma hamatum* and *T. koningii* (Dewan and Sivasithamparam, 1988; Simon, 1989), a sterile red fungus (Dewan and Sivasithamparam, 1989), *Sordaria fimicola* (Dewan *et al.*, 1994) and mycophagous amoebae (Chakraborty and Old, 1982). Andrade *et al.* (1994b) concluded in their studies that different biota and mechanisms were involved in TAD in different soils, and with the range of different take-all suppressive organisms isolated it would also be possible for more than one biotic group to be suppressing take-all in any one soil. This thesis focuses on disease-suppressive fluorescent pseudomonads as a functional group, as this group has been implicated in TAD in the majority of studies.

1.2.4. Suppressive soils have more antagonists

In many examples of take-all suppressive soils there is an increase in the populations of aerobic bacteria and pseudomonads, and the relative number of pathogen and/or disease antagonistic fluorescent pseudomonads are increased when compared to non-suppressive soils. This is a common characteristic of take-all suppressive soil whether suppression was induced by TAD in field soil (Smiley, 1979; Weller *et al.*; 1988; Andrade *et al.*, 1994a), artificially in pots

(Charigkapakorn and Sivasithamparam, 1987) or induced by ammonium nitrogen (Sarniguet *et al.*, 1992b). During take-all decline of turfgrass there is a similar change in the fluorescent pseudomonad community (Sarniguet and Lucas, 1992).

The fluorescent pseudomonad group can be selectively reisolated from soil by plating onto selective media containing ampicillin and chloramphenicol (Simon and Ridge, 1974). Fluorescence is detected under ultra violet light (King, 1954). Fluorescent pseudomonads form a distinct group by traditional taxonomy (Stanier *et al.*, 1966), and by nucleic acid homology (Palleroni *et al.*, 1973). All the isolates listed in Table 1.1 are grouped into a common DNA homology group within rRNA homology group 1 of *Pseudomonas* by Palleroni *et al.* (1973). Their ease of isolation as a group, and their implication in disease suppression, has resulted in the fluorescent pseudomonad group being targeted in population studies in suppressive soils. Other biotic groups which may also be involved in disease suppression are often not examined in detail, but may also play a role. In suppressive soils the increase in the percentage of bacteria antagonistic to Ggt can also be accompanied by a decrease in the percentage of bacteria antagonistic to *Trichoderma koningii* (which suppresses take-all), and a much reduced percentage of *T. koningii* antagonistic to soil bacteria (Simon and Sivasithamparam, 1988).

The differences between suppressive and non-suppressive soils are also found between the same soil with added Ggt or no added Ggt in both tube assays (seedlings; Weller, 1983) and field studies (40 days after tillering; Sarniguet *et al.*, 1992a), ie. there is an increase in the total number of aerobic bacteria, pseudomonads, percentage of pseudomonads, and the relative number of fluorescent pseudomonads which are antagonistic to the pathogen and/or disease. This indicates that changes within the microbial community can occur in a relatively short time, less than one season, and is in response to disease caused by the added pathogen. It does not explain why disease suppression is usually not evident until after three or more years of continuous wheat.

1.2.5. Mechanisms of take-all decline

Shipton (1975) summarised four complementary theories as to the mechanism of TAD, all of which contain common elements, and may act simultaneously: (1) Ggt induces a specific microbial antagonism towards itself in the parasitic and saprophytic stages (Gerlagh, 1968), (2) by microbial antibiotic production which reduces the tropic response of Ggt to roots (Pope and Jackson, 1973), (3) decreasing inoculum survival and infectivity (Vojinovic, 1973), or (4) by modification of the nutritional environment of the rhizosphere (Brown *et al.*, 1973).

Rovira and Wildermuth (1981) put forward a later model to explain the development of TAD. In the first year bacteria multiply on the root lesion, and a small number of these are suppressive. In the second and successive years suppressive bacteria increase in number on new roots and hyphae, and in successive years reduce germination of Ggt from the infection source, and lyse

hyphae which reduces the number of hyphae reaching roots. In respect to the increase in the proportion of suppressive bacteria this model still appears to be suitable. A number of studies have shown though that the initial infection of roots from an inoculum source of Ggt does not appear to be reduced in suppressive soils, but that secondary infection and lesion extension are reduced in suppressive soils (Pope and Jackson, 1973; Wilkinson *et al.*, 1985; Cook *et al.*, 1986). Werker *et al.* (1991) studied the progress of take-all in detail over 9 years and also concluded that the reduction of diseased roots in continuous wheat (TAD) was due to reduced secondary infection from the primary infection site to new roots.

In summary, there is evidence that take-all disease produces a change in the microbial community, increasing antagonists which reduce secondary infection and lesion extension. Multiple biotic components may be involved, and inter-related. Soil population changes could be viewed in evolutionary terms, where at the community level there is selection for antagonists and the evolution of a disease suppressive microbial community.

1.3. Biological suppression of soil-borne disease

The use of rhizobacteria to suppress disease has been reviewed by Weller (1988), and many disease antagonists have been isolated from take-all suppressive soils with the potential for application as biological control agents, including *Pseudomonas corrugata* strain 2140. Strain 2140 inhibits Ggt *in vitro* and reduces take-all in pot tests (Ryder and Rovira, 1993). Disease control by *P. corrugata* 2140 in field trials has been inconsistent (Ryder *et al.*, 1990). Field trials of other pseudomonads and *Bacillus* strains isolated for potential take-all control have also been variable (Capper and Campbell, 1986; Weller, 1988; Wong, 1994), including variable results with isolates previously claimed to be effective (Hornby *et al.*, 1993; Capper and Higgens, 1993).

1.3.1. Mechanisms of pathogen and disease suppression

Pseudomonads produce a range of antimicrobial metabolites involved in disease suppression and pathogen inhibition (Dowling and O'Gara, 1994; Voisard *et al.*, 1994). Some of these metabolites, and their abbreviated names, are listed in Table 1.2. Bacterial induced systemic resistance by plants to disease can also be involved in disease suppression (Zhou and Paulitz, 1994). Not all of the metabolites listed in Table 1.2 may be important with a particular bacterial strain, but where more than one mechanism is involved, the relative importance of an individual mechanism depends on the plant and pathogen involved (Maurhofer *et al.*, 1994a), and the sensitivity of pathogen strains to particular antibiotics (Mazzola *et al.*, 1995). Combinations of mechanisms can also have an additive effect (Ownley *et al.*, 1992). The production of antimicrobial metabolites, however, does not necessarily mean they are involved in disease suppression (Kraus and Loper, 1992). For successful control of a range of pathogens, disease control bacteria need a range of mechanisms (Loper *et al.*, 1994; Homma, 1994).

Table 1.2. Secondary metabolites implicated in disease or pathogen suppression

Metabolite	Isolate	Pathogen	Reference
Volatiles			
HCN	*P. corrugata* 2140	Ggt	Ross and Ryder, 1994
	P. fluorescens CHAO	*Thielaviopsis basicola*	Keel *et al.*, 1989
	P. fluorescens BL915		Lam *et al.*, 1994
	P. fluorescens Pf-5	Ggt	Kraus and Loper, 1992
Siderophores			
pyoverdine (Pvd)	*P. fluorescens* Pf-5	Ggt	Kraus and Loper, 1992
	P. fluorescens CHAO	*Thielaviopsis basicola*	Kloepper *et al.*, 1980; Leong, 1986; Keel *et al.*, 1989; Loper and Buyer, 1991
Antibiotics			
phenazine-1-carboxylic acid (PCA)	*P. fluorescens* 2-79	Ggt	Brisbane and Rovira, 1988; Thomashow and Weller, 1988
	P. aureofaciens 30-84	Ggt	Thomashow *et al.*, 1990
	P. chloroaphis 30-84	Ggt	Mazzola *et al.*, 1995
pyoluteorin (Plt)	*P. fluorescens*	*Pythium ultimum*	Howell and Stipanovic, 1980
	P. fluorescens CHAO	*Pythium*	Maurhofer *et al.*, 1994b
	P. fluorescens Pf-5	Ggt	Kraus and Loper, 1992
pyrrolnitrin (Pyn)	*P. fluorescens*	Ggt	Howell and Stipanovic, 1979; Hill *et al.*, 1994; Homma, 1994
	P. fluorescens Pf-5	Ggt	Kraus and Loper, 1992
2,4-diacetyl-phloroglucinol (Phl)	*P. fluorescens* CHAO	*Thielaviopsis basicola* Ggt	Keel *et al* .,1990
	P. aureofaciens Q2-87	Ggt *Rhizoctonia solani*, *Pythium ultimum*	Vincent *et al.*, 1991
	Pseudomonas sp. F113	range of root pathogens	Shanahan *et al.*, 1992
1,3,6-trihydroxy-2,4-diacetophenone	*P. aureofaciens* Q2-87	Ggt	Harrison *et al.*, 1993
Oomycin A	*P. fluorescens* Hv37aR2	*Pythium ultimum*	Howie and Suslow, 1991
antibiotic 3 (Apd)	*P. fluorescens* Pf-5	Ggt	Kraus and Loper, 1992

It has been suggested that antibiotic production in the soil may not occur, or be reduced, due to insufficient substrate, or if produced would be subject to chemical or biological degradation or would be adsorbed onto soil colloids (Lockwood, 1988). This may be true in some situations, but certainly not all, as the presence or absence of a single antibiotic produced by a PGPR strain can greatly influence the organisms capacity to reduce disease, and antibiotics such as PCA (Thomashow *et al.*, 1990) and Phl (Keel *et al.*, 1992; Shanahan *et al.*, 1992; Bonsall *et al.*, 1997) have been isolated from the root-soil environment, and these compounds contribute to disease suppression by the PGPR producing the antibiotic concerned.

1.3.2. Global regulation of antibiotic production

Genetically diverse groups of bacteria can produce the same antibiotic (Cook *et al.*, 1995). Antibiotic production in pseudomonads is controlled by at least three levels of regulation and these regulatory elements appear to be conserved. *P. fluorescens* strain CHAO produces Plt, Phl, Pvd, HCN, protease and phospholipase, all of which are dependent for expression on a common trans-acting DNA binding transcription regulator coded for by the gene, *gacA* (Laville *et al.*, 1992; Sacherer *et al.*, 1994). *GacA* is the transcription, or response regulator (RR) of a two component regulatory system. *P. fluorescens* strain BL915, another biocontrol strain, contains a gene homologous to *gacA* controlling expression of multiple metabolites, as well as a sensing component containing a histidine protein kinase (HPK) function, which is homologous to the *lemA* gene from *P. syringae* (Gaffney *et al.*, 1994). Loss of function of the regulatory genes, *gacA*, or *lemA*, produced identical phenotypes, and resulted in the pleiotropic loss of all functions controlled by these genes, including disease suppression capability (Gaffney *et al.*, 1994).

LemA and *gacA* type genes are common in many pseudomonads, and examples are listed in Table 1.3, including plant and animal pathogens where virulence factors are regulated. Spontaneous mutation in these regulatory genes during stationary phase is common in pseudomonads, and produces new phenotypes with a pleiotropic change in characteristics. This phenomenon has been called phenotype conversion by Brumbly and Denny (1990). Genes implicated in phenotype conversion are marked with an asterisk (*) in Table 1.3.

Table 1.3. Regulatory genes controlling production of metabolites involved in disease suppression, or pathogenesis, by pseudomonads. Homologous genes are in brackets. CM, colony morphology, DYP, diffusible yellow pigment; EPS, extracellular polysaccharide; PG, endoglucanase; TSO, tryptophan side chain oxidase. * implicated in phenotype conversion.

Isolate	HPK	RR	Regulates	Reference
P. corrugata 2140			HCN, TSO, DYP, CM	Ryder *pers comm.*
P. fluorescens CHAO		*gacA* *	Plt, Phl, Pvd, HCN, Protease, Phospholipase	Laville *et al.*, 1992; Sacherer *et al.*, 1994
P. fluorescens BL915	*lemA* type* (*cheA*)	*gacA* type* (*uvyY*)	Pyn, HCN, Chitinase, Gelatinase, CM	Gaffney *et al.*, 1994
P. fluorescens, Pf-5	*apd* * (*lemA*)		Plt, Pyn, Phl, TSO, HCN	Corbell *et al.*, 1994
P. fluorescens 2-79	*phzP* * (*lemA*)	*phzR* like	PCA	Cook *et al.*, 1995
P. fluorescens Hv37aR2	*afuA**, *afuB**, *afuP**	*afuR*	Oomycin A	Gutterson, 1990
P. chlororaphis 30-84	*phzI* (*luxI*)	*phzR* (*luxR, lasR*)	PCA	Cook *et al.*, 1995
P. syringae pv *syringae*	*lemA* * (*rcsC, phoR bvgS, virA*)	*gacA* type* (*gacA, uvrY*)	Protease, Syringomycin Tabtoxin	Hrabak and Willis, 1992; Rich *et al.*, 1994
P. aeruginosa	*lemA* type		pyocyanin	Rich *et al.*, 1994
P. aeruginosa UCBPP-PA14		*gacA* type	plant and animal virulence	Rahme *et al.*, 1995

Genes required for synthesis of metabolites involved with disease suppression, and regulated at the level of transcription by the *lemA-gacA* regulatory system, are also regulated at transcription by specific sigma factors. Sigma factors are responsible for the specificity of DNA recognition by RNA polymerase. A *rpoS*-like sigma factor in *P. fluorescens* Pf-5 is required for pyrrolnitrin and tryptophan-side chain oxidase activity, and stress resistance. A mutation in this *rpoS*-like sigma factor produces pleiotropic effects (Sarniguet and Loper, 1994). A *rpoD* sigma factor in *P. fluorescens* CHAO regulates production of Plt and Phl (Schnider *et al.,* 1995), and contains conserved regions with homologous genes in other pseudomonads and bacterial genera.

1.3.3. Root colonisation by PGPR

In addition to the loss of antibiotic production by PGPR, poor root colonisation is often given as a reason for lack of disease control by PGPR. In soils where a PGPR can reduce disease a threshold population is required for disease to be controlled (Bull *et al.,* 1991; Raaijmakers *et al.*, 1995; Raaijmakers and Weller, 1998). In sterile soils, the final population of introduced bacteria is independent of the initial population applied, but is determined by substrate availability in the soil (Bennett and Lynch, 1981; Nannipieri *et al.,* 1983; Postma *et al.*, 1990). In non-sterile soil, as when applied in the field, the final population of an introduced PGPR would be determined by substrate availability and microbial interactions (Fukui *et al.,* 1994a and b). Root colonisation and disease control may also be by different mechanisms (Bull *et al.*, 1991), depending on the antibiotic involved (Mazzola *et al.*, 1992). It appears though that PGPR can readily colonise roots and other plant parts under a wide variety of conditions (Milus and Rothrock, 1993; Thompson *et al.*, 1995), and there is no clear evidence that differences in disease suppression between soils is due to poor colonisation in unfavourable soil types. Even with effective root colonisation, biocontrol of one disease may be overshadowed by an increase in damage by another (non-target) disease, which has an added advantage with reduction of the target pathogen (Rovira *et al.*, 1990; Cook, 1992; Pierson and Weller, 1994).

1.3.4. Using mixtures of microorganisms for disease control

The use of a single microbial isolate for disease control under a range of conditions has generally not been successful. Suggestions have been to use local isolates (Hornby *et al.*, 1993), or develop strains which will control disease in a range of soil conditions (Capper and Higgens, 1993). Even so, the use of single isolates to control soil-borne pathogens in the field appears to be unrealistic (Duffy *et al.*, 1996). Using combinations of PGPR to overcome variable edaphic and biotic soil factors has been suggested (Cook *et al.,* 1995), and additive and interactive effects can be important (Andrade *et al.,* 1994b). In recent studies, combinations of pseudomonads increased yield and reduced take-all where individual strains were ineffective (Pierson and Weller, 1994), and combinations of pseudomonads and *Trichoderma koningii* reduced take-all better than bacteria alone (Duffy *et al.*, 1996).

If, however, mixtures of biocontrol agents to control disease are to be used, there is still the problem of where to obtain a compatible mix of isolates suitable to the soil conditions where they are applied. Charigkapakorn and Sivasithamparam (1987) presented evidence for the artificial induction of take-all decline in pots, where pathogen antagonists were increased, and Cook (1992) used diseased microcosms to bioamplify disease antagonists against take-all, with the aim of reisolating these antagonists. If TAD can be artificially induced in microcosms, we should aim to reisolate the disease antagonists as a functional group. Artificially inducing TAD is equivalent to directing the evolution of a suppressive soil microbial community.

1.4. Microbial Plasticity

Since the late 1980's there has been an increased interest in microbial plasticity, or the ability of bacteria to produce new phenotypes, independently of external events such as conjugation, transduction and transformation. The spontaneous production of new phenotypes has been called phenotype conversion (Brumbly and Denny, 1990) or intraclonal polymorphism (Rainey *et al.*, 1994). There is increasing evidence that bacteria may be more "plastic and adaptable than initially imagined" (Terzaghi and O'Hara, 1990). Spontaneous mutants arise in initially pure cultures of bacteria, and Schaaper *et al.*, (1986) found that a wide range of different mutational events could give rise to the same genotype, in this case inactivation of the *lacI* gene. These mutational events included nucleotide sequence deletion, base pair transitions, insertions and deletions. The production of new phenotypes is of great importance to PGPR isolates because they are common, they no longer control disease, and they are difficult to detect on most media. Selection of non performing mutants for the scale up of bacterial inoculum would inevitably lead to failure of the isolate to control disease.

Bacteria undergo many physiological, metabolic and genetic changes, during the transition from logarithmic to stationary phase growth (Kolter *et al.*, 1993). New phenotypes which arise through mutation in stationary phase culture are favoured by the conditions imposed on them, compared to the parent or ancestral type, and these mutants can eventually come to dominate the culture. There has been recent controversy as to whether these mutations in stationary phase cultures are random, or directed by environmental conditions (Cairns, 1988). The directed mutation hypothesis put forward by Cairns (1988) has been disputed (Foster, 1993; Lenski and Mittler, 1993), and does not explain the range of mutations that can occur to produce the same phenotype (Schaaper *et al.*, 1986), or the multiple phenotypes which can arise from a single ancestral cell when cultured under identical conditions. Most of the studies on stationary phase mutants have used *E. coli* as a model, but phenotype conversion is also common in pseudomonads (Table 1.3) and related species such as *Ralstonia solanacearum* (Brumbly and Denny, 1990), *Xanthomonas* spp. (Swords *et al.*, 1996; Rajeshwari *et al.*, 1997) and *Stenotrophomonas* sp. (Nakayama *et al.*, 1997), as well as Gram positive bacteria (Claassen *et al.*, 1986; Bulthuis *et al.*, 1988) and *Streptomyces* (Leblond *et al.*, 1990). The fact that phenotype

conversion occurs in such a range of bacterial groups indicates that phenotype conversion is a common feature of bacteria in the laboratory.

1.4.1. Multiple new phenotypes in response to the same selection pressure

Recent research shows that identical bacterial populations, started from a single ancestral cell, can produce a number of possible phenotypes which are better suited to the laboratory conditions that are imposed on them. Lenski and Bennett (1993) found that replicate cultures of *E. coli* diverged from each other in response to thermal stress, with increasing stress leading to more rapid adaptation. Lenski and Travisano (1994) also found that replicate *E. coli* cultures diverged from each other in relative fitness, and in colony morphology when cultured for 10,000 generations in glucose limited conditions. Bacteria can also diverge in the field, as shown by the small divergence of fatty acid methyl ester (FAME) profiles in descendants of *P. fluorescens* SBW25 inoculated onto sugar beet (Thompson *et al.*, 1995). The divergence of replicate cultures implies that initially identical populations reach different fitness peaks in identical environments (Lenski and Travisano, 1994). This supports the concept that the underlying mutational events which have occurred are random in their occurrence.

The ability of new phenotypes to appear in culture can be dependent on the presence of other new phenotypes (Helling *et al.*, 1987) and new phenotypes can give rise to further variants, (Rainey *et al.*, 1993). Mutations can also have maladaptive effects, conferring varying fitness between isolates (Lenski, 1988a), and can be compensated for by further mutations (Lenski, 1988b). Moxon *et al.* (1994) suggest that plant pathogenic bacteria have 'contingency' genes with high mutation rates and 'housekeeping' genes with low mutation rates to facilitate expansion into new niches and minimising deleterious effects of mutations.

From these examples it can be seen that multiple new phenotypes can arise from a single ancestor, and these may be better suited to the environment in which the organism is cultured The same phenotypes can occur independently in replicate cultures, can be dependent on the presence of other mutants, and can produce further mutants. Identical genomic rearrangements can occur in replicate cultures (Flores *et al.*, 1988), but in contrast, cells with the same phenotype may have different sequence changes in the same gene (Zambrano *et al.*, 1993) or in different genes as in *lemA* or *gacA* mutants which do not control disease (Gaffney *et al.*, 1994). In some cases mutations can be reversible (Claassen *et al.*, 1986; Gill *et al.* 1991).

1.4.2. Cryptic genes

Cryptic genes are genes that are normally inactive in a bacterium, but can be mutationally activated, and these appear to be wide spread in bacterial populations (Young, 1989). Hall and Betts (1987) concluded that in *E. coli* there are at least four sets of genes for catabolism of ß-

glucosides that are normally inactive, but which are potentially functional, and suggest that bacteria possess a reservoir of cryptic genes which are only one mutation away from functioning.

P. fluorescens strains can contain genes which have been shown to be important in disease suppression, but which are not expressed, or expressed at negligible levels. Gaffney *et al.* (1994) introduced a *gacA* type regulatory gene from *P. fluorescens* BL915 to heterologous *P. fluorescens* strains, and the transconjugants were able to produce chitinase, cyanide and pyrrolnitrin which were not detected prior to the addition of *gacA* . Genes for Phl (Vincent *et al.*, 1991) and HCN (Voisard *et al.*, 1989) can be transferred to heterologous pseudomonads, and be expressed and regulated in the new host, increasing disease control in the recipients. These studies show that both structural genes required for the production of secondary metabolites which suppress disease, and their regulatory elements, can be present in pseudomonads, but not detectable by the bacteriums' phenotype under "normal" conditions.

Genes for production of a variety of secondary metabolites involved with disease suppression, and which are coordinately controlled by a *lemA-gacA* type regulatory system, would form a block of cryptic genes when inactivated due to a mutation in one of the regulatory genes. This set of genes may only be one mutation away from being mutationally activated. The pseudomonads of Gaffney *et al.* (1994) which produced secondary metabolites in transconjugants containing an introduced *gacA* gene, may also be close to being mutationally activated. If mutations are random, then there is the possibility of these genes becoming activated, but whether the phenotype persists will depend on the environment. Of interest here is whether genes required for disease suppression become activated, and selected for, during the development of take-all decline.

1.4.3. Microbial plasticity and disease suppression

One particular organism suitable for studying phenotype plasticity in a PGPR isolate is *P. corrugata* strain 2140 as it produces multiple new phenotypes, or variants, when grown in still and shaken broth, and on solid media, during stationary phase for an extended time (Barnett, 1994). New variant phenotypes can be readily detected by altered colony morphology on TZCA medium containing tetrazolium chloride. The distinctive opaque, cream coloured corrugated colony morphology of Pc2140 on TZCA makes variant morphotypes readily detectable compared to other PGPR strains and enhances the usefulness of Pc2140 as a model organism to study phenotype conversion. The most frequently occurring variant phenotypes from Pc2140 generally had a greater loss of pathogen inhibition *in vitro* , compared to less commonly occurring phenotypes. The most common phenotype which occurs in 2140 cultures has a translucent red (TR) colony morphology and is a pleiotropic mutant, with multiple loss of metabolic functions (Ryder, *pers comm* .), and does not suppress take-all in pot tests. The TR type may be due to a mutation in a *lemA-gacA* type gene which is common in phenotype

conversion in other PGPR pseudomonads (Table 1.3). The appearance and detection of these new variants is dependent on the culture conditions. The loss of secondary functions which serve no benefit to the bacteria when grown on a Petri dish also increase its survival compared to the parent type by reducing the metabolic load on the organism, and the loss of production of factors which inhibit pathogens, but may be self toxic after an extended period of time on artificial media, also increases the variants survival. That is, the evolution of PGPR strains on artificial media is directed towards new phenotypes which do not control disease. This is supported by Rich *et al.* (1994) who noted that the *lemA-gacA* regulation system possibly regulates functions which are detrimental to bacterial survival in stationary phase on artificial media.

1.4.4. Environmental selection for new phenotypes

What is often understated in the literature on spontaneous mutants is that we can only detect those mutants that can be selected for. That occurs when the mutants: (1) can grow where the wild type cannot , eg. selection for antibiotic resistance or novel carbon source use; (2) cannot grow where the parent can, eg. selection for auxotrophs by replica plating; or (3) have a higher growth rate or longer survival time than the wild type. Mutations which do not result in the above characteristics cannot be detected, and we have little idea of the underlying mutation rate of the bacterial genome. Single mutant cells cannot be identified without selection from large mixed populations, and of necessity we must work with populations, so it seems we cannot study mutants unless growth conditions influence the survival of the mutants. In the case of strain Pc2140, we can only detect certain mutants due to their better survival during long term stationary phase conditions, and even then we can only tell that they are different phenotypes because of their different colony morphology on a specific medium containing tetrazolium chloride. On other media the different types aren't easily distinguishable from each other, if at all. The phenotypes we detect depend on the environment, with selection being a two way process consisting of: (1) selection for favourable phenotypes; and (2) selection against less favoured phenotypes.

In Wright's model of bacterial evolution, gene pools become coadapted, with frequency dependent selection important, and with many different optimal genotypes (Wright, 1980). Evolution however is limited to the organism's underlying genetic potential to adapt (Hartl *et al.*, 1985; Hall, 1995). In the laboratory, bacteria can diversify by generating multiple new phenotypes in response to relatively simple, and identical, environmental conditions where the criteria for fitness against some single imposed stress can be reasonably be determined. Diversification within and between replicate populations is greater the more physically complex or heterogenous the medium (Korona *et al.*, 1994; Rainey and Travisano, 1998). In the complex soil environment we would expect even greater diversity in response to the more heterogeneous environment compared to the laboratory, and differential selection between new mutants within each niche.

1.4.5. The heterogeneity of the root-soil environment

Electron microscopy provides details of where bacteria are actually located in the soil, rhizosphere and root (Foster *et al.*, 1983; Foster, 1986). These studies show that the root changes in form along its length with a corresponding change in the microflora and its association with the root. Bacteria exist as single cells, and as colonies, in the soil, rhizosphere, in the mucigel covering the root surface, and within and between epidermal and cortical cells (Foster *et al.*, 1983; Assmus *et al.*, 1995). On the root surface infected with Ggt, the mucigel disintegrates at the lesion site and bacteria are more abundant than on healthy parts of adjacent roots, and in some cases there is a massive proliferation of bacteria (Rovira and Wildermuth, 1981; Foster *et al.*, 1983). Electron microscopy, however, provides few details of ecological functions of the microorganisms studied.

There is increasing evidence that plant species, and location on plant roots has an important selection influence on rhizobacteria. Populations of fluorescent pseudomonads are differentially selected for from the soil population depending on the host plant (Glandorf *et al.*, 1993; Lemanceau *et al.*, 1995). Populations of fluorescent pseudomonad groups can vary with location in, on or near roots (Miller *et al.*, 1990; Van Peer *et al.*, 1990; Lemanceau *et al.*, 1995). Bacterial groups also vary along the length of plant roots, with root tips selecting for monosaccharide utilisers where the concentration of these sugars is higher (Liljeroth *et al.*, 1991). The hypothesis that the difference in root exudates selects for different groups according to substrate utilisation (Lemanceau *et al.*, 1995) seems reasonable, but it is not known how this relates to selection for disease antagonists.

1.5. Evolution of disease suppression

Ggt is a highly evolved pathogen, being crop specific, parasitic and has a K life strategy with a long life and persistence in soil (Lockwood, 1988). Plant diseases have also been important in plant evolution (Harlan, 1976). The evolution of disease would have proceeded in parallel with, and dependent on evolution of the plant and pathogen. It would be expected that rhizobacteria which suppress disease have also evolved along with the disease interaction. Darwinian evolution is based on the selection of favourable variants that exist within a population or community. At the community level it would appear that a high level of take-all disease selects for a suppressive soil biota (Smiley, 1979; Sarniguet *et al.*, 1992a).

That some naturally occurring soil microbial isolates that suppress plant diseases exist now demonstrates that suppressiveness has already evolved. What level of suppressiveness a microbial isolate has against a particular pathogen would depend on the multiple environmental pressures which the organism faced during its evolution. We have no reason to assume, however, that it is at its maximum potential in regard to disease suppression. The phenotype of an organism is presumably the best the organism could derive for optimal fitness in the

environment that it was in, and may not be the optimum for any particular characteristic, or for a different environment. We know that plant and animal pathogens can change virulence rapidly, but what about disease suppressiveness? Much work has centred on the evolution of plant-pathogen relationship, but little on the plant-pathogen-suppressive bacteria relationship. The plant-pathogen-suppressive bacteria relationship is important because the suppression built up during TAD can be destroyed by one year of cropping with another species, such as soy beans (Cook, 1981). Virulence and suppressiveness factors (or metabolites) in pseudomonads both have similarities. They are both controlled by two or more component regulatory systems controlling multiple functions, and groups of these functions can be lost simultaneously by mutation in the controlling regulatory genes during stationary phase growth on lab media. Some avirulent forms of plant and animal pathogens produced *in vitro* can revert back to the wild type virulent pathogen on a host (Deretic *et al.*, 1987; Brumbley *et al.*, 1993; Swords *et al.*, 1996), but this has not been reported for *in vitro* mutants produced by a PGPR.

1.5.1. The origin of disease suppressive bacteria

The origin of disease antagonists is not discussed adequately in the literature. It is usually assumed that the antagonists present in suppressive soils were already present in the non suppressive soil, and that they increase in relative number as take-all decline develops (Rovira and Wildermuth, 1981). Individual antagonists can be isolated from non-suppressive soil (Castejonmunoz and Oyarzun, 1995), and non-suppressive soil can induce suppression in sterile suppressive soil (Andrade *et al.*, 1994a). Pseudomonad communities are dynamic (Rainey *et al.*, 1994), so if selection is for existing antagonists within the soil community during TAD, why does suppression take so long (several years) to develop? It seems clear that the community structure of bacterial groups in the rhizosphere and phylloplane change in response to disease or seasonal changes respectively. Bacteria can have high growth rates, and in the soil environment they are subject to growth flushes interspersed with stationary phase conditions. This combination should mean that bacterial populations have the potential to reach a new equilibrium state in a relatively short time.

Studies of the flux of microorganisms during development of disease suppression are lacking. Those that have been done have concentrated on changes within sub-groups of the soil community. Charigkapakorn and Sivasithamparam (1987) artificially induced TAD in pots with successive cycling of take-all diseased roots, and studied changes in the pseudomonad community which was grouped into sets, biovars and species. There were differences in the occurrence of groups between healthy and diseased roots, with sets of isolates belonging to *P. fluorescens* biovar V present on diseased, but not healthy roots and *P. putida* isolates generally occurring only on healthy roots, and not diseased roots.

1.5.2. Selection within and between bacterial populations

The same disease pressure which selects for a disease suppressive soil community from the existing soil microbiota would also select for favoured variants which arose within each individual population. However, the role of microbial plasticity, or change within populations, does not appear to have been considered in relation to the development of disease suppression. Where studies of microbiological changes between suppressive and non-suppressive soils have been made, they have analysed differences between the soil community structure at a single point in time. When the temporal changes in biota that occur during the development of suppressive soil have been studied, a subset (pseudomonads) of the soil community has been analysed (Charigkapakorn and Sivasithamparam, 1987). No one has yet monitored the changes that occur within one particular lineage of cells, starting from a single ancestor, during the development of disease suppression. Thus it is not possible to determine whether bacteria isolated after successive generations wheat are: (1) descended from existing isolates within the microflora and reach higher relative numbers due to shifts in different genotype populations; (2) are descendent from phenotype variants within a genotype through mutation; or (3) a combination of 1 and 2.

1.5.3. Diseased root lesions: a selection site for antagonists?

Shipton (1972) suggested that maximum disease development in the first 2-5 years of continuous wheat monoculture is a prerequisite to the appearance of take-all decline. Cook *et al.* (1995) suggested that selection of antagonistic biota by plant roots is the preferred strategy evolved for suppression of soil borne diseases. It would follow that disease is the environmental pressure that selects for the increased antagonists found in suppressive TAD soils.

The most obvious manifestation of take-all disease on wheat roots is the black necrotic lesion which develops following infection by Ggt. The lesion site would present a unique niche on the plant root. Carbon substrate is often the limiting factor for microbial growth in soils, and root exudates are quickly utilised by microorganisms (Barber and Martin, 1976). Access to root cell contents are largely denied to the soil microbiota while the root epidermis remains intact. In a diseased root lesion produced after infection by the pathogen, the cellular integrity of the root is disrupted providing a site of increased, readily available, substrate. Phenotypes more able to colonise this increasing niche would be favoured. Increasing plant growth, pathogen antagonism or competition with the pathogen may also favour lesion colonisation, growth or survival of bacteria which suppress disease.

We know that bacteria can change on laboratory media to generate new populations better suited to the growth conditions imposed upon them, and in the pseudomonad group, the production of secondary metabolites that are not needed *in vitro* can be quickly lost. In the much

more complex and dynamic soil environment the criteria for fitness would be determined by a great many factors, and functions that are lost on a Petri dish may well be important for the cell to survive in the more complex and competitive soil environment. Indeed, if these secondary metabolites were not important, the functional genes would not be maintained in the population.

Based on the evidence presented in this review of the literature, it is proposed that the diseased root lesion which develops after infection by Ggt is a unique niche that selects for disease suppressive pseudomonads. Selection for disease suppressive pseudomonads could be between populations of existing isolates, or within a population for new phenotypes which arise.

1.6. Research aims

The aims of this research were to investigate (1) the role of take-all root lesions in directing the evolution of a bacterium (Pc2140), and a native pseudomonad population for increased disease suppression and (2) further investigate the diversification of Pc2140, *in vitro* and *in vivo*, and the phenotypic characterisation of variant colony types derived from a known ancestor (Pc2140) and test the effect of phenotype changes on the ability of variant isolates to control take-all.

The aim was to shed light on the following questions raised in the Introduction: (1) are root lesions a distinct niche compared to non-lesioned sections of diseased roots? (2) do root lesions preferentially select for pseudomonads, specifically disease suppressive pseudomonads? (3) does phenotype conversion occur in the root-soil environment, and if so, do diseased roots preferentially select for variant phenotypes with increased ability to control disease? and (4) what is the possible range of different phenotypes produced by a single PGPR isolate?.

Chapter 2. General Materials and Methods

2.1. Introduction

This chapter contains general materials and methods which are used in this work and apply to more than one chapter.

2.2. Materials and Methods

2.2.1. Source of microorganisms

2.2.1.1. *Pseudomonas corrugata* 2140 strains

The ancestral *P. corrugata* strain 2140 (Pc2140) was isolated from wheat roots (grown in soil collected from Wagga Wagga NSW; Ryder and Rovira, 1993). *P. corrugata* strains 2140R (Pc2140R) and 2140RL3 (Pc2140RL) are a spontaneous rifampicin 100 μg/ml (rif) resistant and a rif resistant *lacZY* marked strain of Pc2140 respectively (Ryder *et al.*, 1994). Strains Pc2140R and Pc2140RL were used in this study. Variant Pc2140 colony types are described in Table 2.1.

Table 2.1. Description of Pc2140 colony morphology types on TZCA after 48 h at 25°C.

Colony description	Colony type[a]	Diam. mm	Colour	Opacity	Form	Elevation	Edge	YDP[b]
Opaque	OP (WT)	2	cream	op[c]	irregular	corrugated	wavy	+
Translucent-red	TR	2	red	tr	circular	convex	entire	-
TR YDP	TRY	2	red	tr	circular	convex	entire	+
Faint	FNT	2	yellow	tr[d]	circular	flat	entire	-
Faint-orange	FNTO	2	orange	tr	circular	flat	entire	-
Faint-orange	FNTOL	4	orange	tr	circular	flat	entire	-
Furry	Fur	2.5	red	tr	irregular	convex	furry	-
Pin prick size	PP	0.5	cream	tr	circular	convex	entire	-
Pin prick size-red	PPr	0.5	red	tr	circular	convex	entire	-
Large-flat	LF	4	cream	op	irregular	raised	jagged	-
Corrugated-red	CR	2	red	op	roughly circular	corrugated	wavy	-
Translucent-spreading	TRS	2	red	tr	circular-spreading	flat	entire	-
Red centre	RC	2	cream-red	op	circular	convex	entire	+/-
Domed	DM	2	light pink	op	circular	domed	entire	-
Star	OPS	2	cream-red star shaped centre	op	roughly circular	umbonate	wavy	-

[a]abbreviated description of colony morphology used in this study, [b]yellow diffusible pigment, [c]opaque, [d]translucent.

Variants are labelled by their colony type and isolate number, followed by the parent (R = Pc2140R; RL = Pc2140RL) and whether isolated *in vitro* (i) or from soil (s). For example, TR4.Ri is a translucent (TR) colony type, no. 4, from Pc2140R *in vitro* cultures.

2.2.1.2. Wheat pathogens and other microorganisms

The source of wheat pathogens and other soil organisms used for *in vitro* inhibition assays is shown in Table 2.2.

Table 2.2. Source of wheat pathogens and other soil microorganisms used in *in vitro* inhibition assays and pathogen inoculum for pot microcosms.

Microorganism	Isolated by[a]	Isolated from	Comment
Ggt-8	H. McDonald	wheat roots, Avon S.A.	wheat pathogen, intermediate virulence (Rengel *et al.*, 1993)
Rhizoctonia solani AG-8, Zg-1, rh21	H. McDonald	wheat roots, Avon S.A.	wheat pathogen
Pythium irregulare BH25	B. Hawke	soil	wheat pathogen
Trichoderma koningii 7a	A. Simon	wheat roots	biological control fungus
Rhizopus oryzae fU1	S. Barnett	wheat roots, Urrbrae S.A.	rhizoplane fungus
Pseudomonas putida K4	S. Barnett	wheat roots, Kapunda, S.A.	Gram -ve rhizoplane bacterium
Bacillus cereus bU3	S. Barnett	wheat roots, Urrbrae S.A.	Gram -ve rhizoplane bacterium

[a]address: CSIRO Land and Water, PMB 2 Glen Osmond 5064, South Australia.

2.2.2. Media

Media were sterilised by autoclaving at 121°C for 15 minutes. Agar media were cooled to 50°C prior to pouring into sterile 9 cm Petri dishes. Filter sterilised antibiotics (0.2 μm Gelman Acrodisk), when used, were added to the agar after cooling and immediately prior to pouring.

2.2.2.1. DB, Defined broth medium

This medium was used as a defined broth (DB) medium with known nutrients for generation of multiple phenotype variants from Pc2140, and for some *in vitro* inhibition assays. This medium was developed by M. Ryder (unpublished) based on fungal growth medium. This medium was used with an ammonium (A-DB) or nitrate (N-DB) nitrogen source and contained $(NH_4)_2SO_4$ (5 g/L, A-DB), or KNO_3 (7.7 g/L, N-DB); D-glucose (20 g/L); KH_2PO_4 (2 g/L); K_2HP0_4 (4 g/L); $MgSO_4.7H_2O$ (1 g/L); $NaSO_4$ (0.2 g/L); $CaCl_2$ (0.1 g/L) and $FeCl_3$ (2.5 mg/L). pH 7.0.

For solid media, ammonium defined agar (A-DA) and nitrate defined agar (N-DA), Oxoid no.1 Agar (12 g/L) was added.

When fungi were grown on this medium, filter sterilised (0.2 μm Gelman Acrodisk) vitamins and trace element stock solutions were added. Vitamins (thiamine 200 μg/L; biotin 5 μg/L) and trace elements (H_3BO_3 10 μg/L; $CuCl_2$ 100 μg/L; $MnSO_4$ 20 μg/L; $(NH_4)_6Mo_7O_{24}.4H_2O$ 20 μg/L; $ZnSO_4$ 2 mg/L; $CoCl_2.6H_2O$ 100 μg/L) were added.

Nutrients were added as sterile stock solutions of each nutrient to the basic medium containing glucose and agar. These were added after autoclaving and cooling the medium.

2.2.2.2. LB, Luria Bertani medium

This medium is a general bacterial growth medium (Maniatis *et al.*, 1982), and contained: Bacto tryptone (Difco, 10 g/L); Bacto yeast extract (Difco, 5 g/L); NaCl (10 g/L); pH 7.5. For solid medium, Difco Bacto Agar was added (15 g/L).

2.2.2.3. MM, Minimal medium

This is a selection medium for autotrophic bacteria. Glucose was added here to allow growth of pseudomonads. This medium contained: Glucose (20 g/L); $(NH_4)_2HPO_4$ (1 g/L); KCl (0.2 g/L); $MgSO_4.7H_2O$ (0.2 g/L); Oxoid no.1 Agar (12 g/L), pH 7.

2.2.2.4. MNA, Modified Nutrient Agar

This is a common short term storage medium for bacteria in China (Yufa Peng, *pers comm.*), containing: Nutrient Broth (Difco, 8 g/L); Yeast extract (Difco, 2 g/L); Glucose (2 g/L); KH_2PO4 (0.5 g/L); K_2HPO_4 (2.5 g/L); NaCl (1 g/L); Difco Bacto Agar (15 g/L); pH 7.5.

2.2.2.5. NB, Nutrient Broth

This medium was used as a general bacterial growth medium for scale up of bacterial cells for storage and as inocula for *in vitro* cultures and inhibition assays. Medium contained Nutrient Broth (Difco, 8 g/L).

2.2.2.6. NA, Nutrient Agar

This medium was used as a general bacterial growth medium for scale up of Pc2140 isolates for inoculation into pot microcosms. Constituents were: Nutrient Broth (Difco, 8 g/L); and Difco-Bacto Agar (15 g/L).

2.2.2.7. PDA/4 Potato Dextrose Agar, 1/4 strength

This medium was used as a general fungal growth medium and used at 1/4 strength. Medium contained: Potato Dextrose Agar (Difco, 9.75 g/L); Difco-Bacto Agar (11.25 g/L).

2.2.2.8. TB-T, Trypan Blue, Tetracycline medium

This medium has been described as a selective medium for *Burkholderia cepacia* strains (Hagedorn *et al.*, 1987), and was used in Chapter 3 to further subdivide the bacterial populations from wheat roots. This medium contained: Difco-Bacto Agar (20 g/L); glucose (2 g/L); L-asparagine (1 g/L); $NaHCO_3$ (0.5 g/L); $MgSO_4.7H_2O$ (0.1 g/L); trypan blue (TB, 50 mg/L); tetracycline (T, 20 mg/L); and the pH adjusted to pH 5.5 with 10% phosphoric acid. Filter sterilised (0.2 µm Gelman Acrodisk) trypan blue and tetracycline were added after cooling the medium immediately prior to pouring into plates.

2.2.2.9. TSA, Tryptic Soy Agar

This medium was used as a general bacterial growth medium for isolation of bacterial populations from roots and soil in Chapter 3. Full strength medium contained: Tryptic soy broth (Difco, 30 g/L); and Difco-Bacto Agar (15 g/L). Bacterial populations were isolated on 1/10 strength (TSA/10) medium containing: Tryptic soy broth (Difco, 3 g/L); and Difco-Bacto Agar (15 g/L).

2.2.2.10. TSBA, Trypticase Soy Broth Agar

This is a specific culture medium for growth of bacterial cells for GC-FAME analysis (Section 2.2.13), and contained Trypticase soy broth (BBL, 30 g/L); and Granulated agar (BBL, 15 g/L).

2.2.2.11. TZCA, Tetrazolium chloride Agar

This medium is based on Kelman (1954) and used for detection of colony morphology variants from Pc2140. This medium contained: Proteose Peptone no. 3 (Difco, 10 g/L); Casamino acids (Difco, 1 g/L); D-glucose (5 g/L); Difco-Bacto agar (15 g/L) and 2,3,5, triphenyl tetrazolium chloride (Sigma, 0.05 g/L) added as 5 ml/L of a 1% filter sterilised (0.2 µm Gelman Acrodisk) solution after autoclaving and cooling.

2.2.2.12. X-Gal MM, X-Gal Minimal Medium

This medium is used for detection of *lacZY* marked bacteria. Isolates expressing the *lacZY* gene form blue colonies on this medium (Drahos *et al.*, 1986). Glucose (5 g/L); $(NH_4)_2HPO_4$ (1 g/L); KCl (0.2 g/L); $MgSO_4.7H_2O$ (0.2 g/L); Oxoid no.1 Agar (12 g/L); 5-Bromo-4-Chloro-3-indolyl-ß-D-Galactopyranoside (X-Gal, 40 mg/L), pH 7. X-Gal was added as 1 ml of 40 mg/ml dimethyl-formamide solution after cooling agar and prior to pouring.

2.2.3. Growth conditions of *in vitro* cultures

All fungal and bacterial cultures were incubated at 25°C, except for culture of cells for GC-FAME and BIOLOG analysis which were incubated at 28°C. Overnight broth cultures for scale up of bacterial isolates for storage or inhibition assays was carried out in 10 ml screw capped glass tubes with 3 ml broth in an orbital (shaken, 120 revolutions/min) incubator at 25°C.

2.2.4. Purification and storage of bacterial cultures

The ancestral Pc2140 isolates and variant colony types, were sub-cultured twice by streaking onto TZCA (48 h at 25°C) and a single colony was scaled up overnight in 3 ml NB. The cells (1 ml aliquots) were pelleted by centrifugation (12,000 x g, 5 min), resuspended in 15% glycerol (1 ml) and stored at -70°C. Mixed populations of Pc2140 and soil bacterial populations were prepared for storage at -70°C after harvesting the population from agar plates, pelleting the cells and resuspending in sterile 15% glycerol as before. Bacteria were suspended in glycerol at room temperature for 1 h prior to freezing.

2.2.5. Pathogen (Ggt) inoculum for pot microcosms

To provide consistent levels of take-all disease in pot microcosms the soil-sand mix in diseased treatments were artificially inoculated with Ggt. Ggt was added to pot microcosms on infested ryegrass seeds at the rate of 0.23 g infested ryegrass/kg soil-sand mix (ca. 120 propagules/kg) unless noted. Ggt infested ryegrass inoculum was prepared as described by Simon *et al.* (1987). Gamma irradiated ryegrass seeds (75 g) were soaked overnight in excess sterile water contained in 600 ml milk bottles, well drained, and autoclaved at 121°C for one hour on three consecutive days. Ryegrass seeds were inoculated with Ggt grown for one week on 1/2 strength PDA. The agar from one Petri dish was cut into 5x5 mm squares and mixed with the sterilised ryegrass seeds in one bottle. Ggt inoculated ryegrass cultures were incubated at 25°C for three weeks. Cultures were mixed weekly. After ryegrass seeds were fully colonised by Ggt, seeds were air dried under sterile conditions until no condensation was present. Cultures were stored in sterile airtight containers at -20°C . Each bottle culture was assessed for contamination by plating infested ryegrass seeds onto PDA/4 and contaminated cultures were rejected.

2.2.6. Soil

Take-all disease assays, and cycling of both native soil populations and Pc2140R, was carried out in pot microcosms containing a Kapunda soil-sand mix. Kapunda soil is a sodic red-brown earth (Stace *et al.*, 1968) from Kapunda, South Australia (34° 21' S, 138° 18' E) and is also described as a fine mixed thermic Calcic Natrixeralf (Soil Survey Staff, 1990). This was used unsterilised so as to include the native Kapunda soil biota. Soil was collected from Kapunda (top 10 cm of soil), sieved (2 mm) and air dried before use. The pH of this soil is pH 5.0, 1:5 in 10 mM

$CaCl_2$. Soil composition is 15% clay, 25% silt and 60% sand. The growing medium consisted of sieved (2 mm) Kapunda soil:coarse washed river sand mixed 30:70 and made up to 6.7% w/w with 1/100 strength plant nutrient supplement (PNS, Hoagland and Arnon, 1938). Sterile distilled water or bacterial inoculum (8 ml) was added to pot microcosms after planting to give a final water content of 8.8% w/w (water potential of -2.4 kPa).

Native soil populations were also assessed from wheat roots grown in soil collected from two trial sites at Avon, South Australia (34°14'S, 138°18'E, Chapter 3). These have been described as: (1) suppressive soil (SS) from Phase II trial, wheat-wheat rotation, direct drilled; and (2) non suppressive soil (NS) from Rhizoctonia Monitoring Trial, burnt stubble treatment. These trials are fully described in Rovira (1986) and Roget (1995). The soil type was a solonized brown soil (Stace *et al.*, 1968), also described as a mixed Palexeralf (Soil Survey Staff, 1975). The pH of Avon soil is pH 7.9, 1:5 in 10 mM $CaCl_2$. Soil composition is 16% clay, 7% silt and 77% sand.

2.2.7. Wheat seeds

Wheat (*Triticum aestivum* var Spear) was used for all pot microcosms. Seeds were sorted to remove both larger and smaller size seeds to improve even germination and growth. Selected seeds were pregerminated for 48 h at 25°C on moist filter paper and only healthy well germinated seeds were used. Seven seeds were planted per pot (see Section 2.2.8) and these were thinned to five plants after emergence.

2.2.8. Wheat-soil pot microcosms

The standard pot microcosm used in this work for assessment of take-all disease, and for cycling of the native Kapunda soil biota, consisted of 300 ml non draining pots containing 380 g Kapunda soil:sand mix described by Ryder and Rovira (1993). The soil:sand mix was added to pots in layers. Layer 1, (bottom) 280 g non-infested soil (healthy, non diseased microcosms) or Ggt-infested soil:sand mix (120 Ggt-ryegrass propagules per kg); layer 2, 50 g non-infested soil:sand mix ; layer 3, pre-germinated seeds planted on top of layer 2; layer 4, 50 g non-infested soil:sand mix. Plants were inoculated with 8 ml of either sterile distilled water or bacterial suspension (see Section 2.2.9) for a final soil moisture content of 8.8% w/w. White plastic beads (3 mm) were added (about 35 g) to the top of each pot to minimise evaporation. All pots had an initial total starting weight of 430 g.

2.2.9. Bacterial inoculum for wheat-soil microcosms

The bacterial inoculum for pot microcosms was scaled up on solid medium, harvested, and inoculated into microcosms as a soil drench. Inoculum of single isolates of Pc2140R wild type and variant colony types were prepared by sub-culturing single colonies twice on TZCA (48 h at 25°C) and streaking single colonies onto NA (48 h at 25°C). Inoculum of mixed bacterial

populations cultured on wheat roots were prepared by plating undiluted root extracts onto the appropriate selection media. Bacteria were harvested by scraping bacteria off plates with a sterile glass rod in 3 ml sterile distilled water (SDW). Bacterial suspensions were then diluted to A_{550}=1 to give a final cell concentration of 10^9 cfu/ml. This suspension (8 ml) was applied to the soil surface immediately after planting seeds and adding the final soil-sand layer.

The number of cells and purity of Pc2140R suspensions used to inoculate pots was assessed by plating 10 fold dilutions of the suspension onto TZCA. Populations of native bacteria were plated onto TSA/10 to determine inoculum density.

2.2.10. Growth conditions of wheat-soil microcosms

Pot microcosms were placed in a 15°C water bath after planting and plants grown under natural light in a glass house. Pots were watered to starting weight using 1/10 strength PNS (Hoagland and Arnon, 1938) after losing approximately 10 g in weight.

2.2.11. Estimation of bacterial populations from wheat roots and soil

Bacterial populations on wheat roots were estimated by plating dilutions of macerated root extracts onto selective media. For each microcosm, the roots of the five wheat plants were washed in running tap water to remove adhering soil and pooled. Five cm lengths of seminal roots, taken between two and seven cm from the seed, were excised for estimation of bacterial populations. Root systems affected by take-all were further divided into lesioned and non-lesioned sections of root, with 20 to 50 cm of each root section type assayed from each pot. Lesioned sections contained only black necrotic sections of root, while non-lesioned sections had no obvious evidence of discolouration or infection. Roots of intermediate status were rejected. Secondary roots were removed and root sections washed in 100 ml phosphate buffered saline (PBS; NaCl 8 g/L; KCl 0.2 g/L; Na_2HPO_4 1.15 g/L; KH_2PO_4 0.2 g/L; pH 7.3). Root sections were macerated with a sterilised mortar and pestle in 3 ml sterile PBS. Macerated root extracts were serially diluted 1/10 in PBS and three 20 μl aliquots of each dilution spotted onto the appropriate media. Cultures were incubated for 48 h at 25°C. Populations of bacteria on roots were expressed as colony forming units (cfu) per cm of root (cfu/cm).

Soil bacteria were isolated from the bulk soil not adhering to plant roots. The bulk soil from each pot was well mixed, 5 to 10 g of soil removed and shaken vigorously (180 revolutions/min on a rotary shaker) for 15 min in 100 ml sterile PBS. Soil extracts were diluted tenfold in PBS, and dilutions were plated onto selection media and incubated for 48 h. Populations of bacteria in soil were expressed as cfu per g dry weight of soil (cfu/g DW soil).

2.2.12. Measurement of plant growth parameters

Plant growth parameters (water use, shoot and root dry weight, and percentage of roots affected by lesions), were assessed at the end of each experiment containing wheat-soil microcosms. When wheat plants were harvested, roots were washed free of soil and plants from each pot were pooled and kept separate from other pots. Shoots were cut from roots just above the crown of the plant and separated from roots. Shoots were placed in paper bags, dried at 60°C for 4 days and then weighed for shoot dry weight. Roots were placed into new sealed plastic bags and stored at -20°C until rated for disease. Percentage of the root system affected by black take-all lesions was determined by the average percentage of the primary root system occupied by lesion on the first 12 cm of seminal roots on the five wheat plants in each pot. Percent of the root system with take-all lesions was calculated by the total length of black necrotic root lesion on seminal roots divided by the total length of seminal roots between 0 - 12 cm of root, x 100. After assessment of take-all disease, roots were placed into paper bags, dried at 60°C for 4 days and weighed for root dry weight. Where bacterial populations were measured on roots, percent root lesion was assessed after harvest of the experiment and immediately prior to sectioning of roots for estimation and isolation of bacterial populations. Dry weights of these roots were not measured. The pots used in this work were non draining, and plants were watered to the starting weight after losing ca. 10 g of water. Pots were weighed and the amount of 1/10 PNS solution added was used to calculate the amount of water used by the plants. The results for shoot dry weight and percent root lesions are presented in this work. The results of analysis for water use were similar (in terms of significance and degree of changes) to the results for shoot dry weight. Results for root dry weights followed the same trends as for shoot dry weight but results for root dry weights were much more variable between replicate treatments and only significantly different at P=0.05 when differences between treatments were large.

2.2.13. GC-FAME

Gas-Chromotography of Fatty Acid Methyl Esters (GC-FAME), and analysis of these profiles, was carried out using the Microbial Identification System version 4 (MIS, MIDI Inc. Newark, Delaware). Fatty acids were extracted and prepared according to the MIS procedure. Isolates were sub-cultured twice on Tryptic Soy Broth Agar (TSBA) for 24 h at 28°C. The second sub-culture was streaked in four quadrants as described in the MIS procedure and cells were harvested (40 ± 2 mg) with a sterile 4 mm teflon loop from the second quadrant which had confluent bacterial growth. Cells were wiped off in a sterile 8 mm glass culture tube with a screw cap lid. Bacterial cells were lysed and fatty acids saponified in 1 ml reagent 1 (NaOH, certified ACS, 45 g; methanol, reagent grade 150 ml; deionised distilled water, DDW, 150 ml) at 100°C for 30 min. Fatty acids were methylated by adding 2 ml reagent 2 (6N HCl, 325 ml; ethanol, reagent grade, 275 ml; 10 min at 80°C) and extracted with the addition of 1.25 ml extraction reagent 3 (hexane,

HPLC grade, 200 ml; methyl-tert butyl ether, HPLC grade, 200 ml; 10 min end over end rotation). The bottom aqueous phase was removed with a glass pipette and the remaining hexane phase containing fatty acid methyl esters was washed in 3 ml base wash (reagent 4; NaOH, certified ACS, 10.8 g; DDW 900 ml; 5 min end over end rotation). To help compact the interface layer and clear the hexane layer (containing fatty acid methyl esters), 5 drops of saturated NaCl solution was added at the wash step to each extraction tube, and after washing, extraction tubes were centrifuged at 600 xg for 10 min, or until the interface layer between hexane and aqueous phases compacted. The upper 2/3 of the hexane phase was transferred to 2 ml GC vials. Fatty acid methyl ester profiles were obtained by gas chromatography (Hewlett-Packard model 5890) using an Ultra 2 column (25 m x 0.2 mm, cross linked 5%), H_2 carrier gas (30 ml/min) and a flame ionisation detector.

Fatty acid peaks were identified by the MIS system and assignment of peaks in the gas chromatograph to fatty acids is given in Stead *et al.* (1992) and Siverio *et al.* (1996). GC-FAME profiles of isolates were compared to the Sherlock TSBA aerobe library version 3.8 (MIDI Inc. Newark, Delaware) for taxonomic identification.

A new 2140V library was generated using the Sherlock Library Generation Software containing only Pc2140 parent strains and variant colony types. A separate library entry was made in the 2140V library for each parent type (Pc2140R and Pc2140RL) and each variant isolate where two or more fatty acid analyses were carried out. Comparison of GC-FAME profiles using the Sherlock Library Generation Software (MIDI Inc. Newark, Delaware) was by Principle Component Analyses (PCA) or hierarchical cluster analysis using the unweighted pair-group method using arithmetic averages (UPGMA) and the results presented in a dendrogram.

2.2.14. *In vitro* inhibition of microorganisms

The wild type *P. corrugata* 2140 inhibits a number of fungi and bacteria *in vitro*, and this character was altered in some colony type variants. *In vitro* inhibition by the parent and variant Pc2140 isolates was assayed against five soil fungi and two soil bacteria shown in Table 2.3. Inhibition of each test organism was conducted on solid agar medium in 9 cm Petri dishes. The medium used for inhibition assays against each test microorganism was selected, after initial screening assays, on the basis that the test organism was inhibited by the wild type *P. corrugata* 2140 strains but not by one or more colony morphology variants.

For inhibition of the fungal isolates, one 4 mm plug of actively growing mycelium grown on PDA/4 was placed in the centre of an agar plate. *B. cereus* bU3 was patched to the centre of the plate. *P. putida* K4 was spread (100 µl of 10^7 cfu/ml overnight NB culture) evenly on the test medium as a lawn. Plates were inoculated with Ggt or *R. solani* 48 h prior to inoculation with Pc2140. Plates were inoculated with the other fungal isolates 24 h prior to inoculation with Pc2140. Plates were inoculated with bacterial test isolates *P. putida* and *B. cereus* immediately

prior to inoculation with Pc2140. For the Pc2140 inoculum, a single colony of each Pc2140 isolate was incubated overnight in 3 ml NB, and four 10 µl aliquots (10^9 cfu/ml) of the bacterial suspension were applied an equal distance apart and 30 mm from the centre of the Petri dish. The medium used for each assay, and the time between applying Pc2140 strains and assessment of inhibition, are shown in Table 2.3.

The inhibition characteristics of the wild type and variant Pc2140 types against each of the test organisms could be placed in a distinct category. Inhibition was scored, or rated, as; - no inhibition (ie. the test organism grew over the bacterial patch); or + to ++++ (increasing inhibition) as detailed in Table 2.3.

Table 2.3. *In vitro* inhibition of soil microorganisms by Pc2140 isolates and description of inhibition rating.

			Inhibition rating				
Microorganism	Medium[a]	Days[b]	-[c]	+	++	+++	++++
Ggt-8	N-DA	12	no inhibition	exclusion[d]	mycelium distortion[e]	DIZ[f] with hyphae	DIZ 3-5 mm
Rhizoctonia solani AG-8, Zg-1, rh21	N-DA	6	"	exclusion	DIZ 1 mm	DIZ 2 mm	DIZ 3 mm
Pythium irregulare BH25	A-DA	2	"	exclusion	mycelium distortion	DIZ 1-2 mm	
Trichoderma koningii 7a	N-DA	4	"	exclusion	DIZ with hyphae	DIZ 1 mm	
Rhizopus oryzae fU1	PDA/4	2	"	exclusion	DIZ 2-3 mm	DIZ 3-5 mm	
Pseudomonas putida K4	PDA/4	1	"	DIZ 1-2 mm			
Bacillus cereus bU3	TSA/10	2	"	exclusion	DIZ <4 mm	DIZ 4-5 mm	

[a]test media: N-DA, Nitrate Defined Agar; A-DA, Ammonium Defined Agar; PDA/4, 1/4 strength Potato Dextrose Agar; TSA/10, 1/10 strength Tryptic Soy Agar.
[b]Days till inhibition assessed after inoculating plates with a Pc2140 isolate.
[c]-, no inhibition, test organism grows over Pc2140 patch.
[d]exclusion of fungal or bacterial growth from Pc2140 patch.
[e]mycelium distortion, growth of fungal mycelium distorted but no clear inhibition zone near Pc2140 patch.
[f]DIZ, Distinct Inhibition Zone free of hyphal or bacterial growth.

2.2.15. Statistical analysis

Statistical analysis of data was carried out using GENSTAT 5 statistical analysis package. Analysis of individual experiments is described in the text. Data for bacterial populations was transformed to $\log_{10}$ before analysis. Comparison of treatment means was by Turkeys Honestly Significantly Difference (HSD) at P=0.05 (Equation 1). Where there was unequal replication, means were compared by the Least Significant Difference (LSD) method (Equation 2).

Equation 2.1. $$\text{HSD} = \frac{\sqrt{\text{RMS}} \cdot q_{k,v}(0.05)}{\sqrt{\text{n}}}$$

Equation 2.2. $$\text{LSD} = \frac{t_{v}(0.975)}{\sqrt{\text{RMS}\left(\frac{1}{\text{n}_1} + \frac{1}{\text{n}_2}\right)}}$$

Where RMS is the residual mean square, q is the Studentised range, t is the t distribution, k is the number of treatment means, v is the residual mean square degrees of freedom and n is the number of replications.

Chapter 3. Selection by Root Lesions for Disease Antagonists Within a Native Bacterial Population.

3.1. Introduction

High disease level appears to be a prerequisite for the development of take-all decline (TAD, Shipton, 1972), where take-all disease is suppressed after continuous monoculture of wheat. Typically, take-all disease increases in the first 2-4 years of continuous wheat and then declines (Hornby, 1992). The suppression of take-all disease in TAD soils is known to be due to soil microorganisms (Gerlagh, 1968; Shipton *et al.*, 1973). In particular, *Pseudomonas* spp. which are a major component of the bacterial community on wheat roots (Kleeberger *et al.*, 1983), have been implicated in the suppression of take-all disease (Cook and Rovira, 1976). Many isolates from the fluorescent pseudomonad group have been found to reduce take-all disease of wheat or other soil-borne pathogens (Rovira *et el.*, 1992; Défago and Keel, 1995; Weller *et al.*, 1995) and can be selectively isolated from soil or root samples by plating onto selective media containing chloramphenicol and ampicillin (Simon and Ridge, 1974). Fluorescence on King's medium B (King *et al.*, 1954) is also used as a diagnostic tool for this group, but not all isolates within the fluorescent pseudomonad group produce fluorescent pigments. Due to their potential role in suppressing take-all disease, the ability of single isolates to reduce disease, and their ease of isolation from roots or soil, the fluorescent pseudomonad group have been targeted in many ecological studies of the root microflora involved in the suppression of take-all disease.

Studies on take-all suppressive soils have shown that these soils have a larger population of total aerobic bacteria and fluorescent pseudomonads compared to non-suppressive soils. Also a greater proportion of pseudomonads are antagonistic to the pathogen or disease in suppressive soils (Smiley, 1979; Charigkapakorn and Sivasithamparam, 1987; Weller, 1988; Sarniguet *et al.*, 1992b; Andrade *et al.*, 1994b). The population of total aerobic bacteria and fluorescent pseudomonads, and the proportion of pseudomonads were also increased in the same soil when the pathogen, Ggt, was added, both in pots (Weller, 1983) and in the field (Sarniguet *et al.*, 1992a).

In population studies, fluorescent pseudomonads have been isolated from macerated wheat roots (Bull *et al.*, 1991), roots with adhering soil (Weller, 1983; Weller, 1988) or rhizosphere soil (Sarniguet *et al.*, 1992a). Electron microscopy by Foster *et al.*, (1983) showed that there was a proliferation of bacteria on root lesions caused by the take-all fungus, and Rovira and Wildermuth (1981) postulated that disease antagonists increased on diseased root lesions. Many works have also shown that suppression in TAD soils is due to reduced secondary infection and lesion

extension from the primary infection site (Pope and Jackson, 1973; Wilkinson *et al.*, 1985; Cook *et al.*, 1986; Werker, 1991) suggesting that suppression is occurring after infection at the primary infection site which later develops into the black necrotic root lesion. Despite these observations, there is little information on differences in bacterial populations between diseased root lesions and non-diseased (non-lesioned) sections of the same diseased root system. As the root microflora is not homogeneous it is highly desirable to sample our population of interest (ie. disease antagonists) from the location where suppression is occurring, or where antagonists are selected for, to prevent confounding of results by bacteria from locations of no real relevance to the study.

Although previous works have shown that the microbiota in suppressive and conducive soils are different, these studies are often comparisons at one point in time between two similar soils in the same locality with different cropping regimes. There is less information on the biotic changes which occur during the development of suppression. Charigkapakorn and Sivasithamparam, (1987) simulated the development of TAD in successive generations of wheat grown in pot microcosms, using Ggt infected roots (root lesions) to inoculate successive generations of wheat. Changes in the fluorescent pseudomonad population were assessed at the end of each generation, and they found that infected roots supported higher bacterial populations and higher proportions of fluorescent pseudomonads compared to healthy roots. There was also a trend for more pseudomonad isolates from diseased roots being able to inhibit Ggt compared to isolates from healthy roots after three cycles of infection. Take-all disease was reduced with each successive generation of wheat, but it is difficult to attribute the decline in take-all to (1) the root lesion microorganisms becoming more suppressive, or to (2) reduced inoculum potential (which of course may be due to associated microorganisms on the root lesions). Other microorganisms transferred on root segments may also contribute to reduced disease, but only the pseudomonad population was sampled. Also the effect of bacterial populations from the roots of healthy plants on disease was not reported, so comparison of disease reduction by populations cycled from root lesions and healthy roots cannot be made.

On the basis of these previous studies the hypothesis was formed that the root lesions which develop after infection by Ggt select for a disease suppressive soil biota which later results in TAD, and the fluorescent pseudomonad community is a major contributor to disease suppression. However there is no conclusive evidence that root lesions preferentially select for disease antagonist, or that the fluorescent pseudomonads are responsible for TAD. If TAD can be induced in pot microcosms, and selection for disease suppressive pseudomonads during infection by Ggt is a major contributor to TAD, then TAD should be induced by cycling the pseudomonad community in successive generations of diseased wheat plants. To do this the pseudomonad community can selectively isolated from wheat roots using selective media containing chloramphenicol and ampicillin (Simon and Ridge, 1974) and used to inoculate the

next cycle of wheat in a new wheat-soil microcosm. Bacterial populations from different root locations, and their effect on take-all, can then be compared at the end of each cycle of wheat.

3.1.1. Aims

To determine if TAD can be induced in wheat-soil microcosms by cycling the pseudomonad population on successive generations of wheat roots.

To determine if diseased root lesions select for disease suppressive pseudomonads by comparing the effect on take-all disease by the total aerobic bacterial and pseudomonad populations cycled on successive generations of diseased root lesions with the pseudomonad populations cycled on; (1) non-lesioned sections of diseased roots; and on (2) healthy roots not exposed to disease.

3.2. Materials and Methods

Populations of bacteria were cycled on diseased or healthy wheat roots grown in a non sterilised Kapunda soil-sand mix in 300 ml pot microcosms. This was done for a total four cycles of wheat consisting of C0 (initial cycle) and C1 to C3 (inoculated with bacteria from previous cycle). Each cycle lasted six weeks. Pot microcosms are described in Chapter 2 (Section 2.2.5 to 2.2.10).

3.2.1. Media for selective isolation of bacterial populations

Bacterial populations from wheat roots and soil were partitioned into the total aerobic bacterial (TAB) population and pseudomonads by plating onto selective media containing antibiotics. TAB were isolated on TSA/10 supplemented with cycloheximide (75 µg/ml, TSAcx). Pseudomonads were selected on TSA/10 supplemented with cycloheximide (75 µg/ml), chloramphenicol (12.5 µg/ml) and ampicillin (50 µg/ml) (TSAcca, Simon and Ridge, 1974). Bacterial populations were also isolated on TB-T medium (TB-T isolates) used for selection of *Burkholderia cepacia* (Hagedorn *et al.*, 1987).

3.2.2. Ggt inoculum for *in vitro* inhibition assays

Pseudomonads were assessed for inhibition of Ggt on PDA/4 agar plates evenly sprinkled with Ggt-infected ground rice hulls (Ggt-PDA/4) to provide for rapid assessment of Ggt-inhibition. Ggt infected rice hulls were prepared by the method of Schisler *et al.* (1991). Rice hulls were ground in a kitchen blender and the 290 - 500 µm fraction separated by sieving. To 50 ml of ground rice hulls, 12 ml of sterile distilled water (SDW) was added in a 100 ml autoclavable nylon tube, incubated overnight at 25°C and drained of water. Rice hulls were autoclaved three times at 121°C for 1 h, 24 h apart. Rice hulls were partially dried under sterile conditions to remove excess moisture, and then inoculated with three plugs (4 mm) of actively growing Ggt-8 on

PDA/4. Rice hulls and Ggt were incubated for four weeks at 25°C. Rice hulls and Ggt were well mixed at weekly intervals to produce an even infection of the hulls. Ggt-infected rice hulls were air dried and stored at -20°C until use. For inhibition assays, Ggt-infected rice hulls were sprinkled onto PDA/4 using a sterile plastic container with 1 mm holes in the lid and shaken over the agar plate to give a coverage of about one Ggt-infected ground rice hull per cm^2.

3.2.3. Estimation of bacterial populations

Bacterial populations on wheat roots and soil were estimated by plating 10 fold dilutions of macerated root extracts onto the appropriate selection media (ie. populations of TAB estimated on TSAcx, pseudomonads on TSAcca and TB-T isolates on TB-T media) as described in Section 2.2.11. Populations were assessed at the end of each six week cycle of wheat.

3.2.4. Recovery and scale up of bacterial populations for inoculation in microcosms

For cycling of bacterial populations on wheat roots, populations (TAB, pseudomonads and TB-T isolates) were recovered from wheat roots on selective media, scaled up, and used to inoculate the following cycle of wheat. At the end of the preceding cycle of wheat, wheat roots were treated as for estimation of bacterial populations (Section 2.2.11), with diseased roots being sectioned into lesioned and non-lesioned root sections. Seminal roots between 2 to 7 cm from the crown were macerated in 3 ml PBS (as for estimation of bacterial populations), and 100 µl aliquots of the undiluted bacterial suspension plated onto the appropriate selective media. Bacteria were incubated for 48 h at 25°C to scale up populations for inoculation into the next cycle of wheat. Populations from replicate pots were pooled and kept separate from other populations. Bacteria were harvested in 3 ml SDW, diluted to an A_{550}=1 (ca. 10^9 cfu/ml) and 8 ml applied as a soil drench to the soil surface at planting as described in Section 2.2.9.

The entire microflora from the bulk soil was also cycled in successive generations of wheat. The bulk soil was separated from roots and adhering soil, air dried and sieved (<1 mm) to remove sand and remaining roots. This bulk soil was mixed 4:6 with fresh Kapunda soil to make up for lost weight. This mixture was then mixed 30:70 with sand and used as the soil:sand mix for this treatment. Ggt was added as previously described. This procedure was repeated at the end of each cycle to prepare the soil for the next cycle of wheat.

3.2.5. Native populations of bacteria in Kapunda red-brown earth soil: initial cycle of wheat (C0)

Populations of TAB and pseudomonads were estimated from healthy and diseased roots of six week old wheat seedlings grown in Kapunda soil-wheat microcosms as described in Section 2.2.11. Five replicate pots contained no added Ggt, five contained added Ggt. Diseased roots

were partitioned into the black necrotic lesions and non-lesioned (no visible sign of Ggt infection) sections of root. Bacterial populations from these roots (C0) provided the TAB and pseudomonad populations used to inoculate the following series of pot microcosms.

Bacterial populations (TAB, pseudomonads, and percentage of pseudomonad to the TAB population) from healthy roots, root lesions and non lesioned root section were analysed as a three way ANOVA, RCBD. There were twenty replications consisting of four separate experiments (C0 and control plants for C1 to C3) each with five replications. The interaction between root location and experiment was also assessed.

Populations of soil populations were assessed in two experiments (C2 and C3) from the same microcosms as described above. Populations of TAB, pseudomonads, and percentage of pseudomonads relative to the TAB population were each analysed as a two way (plus or minus Ggt infested soil) ANOVA, RCBD with 10 replications consisting of two experiments with 5 replicate pots.

3.2.6. Cycling of bacterial populations on successive generations of wheat roots and the effect on take-all disease and plant growth

In order to determine the effect of the different bacterial populations from different locations on control of take-all disease over successive cycles of wheat, bacterial populations isolated from wheat roots were inoculated into take-all diseased pot microcosms. To assess the evolution of these populations, the same population (TAB or pseudomonads) from the same location was reisolated on selective media as before and inoculated into the next cycle of wheat for three cycles (C1, C2 and C3). The experimental design of cycling populations on wheat is shown diagrammatically in Fig. 3.1.

Populations assessed for suppression of take-all disease and cycled onto subsequent cycles of wheat were; (1) the pseudomonad population from diseased root lesions; (2) the pseudomonad population from non lesioned sections of diseased roots; (3) the pseudomonad population from healthy roots not exposed to take-all disease; (4) the TAB population from root lesions; and (5) the entire microflora cycled in the bulk soil (Fig. 3.1). For each cycle, populations of pseudomonads from healthy roots were cycled on healthy roots and also inoculated into take-all diseased microcosms for assessment of take-all control. All other populations were cycled in diseased microcosms which were also used to asses control of take-all disease (Fig. 3.1). For each cycle of wheat control treatments were also included. These consisted of identical wheat-soil microcosms with and without added Ggt and no bacterial inoculum. There were five replicate pot microcosms for each treatment for a total of eight treatments for each cycle (C1 to C3).

The pseudomonad population from healthy roots produced a noticeable increase in shoot growth in the first cycle of wheat (C1). In an attempt to further partition this population, the

macerated root extracts from healthy roots inoculated with pseudomonads at the end of C1 were spread plated onto TB-T medium, as well as TSAcca, for inoculation onto healthy roots in C2 as an additional treatment.

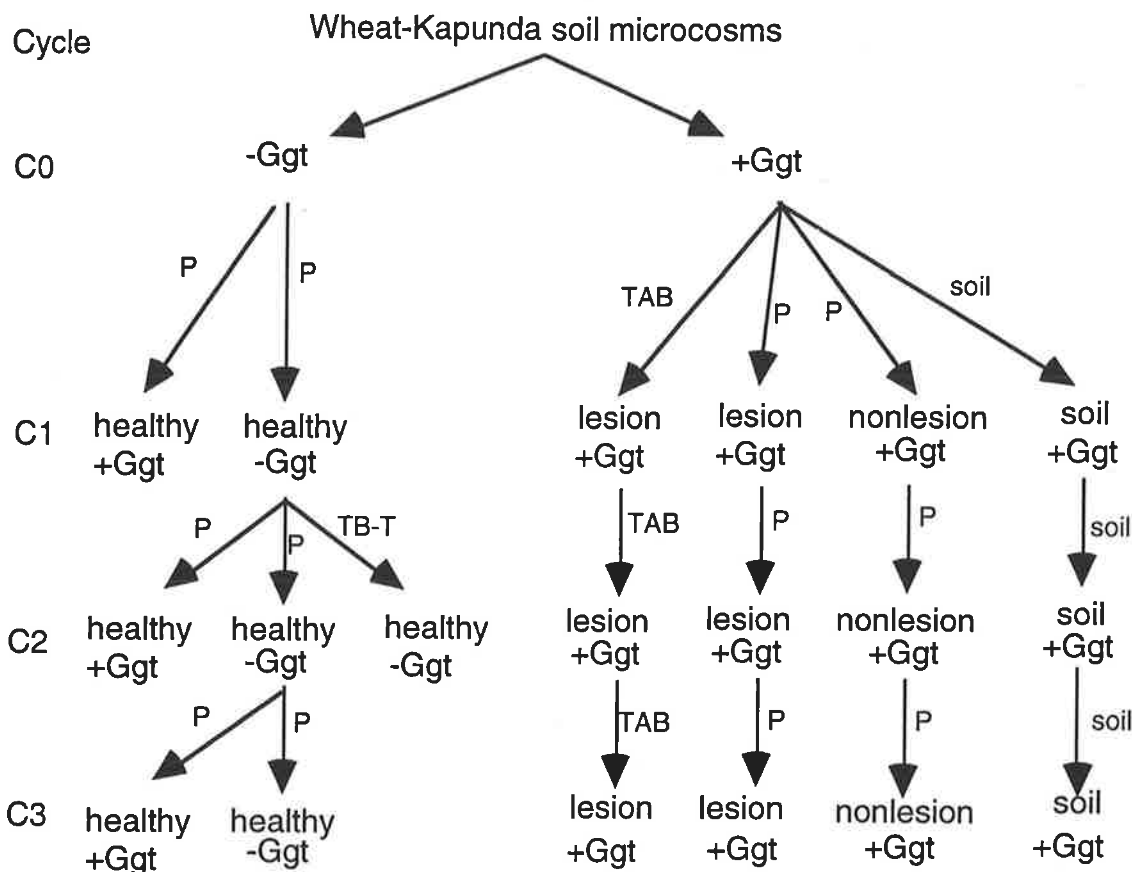


Fig. 3.1. Cycling of native bacterial populations in wheat-Kapunda soil microcosms. Populations of pseudomonads (P) were isolated from healthy roots grown without Ggt (healthy), and from root lesions (lesion) and non-lesioned (nonlesion) sections of diseased roots and cycled in wheat-soil microcosms with Ggt (+Ggt), or no added Ggt (-Ggt, pseudomonads from healthy roots). Total aerobic bacteria (TAB) were isolated from root lesions and cycled in diseased microcosms. The initial cycle (C0) contained no added bacteria. Cycles C1, C2 and C3 were inoculated with bacteria from the preceding cycle. The bulk soil (soil) from diseased microcosms was also cycled in successive cycles of wheat. Bacteria isolated on TB-T medium (TB-T) from healthy roots (C1) were inoculated onto healthy wheat seedlings in C2. Arrows indicate the progress of populations from C0 to C3.

Bacterial populations and plant growth parameters (Chapter 2.2.12) were assessed from each microcosm at the end of each six week growth cycle. Plant growth parameters were analysed as an 8 way (8 treatments) ANOVA, RCBD. Bacterial populations from roots were analysed as a 14 way (8 treatments, plus each of the 6 diseased treatments have root lesions and non lesioned root sections) ANOVA, RCBD. Diseased roots were also analysed as 2x6 split plot, RCBD fitting the two root locations (lesioned and non lesioned root sections) and the 6 diseased treatments.

3.2.7. *In vitro* inhibition and fluorescence by pseudomonads

Fluorescence on King's medium B (KB, King *et al.*, 1954) is used in many studies to select for the fluorescent pseudomonad group, a sub-set of the pseudomonad population. *In vitro* inhibition assays have also been commonly used to assess fluorescent pseudomonads for their ability to inhibit Ggt. It was desirable then to further characterise the pseudomonad populations in this study for their ability to inhibit Ggt and fluoresce on KB medium. *In vitro* inhibition assays were carried out on 1/4 strength Potato Dextrose Agar (PDA/4) and assays for fluorescence on KB medium.

To determine the proportion of pseudomonads which were inhibitory to Ggt *in vitro*, well separated single colonies which grew on TSAcca (on dilution plates used to estimate bacterial numbers) were transferred, using sterile toothpicks, onto PDA that had been sprinkled 48 h previously with Ggt-infected ground rice hulls (Ggt-PDA/4). After the second cycle of wheat (C2) 36 single colonies were transferred onto three PDA-Ggt plates (12 isolates per plate) from each of five replicate pots to give a total of 180 isolates from each root type (healthy, lesioned and non-lesioned sections of root) and from soil (healthy and Ggt-infested). After three cycles of wheat (C3), 24 single colonies from each of five replicate treatments were transferred onto Ggt-PDA/4, 6 colonies per plate, for a total of 120 isolates from each location (roots or soil). Each colony was also patched onto KB medium, 12 isolates per plate and fluorescence under ultra violet light (λ = 366nm) was assessed after 24 h growth at 25°C. The inhibition of isolates towards Ggt after four days was in one of three classes; (1) no inhibition towards Ggt, (ie. Ggt mycelium grew to the edge, or over the bacterial patch), (2) forming a distinct inhibition zone (DIZ) free of hyphae one to five mm from the bacterial patch, or (3) forming a five to 10 mm DIZ. The inhibition score (IS) was calculated as the number of isolates forming a DIZ greater than 5 mm plus 1/2 the number of isolates with a DIZ less than 5 mm to account for the reduced inhibition of these isolates.

The number of isolates inhibiting Ggt (IS), the number of isolates fluorescing on KB medium, and the number of isolates with each combination of plus or minus inhibition or fluorescence was analysed as and ANOVA, RCBD. These characteristics of pseudomonads from diseased roots were also analysed as a 2 x 6 split plot RCBD with two root locations (lesioned and non lesioned root sections) and the six diseased treatments, each with five replications.

The distribution of the two characteristics Ggt inhibition (I) and fluorescence (F) in 120 isolates from the native pseudomonad population from healthy, lesioned and non-lesioned root sections, and from healthy (-Ggt) and Ggt-infested soil from control plants in C3 were analysed for independence using a 2 x 2 contingency table and Pearson's Chi-squared test statistic (χ^2P, Equation 3.1) and compared to the Chi-squared distribution (χ^2, one degree of freedom).

Equation 3.1. $$X^2\mathrm{P} = \sum_{i=1}^{2}\sum_{j=1}^{2}\frac{(f_{ij} - u_{ij})^2}{u_{ij}}$$

Where f_{ij} is the observed number of isolates with the ijth character combination of inhibition and fluorescence, and u_{ij} is the expected number of isolates with ij character combination, and $i=1,2$ (+ or - inhibition) and $j = 1,2$ (+ or - fluorescence).

3.2.8. Take-all control by the total aerobic bacterial population from root lesions

The TAB population from root lesions produced an increase in shoot dry weight and decrease in percentage of root lesions in C2, after being inoculated with this population from C1. This was investigated further in a separate experiment comparing the mixed TAB population with the pseudomonad and non pseudomonad component of this population for take-all control.
The TAB from root lesions in C1, and used for the bacterial inoculum for this treatment in C2, was recovered from storage at -70°C by growing on TSA/10. This population was partitioned into chloramphenicol and ampicillin (cca) resistant (pseudomonads) and cca sensitive (non-pseudomonads). Isolates resistant to cca were selected by plating the TAB population onto TSAcca medium. Isolates sensitive to cca were isolated by replica plating diluted bacterial suspension on TSA/10 onto TSAcca, incubating for 48 h at 25°C, and selecting cca sensitive isolates from TSA/10 plates (total of 43 cca sensitive isolates). The cca sensitive isolates were subcultured onto TSA/10 for inoculation into pots. The cca sensitive and cca resistant isolates were compared to the original mixed population (mix 1), and the mixed TAB population cycled on root lesions for 3 cycles (C3) after being recovered from the pot microcosm which showed the greatest reduction in disease (mix 2). Bacterial populations were scaled up and inoculated into wheat-soil microcosms as before. Control microcosms with and without Ggt were also included, with eight replicate microcosms for each treatment.

3.2.9. Populations of bacteria in Avon soil

Bacterial populations were also assessed in Avon soil to determine if the effect of root lesions in supporting higher bacterial populations with an increase in the proportion of pseudomonads also occurred on wheat roots grown in another soil type. Two soils were collected from Avon, South Australia. These were: (1) suppressive soil (SS) and (2) non suppressive soil (NS) as described in Section 2.2.6. Pot microcosms contained 300 g of soil per pot at 15% w/w final water content (water potential of -5.0 kPa). Wheat plants were grown in each soil without added Ggt and added as Ggt infected rye-grass seeds, 0.23 g (120 propagules) per Kg soil. There were five replicate microcosms for each soil (SS or NS) with and without added Ggt. Populations of bacteria from wheat roots and soil were assessed after six weeks. The pseudomonad population from roots and soil were also assessed for Ggt inhibition and fluorescence on KB medium as

described previously, with 24 pseudomonad isolates from each location (roots or soil) from each microcosm assessed. Populations were analysed as a factorial design, RCBD, with two soil types (SS, NS) and the location of isolation.

3.3. Results

3.3.1. Native populations of bacteria in Kapunda red-brown earth soil

The native populations of TAB (selected on TSAcx) and pseudomonads (selected on TSAcca) were assessed from roots of wheat grown in Kapunda red-brown earth (no bacterial inoculum added) in four separate experiments each lasting 6 weeks. When take-all diseased roots were partitioned into lesioned and non-lesioned sections, lesioned sections consistently had higher populations of TAB and pseudomonads (Fig. 3.2). There was a higher proportion of pseudomonads on root lesions compared to non-lesioned sections of diseased roots (Fig. 3.2). The population size of TAB and pseudomonads on non-lesioned sections of diseased roots was the same as on healthy roots (Fig. 3.2).

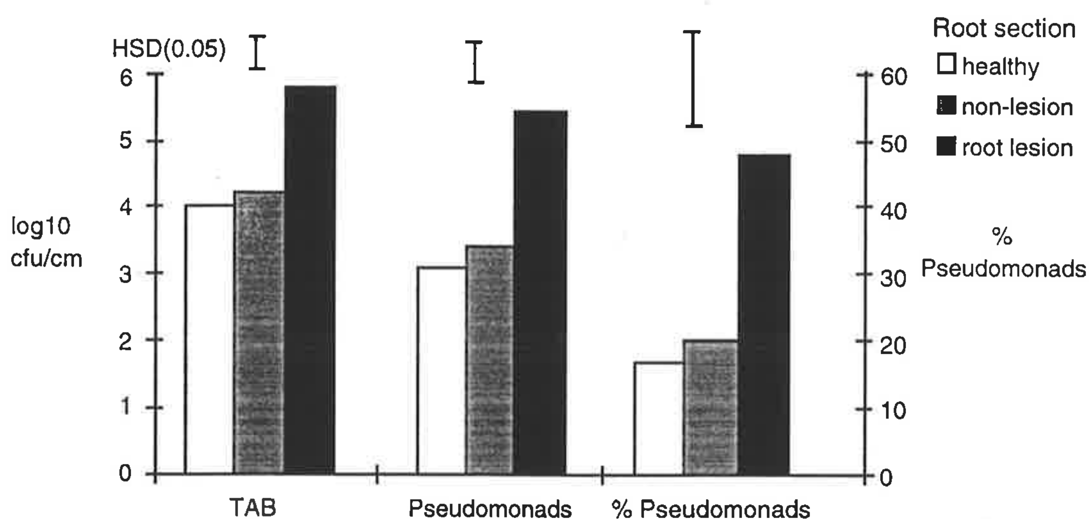


Fig. 3.2. Populations of total aerobic bacteria (TAB), pseudomonads and percentage of pseudomonads to TAB on wheat roots grown in Kapunda soil. Bacteria were isolated from healthy roots (no added Ggt), and from root lesions and non-lesioned sections of diseased roots (added Ggt) of six week old wheat seedlings. Seedlings were from microcosms uninoculated with bacteria in four separate experiments (C0 to C3), five replicate microcosms per experiment, n=20. Populations size is expressed as $\log_{10}$ cfu/cm of root.

The population size of TAB and pseudomonads isolated from bulk soil were not significantly (P=0.05) different between healthy soil (no added Ggt) and Ggt-infested soil. However, TAB and pseudomonad populations and the percentage of pseudomonads were all higher in the Ggt infested soil. (Table 3.1).

Table 3.1. Populations of bacteria in Kapunda bulk soil. Mean populations of total aerobic bacteria and pseudomonads, and the percentage of pseudomonads to total bacteria in the bulk soil after 6 weeks in wheat-Kapunda soil microcosms. Microcosms contained healthy soil (no added Ggt) or Ggt-infested soil. Populations expressesed per g dry weight of soil. Populations were not significantly different at $P = 0.05$, n = 10.

Soil	Pathogen	Total bacteria log_{10}(cfu/g)	Pseudomonads log_{10}(cfu/g)	%Pseudomonads
Healthy soil	0	6.1	5.3	19.6
Ggt-infested soil	+Ggt	6.3	5.6	27.5
Fprob		0.127	0.056	0.240

Inhibition of Ggt by bacterial isolates which grew on TSAcca (pseudomonads) was assessed at the end of the second and third cycles of wheat (36 and 24 isolates per treatment respectively). Bacteria were isolated from the roots of the control plants, and from the bulk soil after the third cycle. Fluorescence on KB medium by isolates from cycle 3 was also assessed. There was no significant (P=0.05) difference in the number of isolates which inhibited Ggt or fluoresced on KB between the sample locations. However, the number of isolates which inhibited Ggt and the number which fluoresced on KB was higher on root lesions and in soil, whether healthy or infested with Ggt, compared to healthy and non-lesioned roots (Table 3.2). When the distribution of the characteristics, Ggt-inhibition and fluorescence was assessed by a 2x2 contingency table, the results indicated that the two characteristics were not independently distributed (P<0.05) for populations from all locations, and for populations from non-lesioned root sections and healthy soil P<0.01 (Fig. 3.3). In populations from roots and Ggt-infested soil, the number of isolates which either did not fluoresce and did not inhibit Ggt (-F-I), or which fluoresced and inhibited Ggt (+F+I) was greater than expected if these characteristics were independently distributed in the population (Fig. 3.3). The number of isolates with only one of the two characteristics (+F-I and -F+I) was less than expected. This result was reversed in bacteria from healthy soil not infested with Ggt, where inhibition of Ggt was positively associated with non-fluorescence (-F+I), and fluorescence was associated with non-inhibition of Ggt (+F-I; Fig. 3.3).

3.3.2. Pseudomonads cycled on healthy roots in Kapunda soil: effect on take-all and shoot growth

Pseudomonad populations from healthy roots, non-lesioned and lesioned sections of take-all diseased roots were grown on TSAcca and were then used to inoculate successive cycles of wheat plants grown in pots. The TAB populations from root lesions were isolated on TSAcx and inoculated in a similar way. The bulk soil from Ggt infested pots was also cycled by mixing with fresh soil used to grow successive generations of wheat.

Table 3.2. Inhibition and fluorescence characteristics of pseudomonads in Kapunda soil. Pseudomonads were isolated from healthy roots, root lesions, non-lesioned roots, healthy soil and Ggt-infested soil from control microcosms (no bacterial inoculum) from the second (C2) and third (C3) cycle of wheat. A total of 180 isolates from C2 were assayed for inhibition of Ggt, and 120 pseudomonad isolates from C3 were each assayed for inhibition and fluorescence. Inhibition score (IS) is the number of isolates forming a greater than 5 mm inhibition zone plus 1/2 the number of isolates with a less than 5 mm inhibition zone. Fluorescence (F) is the number of isolates which fluoresce on KB medium under UV light. ND = not determined. Numbers in brackets indicate the percentage of bacteria which inhibited Ggt or fluoresced.

Source of isolates		C2	C3	
Number of isolates		180	120	120
Location	Pathogen	IS (%)	IS (%)	F (%)
Healthy roots	0	13 (7)	15 (12)	40 (33)
Non-lesion roots	+Ggt	6 (3)	11 (10)	42 (35)
Root lesion	+Ggt	28 (16)	23 (19)	81 (68)
Healthy soil	0	ND	33 (28)	71 (59)
Diseased soil	+Ggt	ND	32 (27)	83 (69)

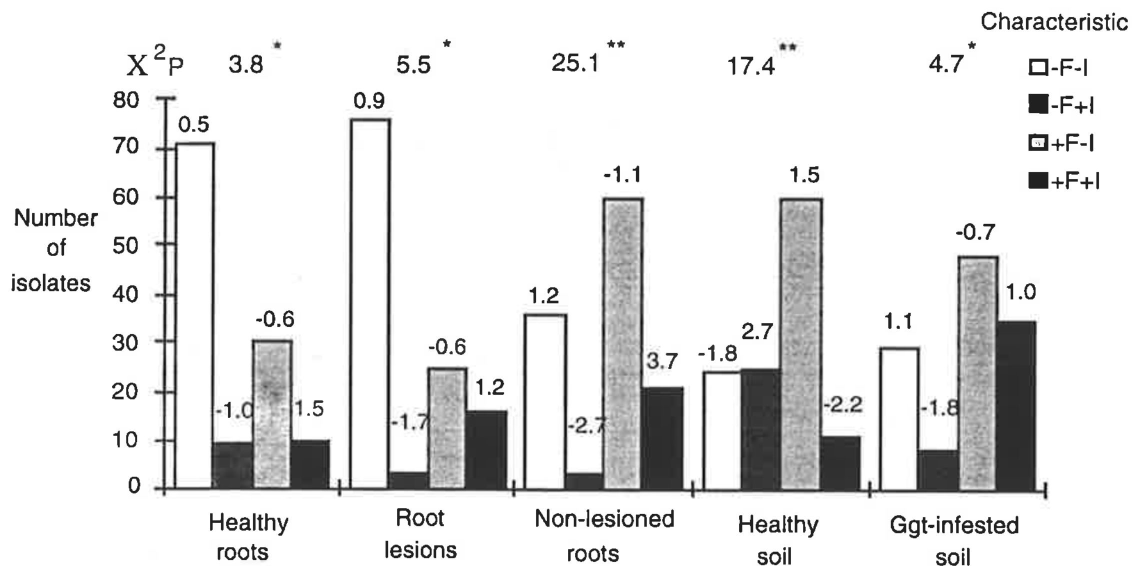


Fig. 3.3. Association between fluorescence and inhibition of Ggt among pseudomonads isolated from various locations on wheat roots and soil. Data are expressed as the number of pseudomonads (out of 120) with each of the characteristics: non-fluorescent non-inhibitory (-F-I), non-fluorescent Ggt inhibitors (-F+I), fluorescent non-inhibitory (+F-I) and fluorescent Ggt inhibitors (+F+I) isolated from each location. The Pearson Chi-squared test statistic (X^2P) for independence is given above the data for each location, $X^2_1(0.05)$ =3.8, $X^2_1(0.01)$ =6.6. Probability that characters are independently distributed is less than *P*=0.05* or *P*=0.01**. Scaled differences for each location are indicated above each character combination. Scaled differences = $(f - \mu)/\sqrt{\mu}$; where f is the observed and μ the expected number of isolates. Positive values indicate a positive association between inhibition and fluorescence. Negative values indicate a negative association between inhibition and fluorescence.

Pseudomonads cycled on healthy wheat roots grown in the absence of Ggt significantly (*P*=0.05) increased seedling shoot dry weight compared to that of control plants uninoculated with Ggt in the first (C1) and second (C2) cycles of wheat (Fig. 3.4). The increases were 20% in C1 and 28% in C2. This group of bacteria also significantly (*P*=0.05) increased shoot dry weight in Ggt-infected plants compared to uninoculated Ggt-infected plants in C1 but not in subsequent generations (Fig. 3.4). In each cycle, the amount of root disease on plants that had been inoculated with pseudomonads from healthy roots was reduced compared to control (uninoculated) plants, but this effect was not significant (*P*=0.05; Fig. 3.5). Inoculation of plants with bacteria isolated on Burkholderia selective medium (TB-T) from the first cycle (C1, pseudomonads from healthy roots) also significantly (*P*=0.05) increased shoot dry weight by 22% compared to healthy (-Ggt) plants without bacterial inoculation (Fig. 3.4).

3.3.3. Pseudomonads cycled on lesioned and non-lesioned sections of diseased roots in Kapunda soil: effect on take-all and shoot growth

Inoculating Ggt-infected plants with pseudomonads that had been cycled on either lesioned or non lesioned sections of diseased roots did not significantly change (*P*>0.05) shoot dry weight or disease severity compared to diseased control plants during cycles C1, C2 or C3. (Fig. 3.4 and 3.5). However, although not significantly different at *P*=0.05, plants inoculated with pseudomonads from root lesions in C2 has a higher disease severity compared to diseased control plants and plants inoculated with pseudomonads from non-lesioned root sections (Fig. 3.5).

3.3.4. TAB cycled on root lesions in Kapunda soil: effect on take-all and shoot growth

Seedlings treated with the total aerobic bacteria (TAB) that had been cycled on lesioned root sections of diseased plants showed increased shoot dry weight compared to diseased controls and plants inoculated with pseudomonads from root lesions in each cycle, but this was only significant in C2 (Fig. 3.4). Root disease severity was also significantly (*P*=0.05) reduced by TAB compared to pseudomonads from root lesions in C2, and plants inoculated with TAB had the lowest disease severity out of all treatments in C3 (Fig. 3.5).

The TAB from C1 (mix 1), and the TAB population from one pot of this treatment from C3 (mix 2) also increased plant growth and decreased root lesion severity compared to control plants after the bacteria were recovered from storage at -70°C (Fig. 3.6). When the TAB from root lesions from C1 were partitioned into cca sensitive (cca^{-}, non-pseudomonads) and cca resistant (cca^{+}, pseudomonads) bacteria, and inoculated into a standard pot test, both groups increased plant growth and decreased root disease severity compared to control plants (Fig. 3.6). Results from the partitioned populations (cca^{+} and cca^{-}) were similar to the two mixed populations (Fig. 3.6).

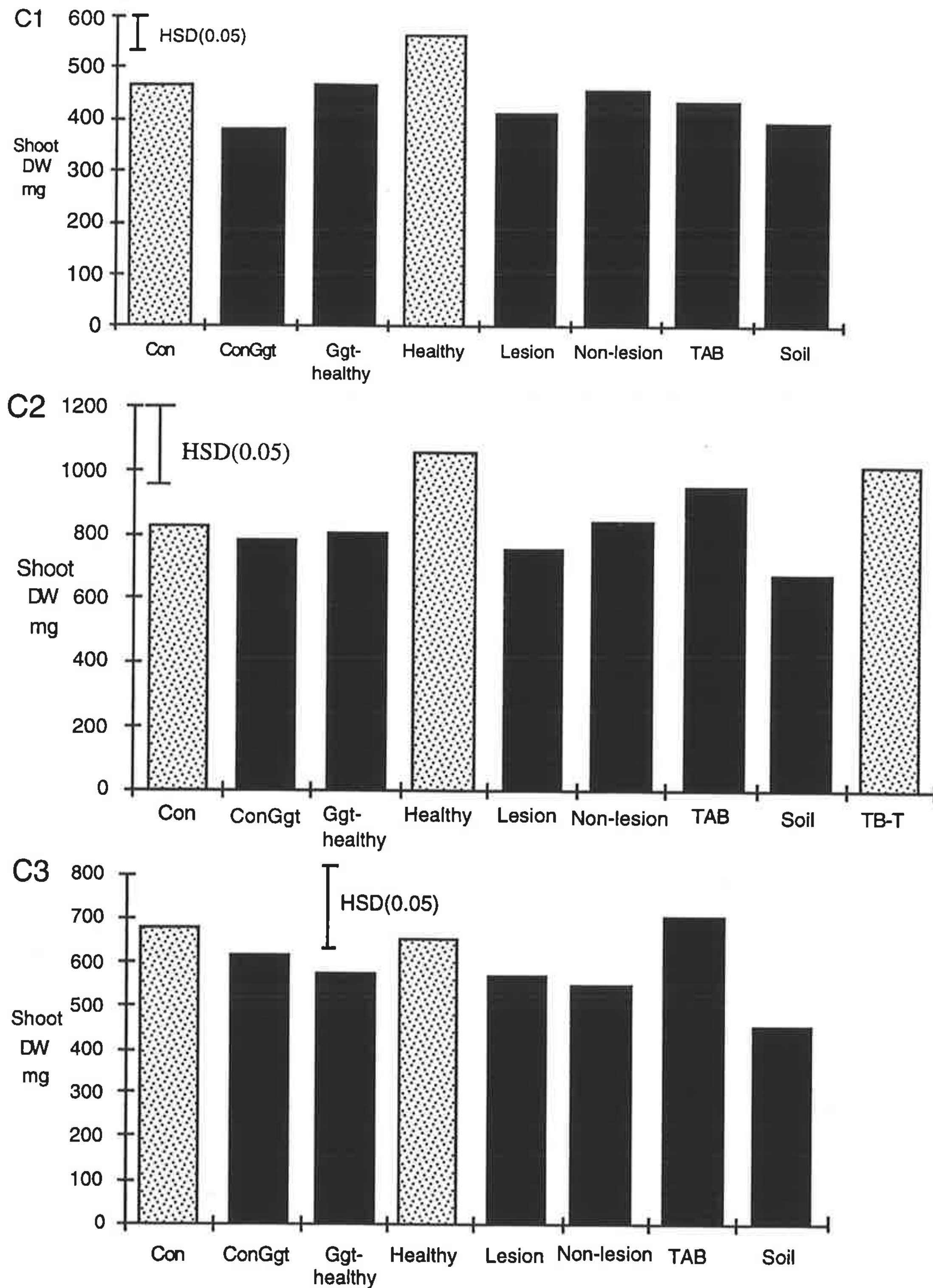


Fig. 3.4. Shoot dry weight (DW, mg) of wheat inoculated with bacterial populations after one (C1), two (C2) and three (C3) cycles of wheat in Kapunda soil. Treatments are: control, no added bacteria or Ggt (Con); control with added Ggt but no added bacteria (ConGgt); and plants inoculated with pseudomonads cycled on healthy roots and inoculated with Ggt (Ggt-healthy); pseudomonads cycled on healthy roots with no added Ggt (Healthy); pseudomonads cycled on root lesions (Lesion); pseudomonads cycled on non-lesioned roots (Non-lesion); TAB cycled on lesions (TAB); cycled Ggt-infested bulk soil (Soil); and TB-T isolates from healthy roots. Black columns indicate microcosms inoculated with Ggt. n=5.

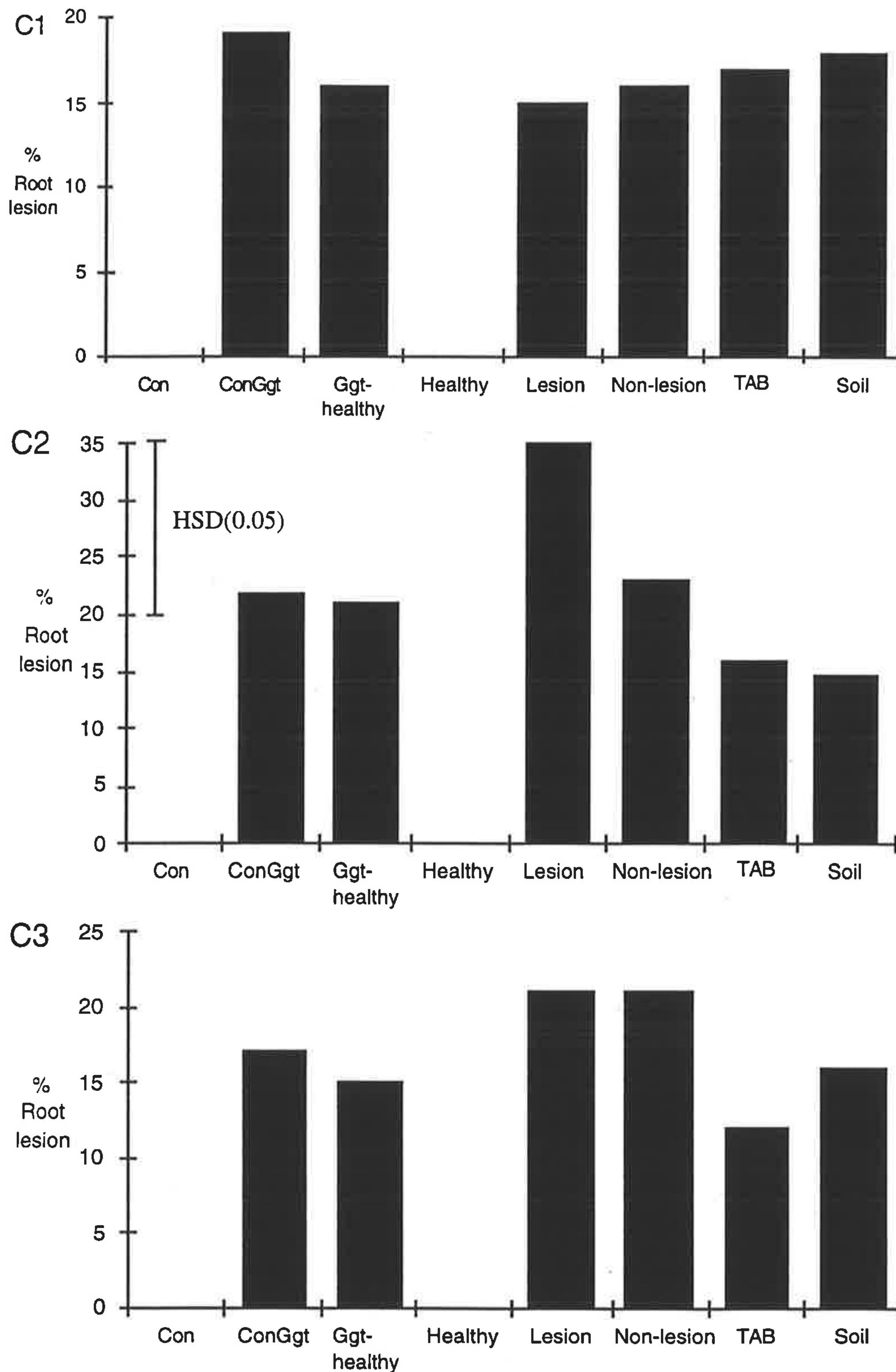


Fig. 3.5. Percentage of root lesions on wheat roots inoculated with bacterial populations after one (C1), two (C2) and three (C3) cycles of wheat in Kapunda soil. Treatments are: control, no added bacteria or Ggt (Con); control with added Ggt but no added bacteria (ConGgt); and plants inoculated with pseudomonads cycled on healthy roots and inoculated with Ggt (Ggt-healthy); pseudomonads cycled on healthy roots with no added Ggt (Healthy); pseudomonads cycled on root lesions (Lesion); pseudomonads cycled on non-lesioned roots (Non-lesion); TAB cycled on lesions (TAB); cycled Ggt-infested bulk soil (Soil). The was no significant difference in percent root lesions between treatments in C1 (P=0.95) and C3 (P=0.10). n=5.

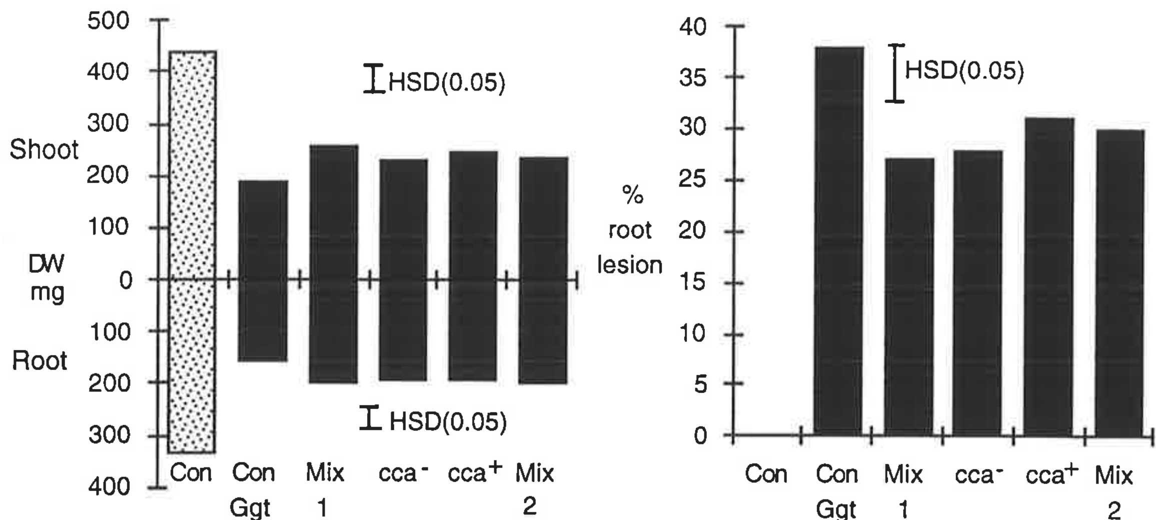


Fig. 3.6. Shoot and root dry weight (A) and percent root lesion (B) of wheat after inoculation with TAB cycled on root lesions, and the pseudomonad and non-pseudomonad sub-populations. Treatments are: control plants with no added Ggt (Con) or with Ggt (ConGgt) and no added bacteria; diseased wheat (added Ggt) inoculated with TAB from root lesions at the end of C1 (Mix 1) and C3 (Mix 2), and with the cca sensitive (cca$^-$, non-pseudomonads) and cca resistant (cca$^+$, pseudomonads) components of the TAB population from C1. HSD is shown for diseased treatments, n=8.

3.3.5. Kapunda soil cycled with successive generations of diseased wheat: effect of take-all and shoot growth

When Kapunda soil infested with Ggt was cycled during successive generations of wheat plants, shoot growth was slightly reduced compared to diseased control plants in the first two cycles (C1 and C2) and was significantly (P=0.05) reduced in the third cycle C3, Fig. 3.4). Root lesion severity was not significantly (P=0.05) different from controls, but was generally lower in each cycle (Fig. 3.5). Percent root lesions on roots grown in cycled soil were significantly (P=0.05) reduced compared to roots inoculated with pseudomonads from lesioned roots in the second cycle, C2 (Fig. 3.5).

3.3.6. Bacterial populations after cycling on wheat roots

Bacterial populations were consistently higher on root lesions compared to non-lesioned sections of root for TAB and pseudomonads after each cycle (Figs. 3.7, 3.8, 3.9). Similarly, populations isolated on TB-T media were also increased on root lesions (Fig. 3.10). When the percentage of pseudomonads on diseased roots was analysed as a 2 (lesioned, non-lesioned roots) x 6 (diseased treatments) split plot design, the percentage of pseudomonads was significantly higher on lesioned sections of roots for all three cycles of wheat (Table 3.3). In contrast, the proportion of TB-T isolates compared to the TAB and pseudomonad populations was reduced on root lesions compared to non-lesioned root sections (Table 3.4).

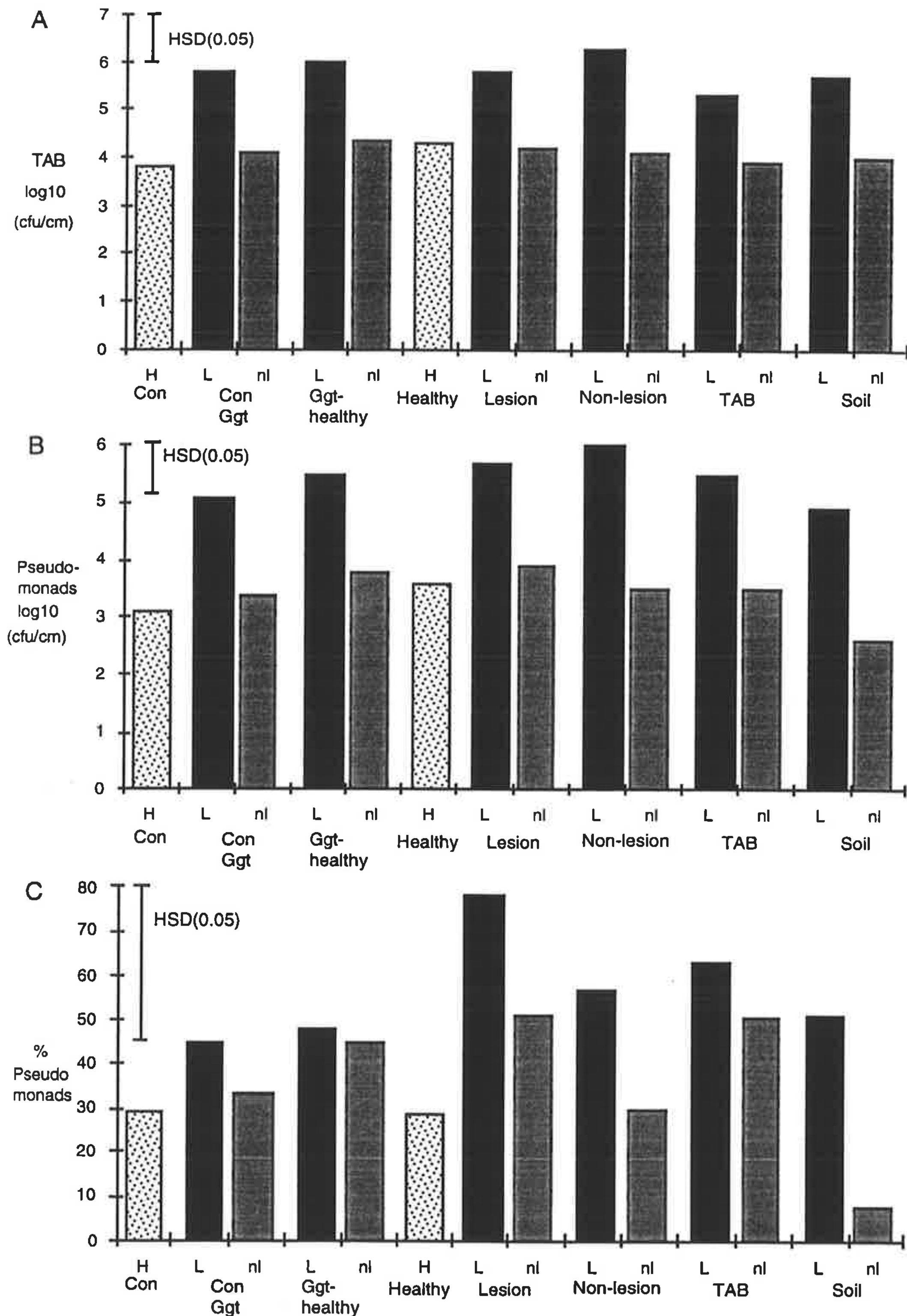


Fig. 3.7. Populations of TAB (A), pseudomonads (B) and percentage of pseudomonads (C) on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after one cycle of wheat (C1). Treatments are: control, no added bacteria or Ggt (Con); control with added Ggt but no added bacteria (ConGgt); and plants inoculated with pseudomonads cycled on healthy roots and inoculated with Ggt (Ggt-healthy); pseudomonads cycled on healthy roots with no added Ggt (Healthy); pseudomonads cycled on root lesions (Lesion); pseudomonads cycled on non-lesioned roots (Non-lesion); TAB cycled on lesions (TAB) and cycled Ggt-infested bulk soil (soil). n=5.

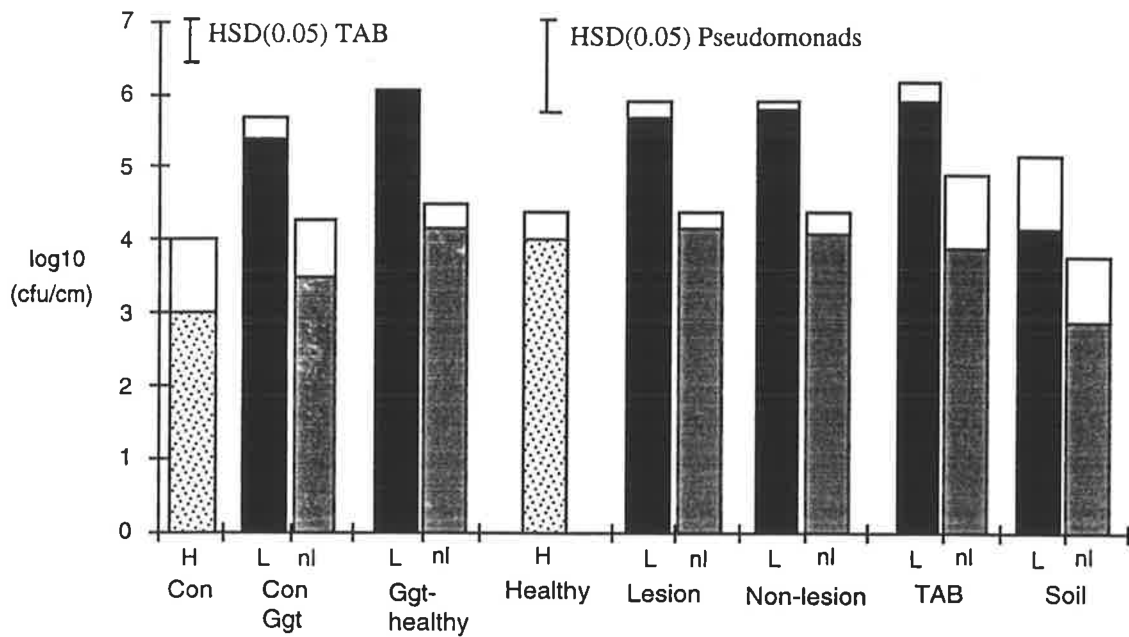


Fig. 3.8. Population size of TAB and pseudomonads on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after two cycles of wheat (C2). Treatments are as before (Fig. 3.7). TAB population shown in clear columns, pseudomonads in shaded columns. n=5.

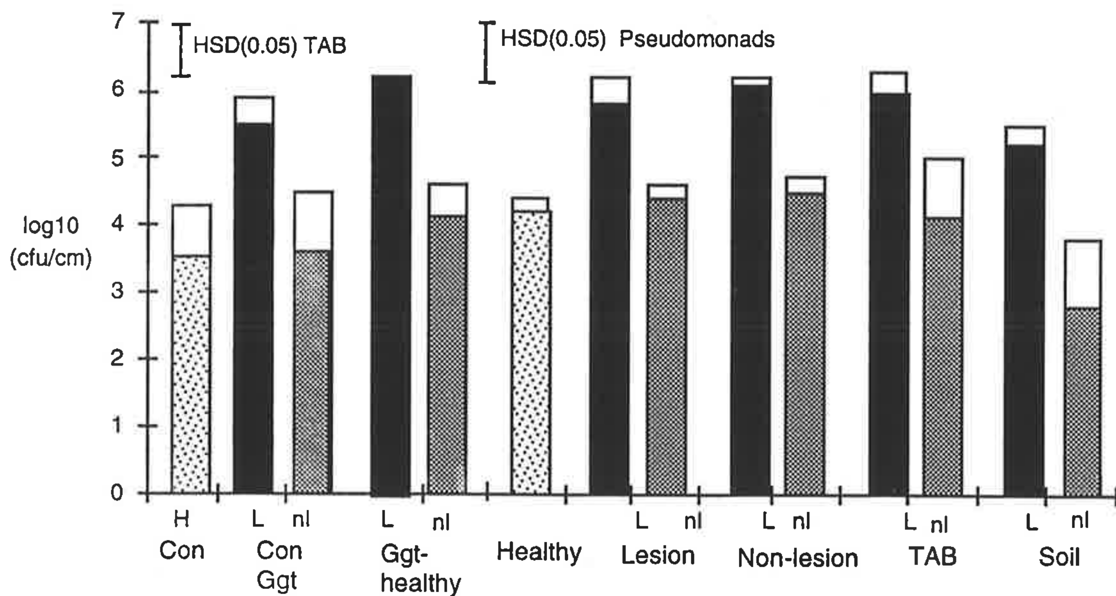


Fig. 3.9. Population size of TAB and pseudomonads on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after three cycles of wheat (C3). Treatments are as before (Fig. 3.7). TAB population shown in clear columns, pseudomonads in shaded columns. n=5.

The differences in bacterial populations (TAB and pseudomonads) on roots inoculated with different bacterial populations, or soil, were only significantly (*P*=0.05) different between the highest and lowest values for all cycles of wheat (Figs. 3.7, 3.8, 3.9). Populations on roots inoculated with bacteria were generally higher than on roots of uninoculated control plants. Diseased plants inoculated with pseudomonads from healthy roots had the highest proportion

of pseudomonads of all treatments in C2 and C3 (Figs. 3.8, 3.9). TAB and pseudomonad populations, and percentage of pseudomonads were lowest on roots grown in cycled soil for all three wheat cycles (Figs. 3.7, 3.8, 3.9).

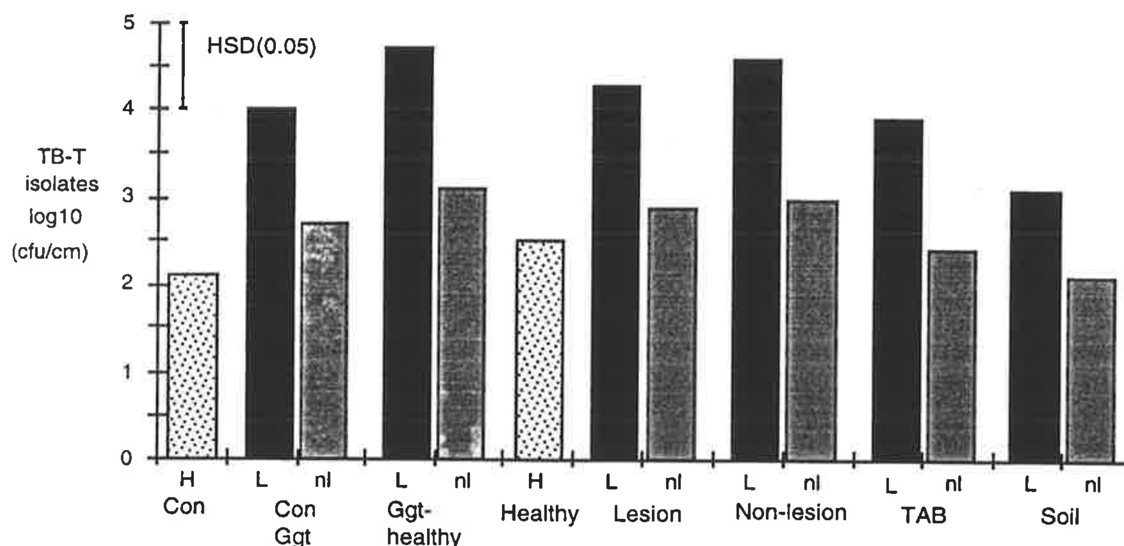


Fig. 3.10. Population size of TB-T isolates on healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after one cycles of wheat (C1). Treatments are as before (Fig. 3.7). n=5.

Table 3.3. Percentage of pseudomonads to TAB in C1, C2, and C3 on root lesions and non-lesioned sections of diseased roots. Percentage of pseudomonads analysed as a 2 (lesion, non-lesion) x 6 (treatment) split plot RCBD. Treatments consisted of the six diseased (added Ggt) treatments in C1, C2, and C3. Main effect of root lesions and non-lesioned root sections shown. n=30.

	Percent Pseudomonads		
Cycle of wheat	C1	C2	C3
Non-lesioned roots	36.2	37.1	34.1
Root lesions	56.9	67.9	67.5
Fprob	0.004	<0.001	<0.001

Table 3.4. Percentage of TB-T isolates compared to TAB and pseudomonads on root lesions and non-lesioned sections of diseased roots after one cycle (C1) on wheat roots. Percentage of TB-T isolates analysed as a 2 (lesion, non-lesion) x 6 (treatment) split plot RCBD. Treatments consisted of the six diseased (added Ggt) treatments in C1. Main effect of root lesions and non-lesioned root sections shown. n=30.

	%TB-T/TAB	%TB-T/Pseudomonads
Non-lesioned roots	9.0	27.8
Root lesions	4.4	11.2
Fprob	0.042	0.001

3.3.7. Inhibition and fluorescence in pseudomonads after the third cycle of wheat in Kapunda soil

Inhibition of Ggt and fluorescence on KB medium was assessed in 24 pseudomonads isolated from each root section per pot after three cycles of wheat (C3). When populations on root lesions and non-lesioned sections of roots from all diseased treatments were analysed as a 2 (lesioned, non-lesioned roots) x 6 (diseased treatments) split plot design, the number of isolates which fluoresced on KB was significantly ($P<0.001$) increased on root lesions compared to non-lesioned sections of root (Table 3.5). There was no significant ($P=0.05$) difference in Ggt inhibition between pseudomonads isolated from lesioned and non-lesioned root sections. When both character combinations (inhibition and fluorescence) were considered together, the number of pseudomonads which fluoresced and did not inhibit Ggt (+F-I) was increased on lesions, and non-fluorescent isolates whether inhibitory to Ggt or not (-F+I and -F-I) were increased on non-lesioned sections of root (Table 3.5). There was no significant difference in number of fluorescent Ggt-inhibitors (+F+I) between the two root locations. The mean inhibition score and number of fluorescent isolates for all treatments and root locations are shown in Figs. 3.11 and 3.12 respectively. Due to the high variability between replicate samples, usually only the values for the highest and lowest number of isolates were significantly different at $P=0.05$. Pseudomonads cycled on healthy roots produced a relatively high proportion of Ggt inhibitors (Fig. 3.11), and low proportion of fluorescent isolates (Fig. 3.12) when inoculated onto both healthy and diseased roots. This was the opposite for TAB cycled on lesions where the inhibition score for pseudomonads isolated from this treatment was relatively low (Fig. 3.11) and the proportion of fluorescent isolates high (Fig. 3.12). Pseudomonads from cycled soil had a relatively high Ggt-inhibition score (Fig. 3.11). Pseudomonads cycled on root lesions and non lesioned roots had a relatively low inhibition score (Fig. 3.11), but similar numbers of fluorescent isolates to pseudomonads from control plants (Fig. 3.12). Pseudomonads isolated from roots inoculated with the TAB from root lesions had the lowest inhibition score and the highest number of fluorescent isolates, as did pseudomonads from cycled soil (Figs. 3.11 and 3.12).

When the number of pseudomonad isolates with each character combination of inhibition and fluorescence from all treatments (C3) was analysed, the number of fluorescent-Ggt inhibitors (+F+I) out of 24 was not significantly different between treatments. The number of isolates with all other character combinations (-F-I; -F+I; +F-I) were significantly different ($P<0.001$) between at least two pseudomonad populations (from healthy, lesioned and non-lesioned roots), but usually only between the highest and lowest values (Fig. 3.13). Pseudomonads and TAB cycled on diseased root had a relatively high number of fluorescent-non inhibitors (+F-I) in the pseudomonad population (Fig. 3.13). Pseudomonads cycled on healthy roots, and from cycled soil, had a relatively high number of non-fluorescent Ggt-inhibitors (-F+I; Fig. 3.13). Pseudomonads from the TAB and cycled soil populations had low numbers of non-fluorescent-non-inhibitors (-F-I; Fig. 3.13).

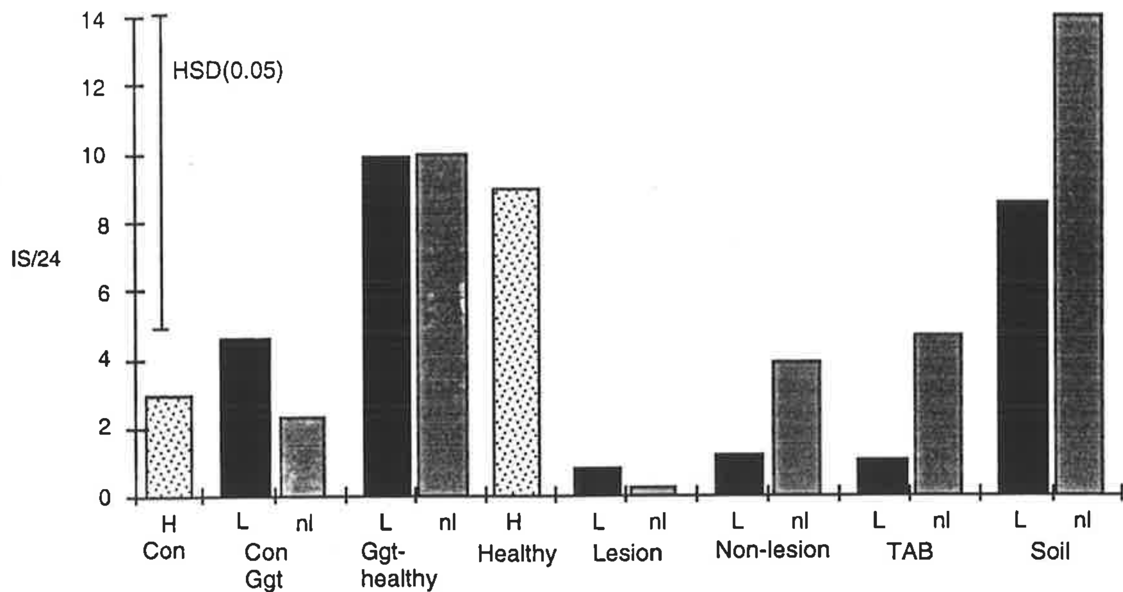


Fig. 3.11. Inhibition score of pseudomonad isolates from healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after three cycles of wheat (C3). Mean inhibition score (IS) out of 24 isolates shown. Treatments are as before (Fig. 3.7). n=5.

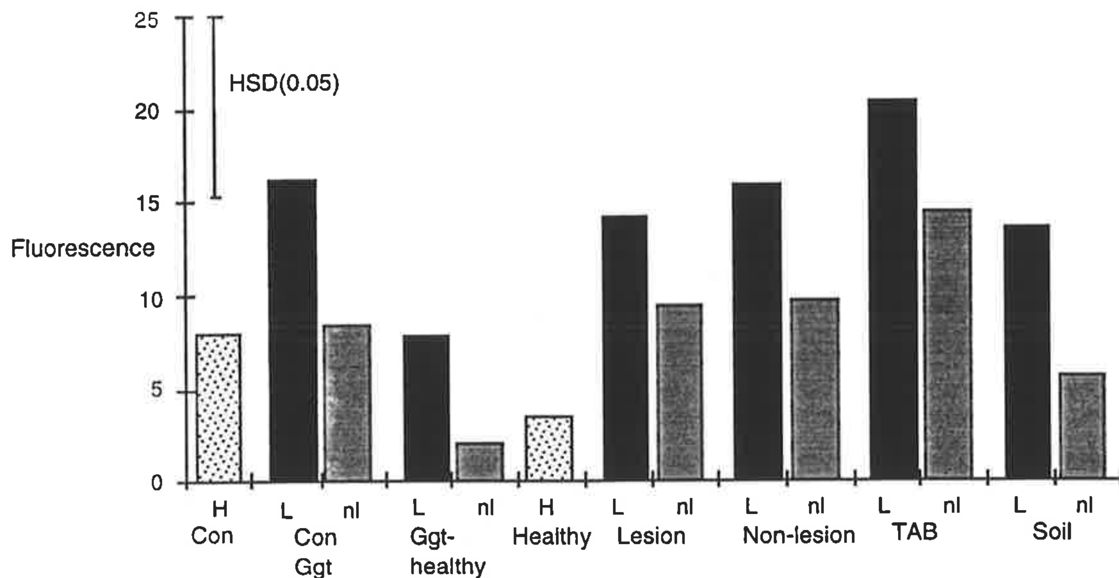


Fig. 3.12. Number of pseudomonad isolates which fluoresce on KB medium after three cycles of wheat. Pseudomonads were isolated from healthy roots (H), and root lesions (L) and non-lesioned (nl) root sections after three cycles of wheat (C3). Mean number of fluorescent isolates out of 24 isolates shown. Treatments are as before (Fig. 3.7). n=5.

Table 3.5. Ggt-inhibition and fluorescence by pseudomonad isolates on root lesions and non-lesioned roots after three cycles on wheat roots in Kapunda soil. The number of isolates out of 24 was analysed as a 2 (lesion, non-lesion) x 6 (treatment) split plot RCBD. Treatments consisted of the six diseased (added Ggt) treatments in C3. Main effect of root lesions and non-lesioned root sections is shown (mean number of isolates with each characteristic). Inhibition and fluorescence characteristics are; Inhibition score against Ggt (IS); fluorescence on KB medium; non-fluorescent non-inhibitory (-F-I); non-fluorescent Ggt-inhibition (-F+I); fluorescent non-inhibitory (+F-I); fluorescent Ggt-inhibition (+F+I). n=30.

	Mean number of isolates out of 24		
Characteristic	Root lesions	Non-lesioned roots	Fprob
IS	4.4	5.9	0.113
Fluorescence	14.6	8.3	<0.001
-F-I	6.4	10.5	<0.001
-F+I	3.1	4.9	0.007
+F-I	12.8	6.7	<0.001
+F+I	1.8	1.5	0.666

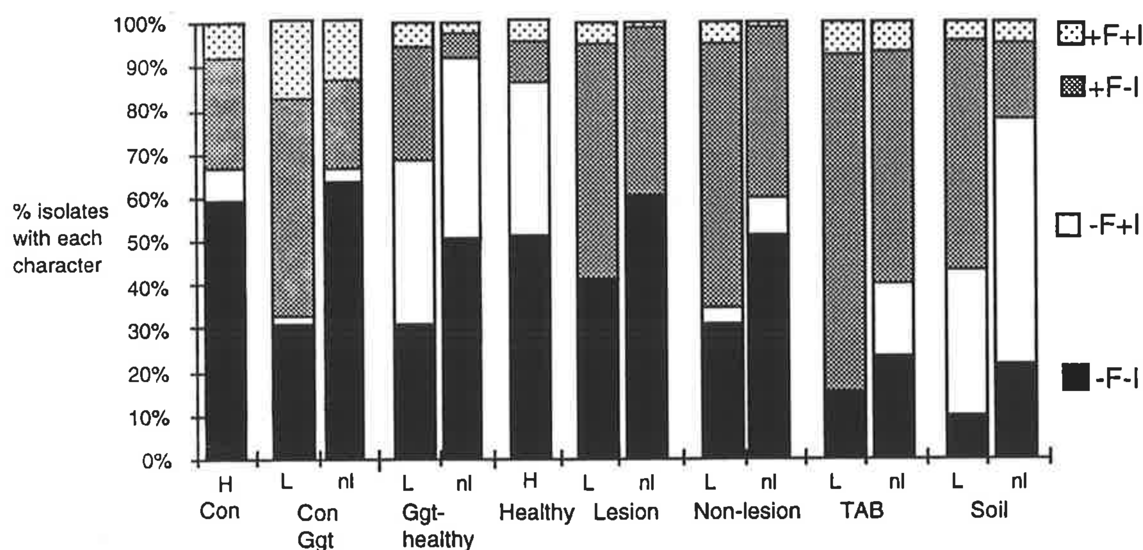


Fig. 3.13. Percentage of pseudomonad isolates with each of the four character combinations of fluorescence (F) and Ggt-inhibition (I) after 3 cycles of wheat (C3). Pseudomonad were isolated from healthy roots (H), root lesions (L) and non-lesioned (nl) root sections. The four combinations of fluorescence and inhibition characteristics are: non-fluorescent non-inhibitory (-F-I); non-fluorescent Ggt-inhibition (-F+I); fluorescent non-inhibitory (+F-I); fluorescent Ggt-inhibition (+F+I). Treatments are as before (Fig. 3.7).

3.3.8. Bacterial populations on wheat roots grown in Avon soil

TAB and pseudomonad populations were assessed from wheat roots grown with and without take-all disease in two soils from Avon. These two soils were described as suppressive (SS) and less suppressive, or non-suppressive (NS) to take-all disease and wheat plants were grown in each soil type with and without added Ggt. There was no significant (*P*=0.05) difference in seedling growth, root disease or bacterial populations (TAB and pseudomonads) between the two soils. When bacterial populations from diseased roots (lesioned and non-lesioned sections) grown in the two soils were analysed as a 2x2 factorial there was a significant (*P*=0.05) increase in the number of pseudomonads and percentage of pseudomonads on root lesions compared to non-lesioned root sections (Table 3.6). TAB populations were also slightly higher on root lesions but this was not significant at *P*=0.05. The population size of TAB and pseudomonads on healthy roots was similar to non-lesioned sections of root. There was no difference in bacterial numbers between healthy and Ggt-infested soil.

Twenty four pseudomonad isolates from wheat roots (healthy, lesioned and non-lesioned) and from the bulk soil (healthy and Ggt-infested) were assessed for fluorescence on KB medium and Ggt-inhibition. The number of isolates which: inhibited Ggt; fluoresced on KB medium; and the number of isolates with each of the four character combinations (fluorescent-Ggt-inhibitory; non-fluorescent non-inhibitory; non-fluorescent Ggt-inhibitory; fluorescent non-inhibitory) were each analysed separately as a 2x5 factorial (2 soils; 5 locations). There was no difference in the number of isolates with any of the fluorescence or inhibition characteristics between the SS and NS soil. The inhibition and fluorescence characteristics of the pseudomonads from wheat roots (healthy, lesioned and non-lesioned) were similar (Table 3.7). The biggest difference in the number of isolates with each characteristic occurred in pseudomonads isolated from the bulk soil. There were fewer Ggt-inhibitors and fewer fluorescent isolates in Ggt-infested soil compared to isolates from wheat roots. This is also reflected in the low number of fluorescent Ggt-inhibitors (+F+I) in Ggt-infested soil (Table 3.7). The number of isolates with these characteristics was also reduced in healthy soil compared to root populations, but to a lesser extent then in Ggt-infested soil (Table 3.7). The number of non-fluorescent non-inhibitory (-F-I) pseudomonad isolates was increased in Ggt-infested soil, and to a lesser extent in healthy soil, compared to the number on wheat roots (Table 3.7). The number of non-fluorescent Ggt-inhibitors (-F+I) was highest in pseudomonads from healthy soil, but this was not significant at *P*=0.05 (Table 3.7).

Table 3.6. Populations of TAB, pseudomonads and percentage of pseudomonads on root lesions and non-lesioned roots grown in Avon soil. Populations were analysed as a 2 (soil type NS, SS) x2 (root lesions and non-lesioned roots) factorial RCBD. There was no difference between SS and NS soils. Mean populations for TAB, pseudomonads and %pseudomonads on diseased roots shown, n=10.

Root location	TAB $\log_{10}$(cfu/cm)	Pseudomonads $\log_{10}$(cfu/cm)	%Pseudomonads
Non-lesioned roots	3.8	3.3	39
Root lesions	4.0	3.8	63
Fprob	0.144	0.004	0.033

Table 3.7. Ggt-inhibition and fluorescence in pseudomonads isolated from roots and bulk soil from wheat-Avon soil microcosms. The number of isolates out of 24 with each Ggt-inhibition and fluorescence characteristics was analysed as a 2 (soil type; SS, NS) x 6 (location of isolation) factorial RCBD. There was no difference between soil types. Pseudomonads were isolated from healthy roots, root lesions, non-lesioned diseased roots, healthy soil and Ggt-infested soil. Inhibition and fluorescence characteristics are; Inhibition score against Ggt (IS); fluorescence on KB medium; non-fluorescent non-inhibitory (-F-I); non-fluorescent Ggt-inhibition (-F+I); fluorescent non-inhibitory (+F-I); fluorescent Ggt-inhibition (+F+I). The mean number of pseudomonads out of 24 with each of the characteristic is shown. There was no difference between soil types. n=10.

	Mean number of isolates out of 24 with each characteristic					
Location	Inhibition	Fluorescence	-F-I	+F-I	-F+I	+F+I
healthy roots	14.1	15.3	2.6	3.3	2.1	12.0
Non-lesioned roots	11.8	15.1	3.6	4.6	1.3	10.5
Root lesions	13.1	16.1	1.9	5.0	2.0	11.1
Healthy soil	11.6	10.4	5.6	2.7	3.9	8.7
Ggt-infested soil	7.6	9.2	7.4	4.8	3.3	4.3
Fprob	0.026	<0.001	0.011	0.512	0.057	0.001
HSD(0.05)	5.7	5.2	4.7	ns	ns	5.3

3.4. Discussion

These results clearly show that bacterial populations on the black necrotic lesions produced on wheat roots after infection by Ggt are both quantitatively and qualitatively altered compared to root sections not infected with Ggt. When wheat was grown in a Kapunda soil:sand mix, populations of TAB were consistently increased 40 fold, and pseudomonads 100 fold higher, on root lesions compared to non-lesioned sections of diseased roots. This occurred in both the native bacterial populations and after cycling bacterial populations on wheat roots (Figs. 3.7, 3.8 and 3.9). The proportion of pseudomonads present in the TAB population was also increased on root lesions (Fig. 3.2 and Table 3.3) and a greater proportion of these pseudomonads

fluoresced on KB medium (Fig. 3.12; Table 3.5). The population size of TAB and pseudomonads on non-lesioned sections of diseased roots was similar to populations on healthy roots not exposed to disease (Fig. 3.2), and the size of populations in soil not infested with Ggt (healthy soil) was similar to populations in Ggt-infested soil (Table 3.1). Pseudomonad populations were also preferentially increased on disease lesions on roots grown in Avon soil (Table 3.6), but to a lesser extent than in Kapunda soil.

These data support the hypothesis that root lesions are a unique niche which preferentially selects for pseudomonads (Fig. 3.2). A greater proportion of these were also fluorescent on KB medium (Fig. 3.12). Root lesions also selected against isolates able to grow on TB-T medium (Table 3.4). Previous studies comparing bacterial populations on take-all diseased and non-diseased roots reported a less than ten fold increase in total aerobic bacteria and pseudomonads, and a relatively small increase in the relative number of fluorescent pseudomonads on diseased roots (Weller, 1983; Sarniguet *et al.,* 1992a). This is much less than the difference found in this study between lesioned and non-lesioned sections of the same roots indicating that differences found in previous studies between populations of bacteria on non-diseased and Ggt-infected roots are likely to be due to the influence of the disease lesions produced on the roots after infection by the fungal pathogen. These lesions will support high populations even though they may form only a small proportion of the total root system. Other studies have demonstrated that the community structure of the fluorescent pseudomonad group varies according to host plant, location in, on or near roots, and position along roots (Miller *et al.*, 1990; Van Peer *et al.*, 1990; Liljeroth *et al.*, 1991; Glandorf *et al.*, 1993; Lemanceau *et al.*, 1995). Root lesions should also be considered as a unique site which alters the bacterial community on plant roots.

Although in this study root lesions selected for pseudomonads, it is less clear whether disease antagonists are selected for within the pseudomonad group on root lesions. *In vitro* inhibition of Ggt was variable between replicate treatments but generally higher on lesions of roots grown in soil uninoculated with bacteria (Table 3.2). The high variability between replicate treatments when fluorescence and inhibition was assessed may be due to the natural soil heterogeneity and the small sample size where only the predominant isolates were selected from each pot microcosm after plating on TSAcca. Cycling of the pseudomonad population on root lesions, however, resulted in the lowest proportion of Ggt-inhibitors after 3 cycles (Fig. 3.11), and resulted in the highest level of disease for all treatments after two cycles of wheat (Fig. 3.5). In contrast, the pseudomonad population cycled on healthy roots had a noticeable plant growth promoting effect in C1, even in the prescence of disease (Fig. 3.4). This plant growth promoting effect was repeated in C2 by pseudomonads cycled on healthy roots, and by another sub-set of the TAB population isolated on TB-T medium. The population size of TB-T isolates on healthy roots was similar to non lesioned sections of roots (Fig. 3.10), and the proportion of TB-T isolates was decreased on root lesions compared to non-lesioned sections of diseased roots (Table

3.4). Pseudomonads cycled on healthy roots also had a much greater number of non-fluorescent Ggt-inhibitors after three cycles of wheat (Fig. 3.13) and diseased plants inoculated with pseudomonads from healthy roots had a lower percentage of root lesions. These results suggest that healthy roots not exposed to disease provide a greater selection pressure for isolates which promote plant growth in the presence and absence of Ggt compared to diseased root lesions.

Many studies have targeted the fluorescent pseudomonad group as the functional group of interest in TAD (Weller, 1983; Charigkapakorn and Sivasithamparam, 1987; Weller, 1988; Sarniguet *et al.*, 1992a and b). Pseudomonads were also the target group of interest in this work, and as the pseudomonads are a sub-set of the TAB population, the TAB from root lesions were also cycled on root lesions to allow comparisons between the two populations to be made. However, whereas the pseudomonad group cycled on root lesions failed to reduce the effect of take-all disease, the TAB both increased plant growth and reduced root infection compared to the pseudomonad group and control plants in C2 and C3 (Figs. 3.4 and 3.5). This result was repeated when the TAB from root lesions (C1) were recovered from freezer storage and inoculated into a separate pot test, along with the TAB from the pot with the best plant growth from this treatment in C3 (Fig. 3.6). From this it was hypothesised that the non pseudomonad (chloramphenicol and ampicillin sensitive) component of the TAB was responsible for the reduction in take-all. However when the TAB were partitioned into chloramphenicol- and ampicillin-sensitive (cca^-) and resistant (cca^+) components, both groups increased plant growth and reduced disease compared to control plants, and to a similar degree to the mixed TAB population (Fig. 3.6). This might be due to beneficial pseudomonads being selected for on root lesions when they are part of a more complex community (ie. the TAB population), and may involve interactions with non-pseudomonads.

The fluorescent pseudomonad group has been selectively isolated from roots and soil in a number of studies based on antibiotic resistance and fluorescence on KB medium (Weller, 1983; Charigkapakorn and Sivasithamparam, 1987; Weller, 1988; Sarniguet *et al.*, 1992a and b). However, the association between fluorescence and Ggt-inhibition can be highly biased depending on where the sample is taken from, and these studies would have excluded potential antagonists if they were not fluorescent. In this work, Ggt-inhibition was associated with non-fluorescence in pseudomonads from healthy soil (Fig. 3.2) and non-fluorescent Ggt-inhibitors were also increased in the pseudomonad population cycled on healthy roots compared to other treatments (Fig. 3.13). There is also evidence that the pseudomonads may not have been the right target group in Kapunda soil. Firstly there was a greater selection pressure for pseudomonads on root lesions compared to healthy roots, but the pseudomonad group from healthy roots increased plant growth and reduced disease compared to the pseudomonads from lesions. Secondly the TAB from lesions had a positive effect on growth of diseased plants whereas the pseudomonad group from the same location did not after they had been cycled on

diseased wheat roots. This highlights the problem of knowing what is the actual group, or groups, involved in disease suppression so that population studies on disease suppression can target the appropriate biotic group.

In these experiments, bacteria were inoculated into pot microcosms at a density of 10^7 to 10^8 cfu/g soil, which is much higher than the native populations in the soil-sand mixture after 6 weeks growth of wheat (Table 3.1). The added bacteria were therefore expected to be the dominant bacteria at the start of each cycle. Bacterial inoculation of microcosms, though, had little influence on gross bacterial numbers when treated and control plants were compared after six weeks (Figs. 3.1, 3.7, 3.8 and 3.9). Ggt-infested soil cycled in successive wheat microcosms had the greatest impact on total numbers of TAB and pseudomonads in all three cycles with reduced numbers of both TAB and pseudomonads.

Cycling of bacterial populations did however produce changes in the composition of the pseudomonad population with respect to inhibition of Ggt and fluorescence (Fig. 3.13). Pseudomonads cycled on both lesioned and non-lesioned sections of diseased root were similar to infected control plants in the number of isolates with each combination of fluorescence and inhibition characteristics. In contrast, pseudomonads cycled on healthy roots had a greater number of non-fluorescent Ggt-inhibitors, and in the TAB population cycled on root lesions the pseudomonad population was increased in fluorescent non-inhibitors, and these two treatments had the best response in terms of plant growth and disease control.

3.5. Conclusions

The results of this section show that the bacterial microflora on wheat roots can be partitioned by bacterial group and location on wheat roots and can produce a response in terms of plant growth and disease severity when inoculated into wheat-soil microcosms. By partitioning diseased roots into lesioned and non-lesioned sections these results show that root lesions are a distinct niche supporting higher bacterial populations and preferentially selecting for pseudomonads, in particular fluorescent pseudomonads, compared to non-lesioned and healthy roots. Cycling of just the pseudomonad population on root lesions did not select for a take-all suppressive pseudomonad population, but the pseudomonad population can become suppressive when cycled with the TAB population. Healthy roots grown without disease selected for plant beneficial pseudomonads which also reduce disease severity. The two hypotheses that the fluorescent pseudomonads are the most important group involved with TAD, and that root lesions select for disease antagonistic fluorescent pseudomonads are not supported by these results. Other bacterial groups, (eg, non-fluorescent pseudomonads, non-pseudomonads) may be of more importance in suppression of take-all in Kapunda soil.

Chapter 4. *In vitro* Diversification of *Pseudomonas corrugata* strain 2140

4.1. Introduction

Phenotype conversion has been reported in many bacteria, commonly occurring in stationary phase cultures grown on or in artificial media (reviewed by Rainey *et al.*, 1993). This phenomenon has been best studied in *Escherichia coli*, and there has been controversy over whether the mutations which give rise to the new phenotypes were random or whether they are directed by environmental conditions (reviewed by Foster, 1993; and Lenski and Mittler, 1993). Usually in these other studies only one, or few, new phenotypes are characterised in detail, and a major problem has been to separate the effects of environmental (ie. culture) conditions in either selecting for new phenotypes, or directing which mutations occur. This difficulty is in part due to the small size of bacteria which limits researchers to studying populations (colonies), not individual cells. New phenotypes can only be studied if there is some means of detecting them. This usually means the new phenotypes must have a selective advantage over the parent type under the conditions in which they are being cultured. For example, antibiotic resistant mutants are easily recovered on media containing the appropriate antibiotic. Similarly, mutants which utilise a carbon source not used by the parent type can be recovered on that carbon source. In these examples only one phenotype is selected for. Other phenotype variants can also be recovered if they grow to relatively high numbers in relation to the parent type such that they can be detected when plated onto conventional agar media, or if they produce sectoring or outgrowths in colonies grown on agar media. Again, only those phenotypes which have a selective advantage are selected for. None the less, it is clear that bacteria can rapidly diversify in the laboratory (Terzaghi and O'Hara, 1990; Rainey *et al.*, 1993). However, it is not known how previous studies on the *in vitro* diversification of laboratory isolates such as *E. coli*, or even *P. fluorescens* SBW25 isolated from the sugar beet phylloplane (Rainey and Travisano, 1998), relates to the *in vitro* diversification of PGPR strains isolated for disease control.

Pseudomonas strains isolated as PGPR have been reported to produce new variant phenotypes *in vitro*, and these are of great importance as the ability to control disease is reduced or lost in these new phenotypes due to mutations in regulatory genes which control the expression of multiple metabolites involved with disease control (see Table 1.3). The wild type Pc2140 produces an opaque, distinctly corrugated colony morphology (OP) on TZCA medium. When cultured on most media, Pc2140 produces only one variant colony type, a translucent red (TR) colony type when plated onto TZCA. The TR colony variant does not control take-all disease (Barnett, 1994) and appears to be a pleiotropic mutant analogous to the *gacA-lemA* mutants described in studies on other pseudomonad PGPR strains (Laville *et al.*, 1992; Gaffney *et al.*, 1994). The loss of metabolite production in these TR variants appears to

confer a selective advantage in stationary phase cultures compared to the parent Pc2140 type when grown on laboratory media. The TR type can be readily distinguished from the parent type by its altered colony morphology on TZCA medium (Barnett, 1994), but differences in colony morphology are not apparent or only slightly different on other media. For example, the wild type Pc2140 produces a cream coloured opaque corrugated colony type on TSA, and on the same medium the TR type produces a cream coloured smooth colony which is slightly more translucent than the wild type. Both types produce the same colony morphology on NA over a 48 hour period with the less mucoid growth of the TR type only becoming apparent in stationary phase cultures a week or more in age. TZCA was developed by Kelman (1954) to detect colony morphology variants of *P. solanacearum* (now *Ralstonia solanacearum*; Kersters *et al.*, 1997) but it does not appear to be used by researchers working with other *Pseudomonas* species, despite the importance of detecting mutant phenotypes in PGPR cultures. Other *P. corrugata* strains have also been reported to produce colony variants *in vitro* , from a corrugated wild type colony to a smooth variant colony type as observed on peptone yeast extract agar, and these new smooth colony types have altered GC-FAME profiles compared to the wild type. Thompson *et al.* (1995) also reported a divergence in FAME profiles in a genetically marked *P. fluorescens* strain when reisolated from sugar beet leaves.

In a previous experiment, multiple colony morphology variants (4) of Pc2140 (wild type, no antibiotic or marked genes) were detected after culture in an unaerated defined broth medium with an ammonium nitrogen source (A-DB), and plating diluted suspensions onto TZCA (Barnett, 1994). Most of these colony types had not been previously detected. Given the importance of phenotype variation in the biocontrol abilities of *Pseudomonas* PGPR strains in general, and the ease of detection of multiple Pc2140 variant it was decided to use Pc2140 as a model PGPR to further investigate the phenotype variation phenomenon. Marked strains of Pc2140 were used to ascertain the parentage of any new colony type variants.

4.1.1. Aims

To investigate the production of multiple variant phenotypes from marked strains of Pc2140 (Pc2140R, rif resistant; and Pc2140RL, rif resistant and *lacZY* genetic marker) and to use GC-FAME profiles to compare isolates.

To compare the same colony types within and between replicate cultures to determine if the same phenotype occurs in replicate cultures and to isolate colony type variants to determine if they produce further variant colony types.

To compare common laboratory culture media for the frequency of appearance of TR colony types, and to determine if other pseudomonad PGPR isolates also produce multiple phenotypes when cultured in A-DB.

4.2. Materials and Methods

4.2.1. Production of variant phenotypes from Pc2140R and Pc2140RL

In vitro variants of *P. corrugata* strains Pc2140R and Pc2140RL were generated, in separate experiments, in an ammonium unshaken defined broth medium (A-DB, Section 2.2.2.1). Pc2140 had previously been noted to produce multiple phenotypes in this medium (Barnett, 1994). Variant phenotypes were detected by altered colony morphology on TZCA medium (Section 2.2.2.11). Pc2140R or Pc2140RL were sub-cultured twice on TZCA, and a well isolated single colony was used to inoculate 3 ml of NB. This was incubated overnight at 25°C, and 100 µl aliquots of overnight NB culture (ca. 10^9 cfu/ml) were added to 8 ml A-DB in sterile 10 ml glass culture tubes with screw cap lids. For strain Pc2140R, 50 replicate tubes were inoculated, and for Pc2140RL, 25 replicate tubes were inoculated and incubated unshaken at 25°C. In the Pc2140R experiment, six blank tubes containing A-DB inoculated with 100 µl sterile distilled water were included. Ten blank tubes with added 100 µl sterile NB were included in the Pc2140RL experiment. Blanks were sampled as per the inoculated tubes. At each sampling, bacterial suspensions of ten-fold dilutions were spotted onto TZCA, (three 20 µl replicates for each dilution). Cultures of Pc2140R were sampled at 25 days, and Pc2140RL cultures at 11, 18 and 25 days. A description of variant colony morphologies is given in Chapter 2, Table 2.1.

4.2.2. Confirmation that variant isolates are derived from Pc2140

Variants from cultures of Pc2140R were initially determined to be derived from Pc2140R from their rifampicin resistance. The origin of variants from Pc2140RL cultures was confirmed by rifampicin resistance, and formation of blue colonies (Drahos *et al.*, 1986) on X-Gal MM with glucose as the sole carbon source (Section 2.2.2.12), which indicates the constitutive expression of the *lacZY* gene in Pc2140RL (Ryder *et al.*, 1994) This was carried out by patching isolates onto TZCA supplemented with rifampicin (100 µg/ml), or onto X-Gal MM to determine rifampicin resistance or *lacZY* gene activity, respectively. Selected variants were further confirmed as Pc2140 by DNA fingerprint as described in Chapter 7.

4.2.3. Comparison of variant colony types by GC-FAME profiles

To determine if colony morphology variants were altered in their GC-FAME profiles compared to their wild type parent, five variant isolates of Pc2140R from static A-DB cultures (25 d) with different colony morphology were assessed by GC-FAME and compared to Pc2140R. These variants are shown in Fig. 4.4. GC-FAME analysis was carried out as described in Section 2.2.13. Three independent extractions and analyses were performed for each variant, eight for Pc2140R, and entries made in the new 2140V library for each isolate. GC-FAME profiles were

compared by the MIS Sherlock Library Generation Software using UPGMA hierarchal cluster analysis (Section 2.2.13).

4.2.4. Comparison of similar colony types by GC-FAME profiles

To determine if variants with the same colony type were the same phenotype (ie. the same characteristics), GC-FAME profiles of the two main variant colony types, TR and FNT, from the same and replicate Pc2140RL cultures were compared. FNT8.RLi and FNT9.RLi were isolated from tube 9, and FNT12.RLi from tube 19 at 11 days. TR1.RLi and TR2.RLi were isolated at 11 days, and TR30.RLi at 18 days, from tube 10, and TR6.RLi from tube 6 at 11 days. TR types from some replicate cultures produced a yellow diffusible pigment (TRY colony type). TRY19.RLi and TRY45.RLi were isolated from tube 6 at 11 and 25 days respectively, and TRY33.RLi from tube 3 at 18 days. Three replicate fatty acid extractions and GC-FAME analysis of each variant were performed, with four replications for the ancestral Pc2140RL. The mean GC-FAME profiles for each variant were compared to the ancestral Pc2140RL isolate by hierarchical cluster analysis.

4.2.5. Production of further variants from variant colony types

To determine if variants colony types give rise to further variants, three variant types were isolated from A-DB cultures of Pc2140RL and introduced into filter sterilised used culture media of the same age, and at an equivalent cell concentration to the medium they were isolated from. Used culture medium was used to maintain the same selection pressures as present in the original culture. Variants from Pc2140RL (TR1.RLi, FNT8.RLi and FNTO22.RLi), were isolated from 11 day old A-DB cultures. Single colonies from 1/10 dilution series plated onto TZCA (48 h) were subcultured twice on TZCA and a single colony transferred to NB and cultured overnight at 25°C. Cells were pelleted by centrifugation and the supernatant discarded. The bacterial pellet was resuspended in 8 ml (10^9 cfu/ml) of filter sterilised (0.2 µm, Gelman Acrodisc) supernatant from 11 day old cultures of Pc2140 (non rif resistant, no *lacZY* genes) after previous removal of cells by centrifugation (12,000 x g for 5 min). Three replicate cultures of each variant isolate were incubated at 25°C and sampled at 10, 17, and 24 days for appearance of colony types differing from the parent (or primary) variant on TZCA. Variants descendent from Pc2140RL can be differentiated from any Pc2140 cells not removed from the 11 day old medium supernatant because Pc2140RL and descendants are rifampicin resistant and contain the *lacZY* genes (blue colonies on X-Gal MM).

4.2.6. Appearance of variants on common laboratory media

It had previously been observed that the appearance of TR type colony variants from Pc2140 cultures differed between media (Ryder, unpublished data). The TR type is the most common variant colony type found on most media. To assess the production of TR types on solid media,

Pc2140RL was cultured on common solid media (NA, LB agar and TSA) at full and 1/10 strength (Section 2.2.2). The appearance of variants was also assessed on a minimal medium (MM) and modified nutrient agar (MNA; Section 2.2.2). MNA is a common medium for culture and short term (1 to 3 months) storage of *Pseudomonas* strains in China (Y. Peng, *pers comm.*).

Pc2140RL was sub-cultured twice on TZCA and a single colony transferred to 3 ml NB and cultured overnight at 25°C. Nine aliquots (10 µl, 10^6 cfu) of overnight Pc2140RL cultures were spotted onto each medium to form nine discrete bacterial patches, four replicate agar plates for each medium. At each sampling (every 3 to 5 days for 31 days) one patch per plate was wiped off with a sterile cotton bud and suspended in phosphate buffered saline (PBS), serially diluted 1 in 10 and plated onto TZCA to detect TR variant colony types.

4.2.7. Production of variants from other *Pseudomonas* isolates

To determine if other pseudomonad isolates also produce new variant types which are detectable on TZCA, three *Pseudomonas* strains (*P. fluorescens* 2-79, *P. fluorescens* Pf-5 and *P. putida* 879) and the closely related *Burkholderia cepacia* type strain were cultured in A-DB culture as described previously (Section 4.2.1). For each isolate, 10 replicate cultures were incubated at 25°C for 20 days and the presence of colony variants in each culture was assessed by plating 1 in 10 dilutions onto TZCA. The source of these isolates is shown in Table 4.2.

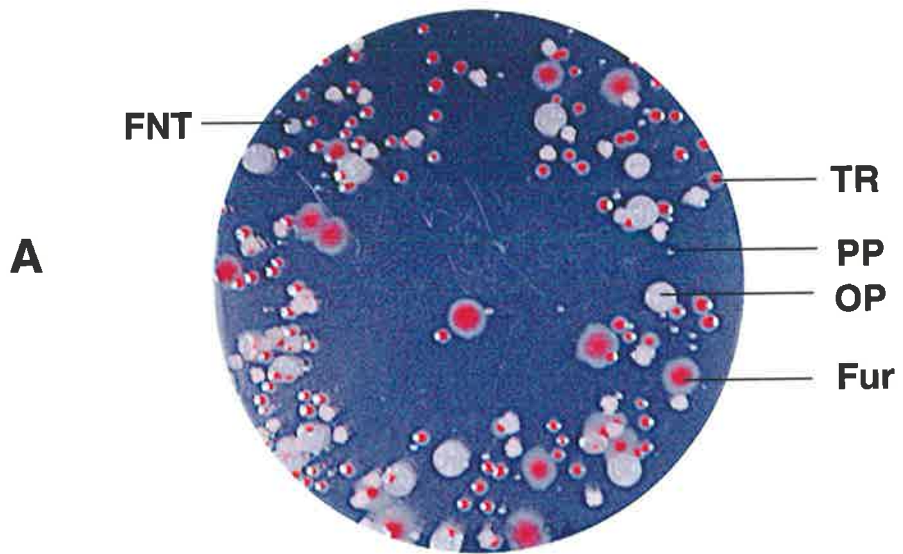
4.3. Results

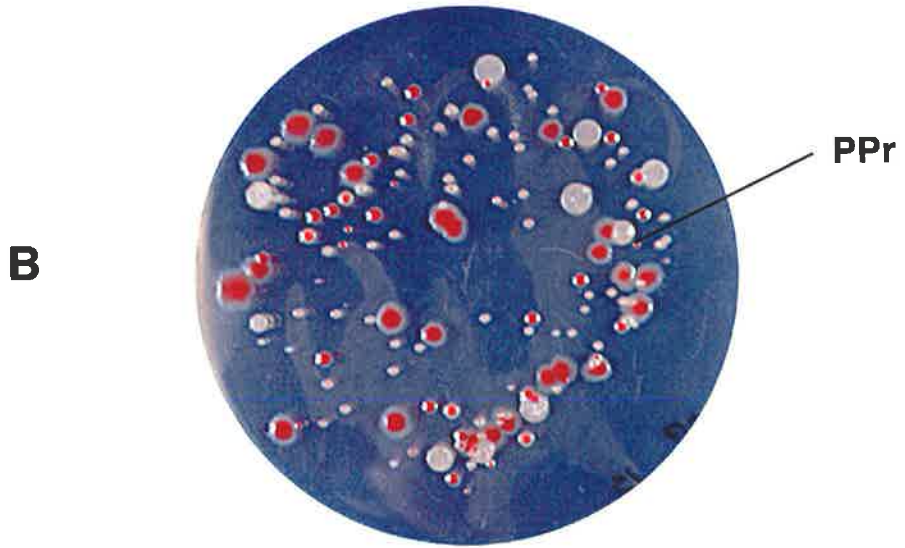
4.3.1. Production of variant phenotypes from Pc2140R and Pc2140RL

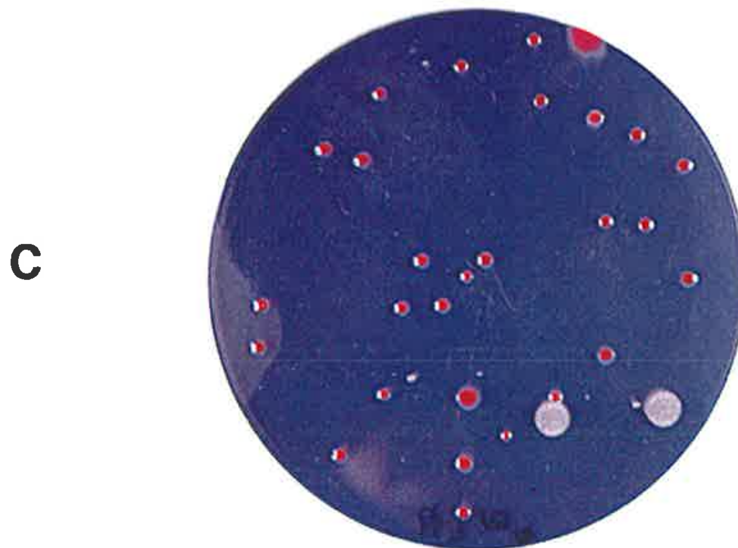
Isolates which differed in colony morphology from the wild type *P. corrugata* 2140 were isolated from stationary phase static A-DB cultures started from pure wild type isolates of Pc2140R and Pc2140RL. The wild type Pc2140 produces distinctive cream coloured, opaque, irregular, corrugated, wavy edged, 2 mm diam. (OP) colonies on TZCA after 48 h at 25°C (Table 2.1, Fig. 4.1). Variants present at a density of less then 1/500 were not detectable on dilution plates. Pc2140R and Pc2140RL both produced a similar spectrum of variant colony types, with similar colony types produced *in vitro* by both parents, and in replicate cultures of the same parent. Mixed populations in three replicate cultures of Pc2140R after 25 days in A-DB are shown in Fig. 4.1 after plating onto TZCA medium. At least 11 distinct colony types were isolated in total from these cultures, and from variants sub-cultured to A-DB. Variant colony types are described in Table 2.1. The colony morphology of variant types was maintained when regularly subcultured onto TZCA, and after storage at -70°C, or after growth on other media and replating onto TZCA.

Of 25 replicate cultures of Pc2140RL, 19 contained colony morphology variants at 11 days, increasing to 23 cultures at both 18 days and 25 days. There were one to four different variant colony types per culture tube in addition to the wild type. The number of variant types in each

Fig. 4.1. Mixed populations of Pc2140R and variant colony types on TZCA from three replicate cultures of Pc2140R after 25 days in static A-DB medium. Populations are from culture tube 18 (A), tube 31 (B) and tube 43 (C). Shown are diluted suspensions (100 µl) of each culture on TZCA in 9 cm Petri dishes after incubation for 72 hr at 25°C. The wild type (OP), TR, FNT, PP, PPr and Fur colony types are indicated.


A
FNT
TR
PP
OP
Fur


B
PPr


C

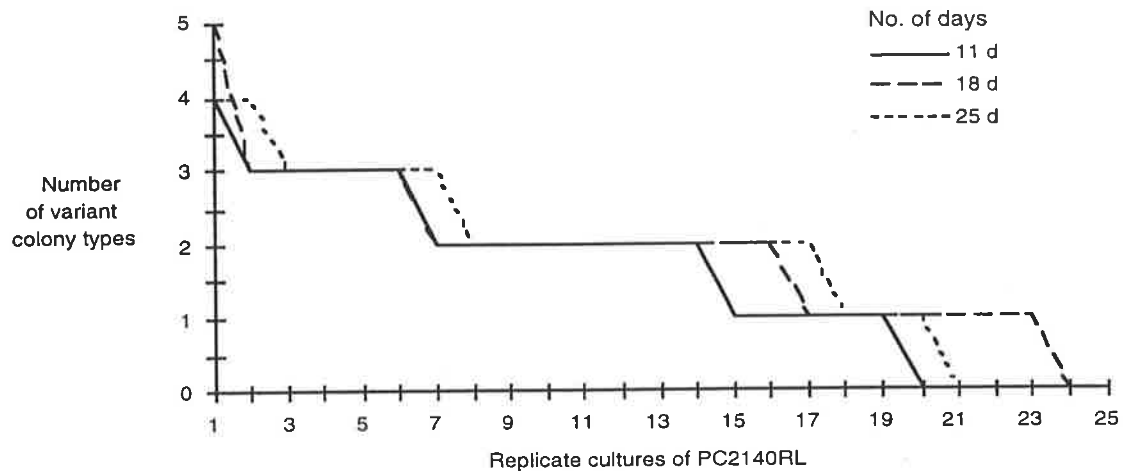


Fig. 4.2. Number of variant colony types in each of 25 replicate cultures of Pc2140RL after 11, 18 and 25 days growth in static A-DB medium. Replicate cultures are shown in ranked order from the highest to lowest number of variant colony types.

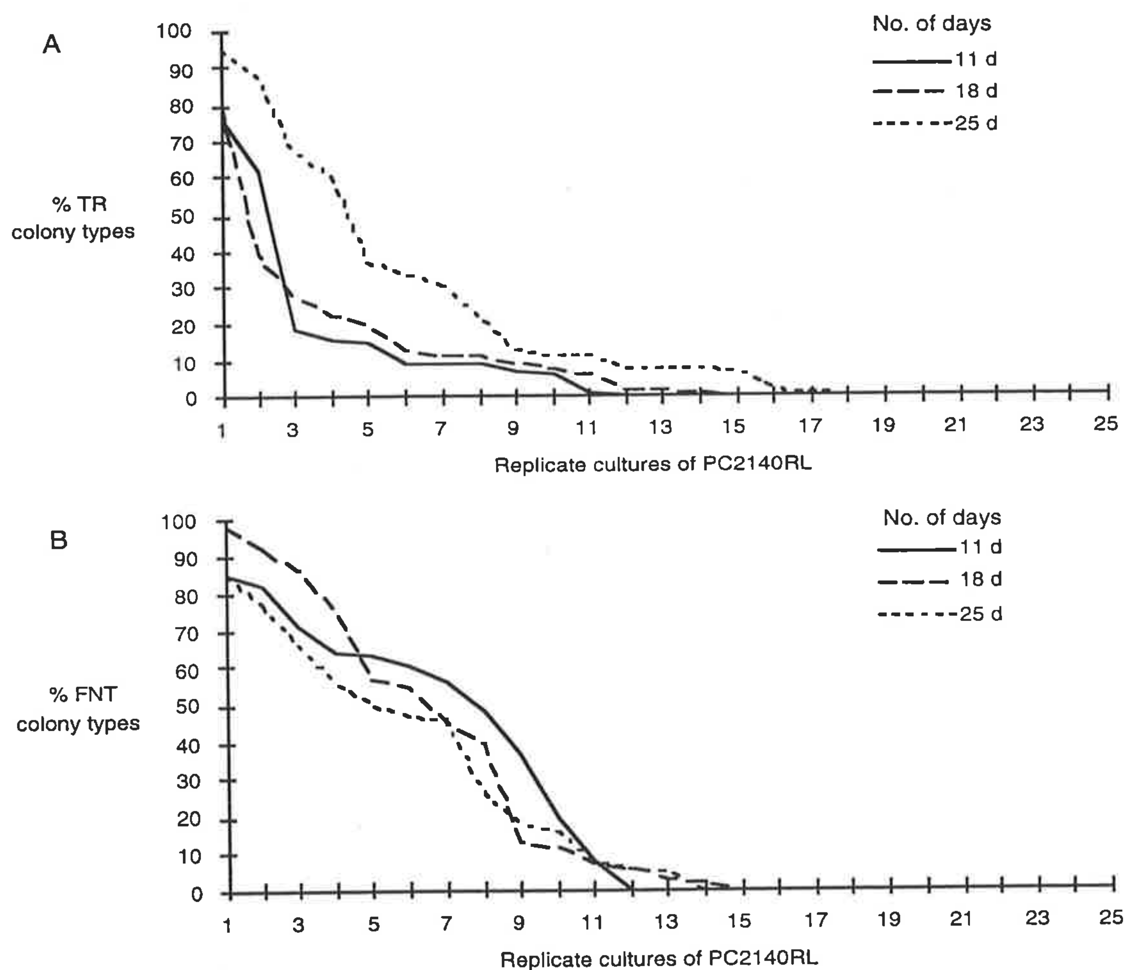


Fig. 4.3. Percentage of TR (A) and FNT (B) colony types in each of 25 replicate cultures of Pc2140RL after 11, 18 and 25 days growth in static A-DB medium. Replicate cultures are shown in ranked order from highest to lowest percentage of the variant colony type.

culture (in ranked order) is shown in Fig. 4.2. The proportion and range of variant types varied between replicate cultures. The most common variants were a red translucent convex (TR) colony type and a faint yellow, flat (FNT) type. TR and FNT colony types were detected in 14/25 cultures at 18 days, but not necessarily both in the same culture. The TR and FNT colony types were often present in higher numbers than the parent type. The percentage of TR and FNT colony types in the total population is shown in Fig. 4.3, with cultures in ranked order. Similar results were obtained using Pc2140R, with 42/50 cultures having variant colony types after 25 days. One culture no longer contained detectable wild type colonies. Cultures contained between 10^6 to 10^8 cfu/ml at sampling times (11 to 25 days). Cultures with a higher density of cells generally contained a higher proportion of variants and more variant colony types. No bacteria were isolated from blank control tubes.

4.3.2. Confirmation that variant isolates are derived from Pc2140

All variants from Pc2140R and Pc2140RL cultures were resistant to rifampicin (100 µg/ml). All variants arising from Pc2140RL cultures were positive for constitutive ß-galactosidase activity (blue colonies on X-Gal MM), indicating the presence of the *lacZY* genes. Pc2140R variant colony types exhibited the same total DNA restriction enzyme and ERIC-PCR fingerprints as the wild type (see Chapter 7).

4.3.3. Comparison of variant colony types by GC-FAME profiles

To determine if variant colony types differed in their GC-FAME profiles compared to their parent Pc2140 strain, five isolates with differing colony morphologies from 25 day old A-DB cultures of Pc2140R were analysed for their GC-FAME profiles, and compared to Pc2140R by hierarchical cluster analysis. These variant isolates and the similarity of their GC-FAME profiles to Pc2140R are shown in Fig. 4.4. The MIS manual suggests that a Euclidean Distance (ED) of less than 2 indicates isolates are the same strain, a ED<6 the same biovar and a ED<10 the same species. Replicate extractions and analysis of Pc2140R and variant isolates varied by less than 2 ED, but were usually less than 1 ED different. One variant, FNT8.Ri, was 4 ED units from Pc2140R, and both of these (Pc2140R and FNT8.Ri) an ED of 12 from the other variants (Fig. 4.4). The MIS using Sherlock aerobic library version 3.8 identified the wild type Pc2140R as *P. putida* bv. A, one variant (FNT8.Ri) as a different biovar and one variant (PPr6.Ri) as a different species. FNT8.Ri was identified as having closest similarity to *P. putida* bv. B. PPr6.Ri was identified as having closest similarity to *P. fluorescens* bv. B (Chapter 6, Table 6.4).

4.3.4. Comparison of similar colony types by GC-FAME profiles

TR and FNT colony types were selected from the same and from replicate cultures and compared by GC-FAME profiles. The results are represented in a dendrogram (Fig. 4.5). The three FNT variants were grouped together with the wild type ancestor Pc2140RL. The

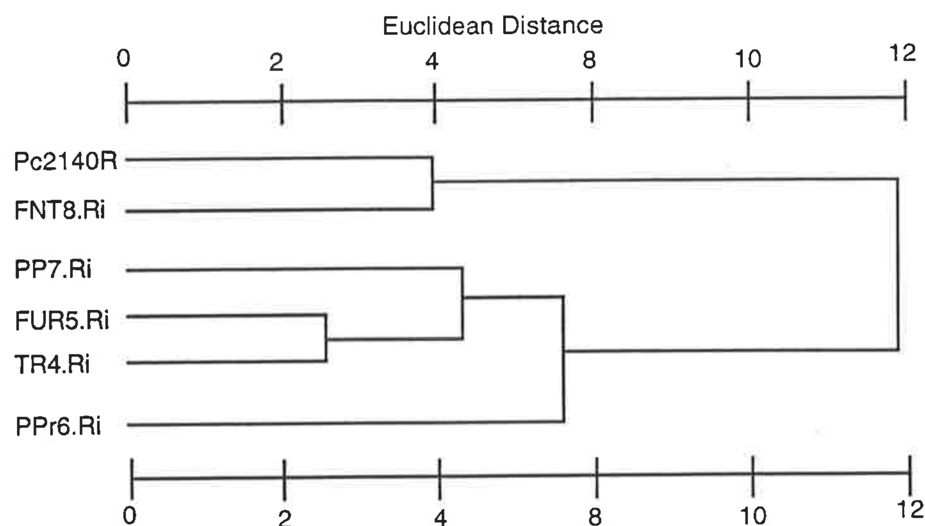


Fig. 4.4. Comparison of five different variant colony types by GC-FAME profiles. Variant colony types were isolated from Pc2140R cultures after 25 days in static A-DB medium.

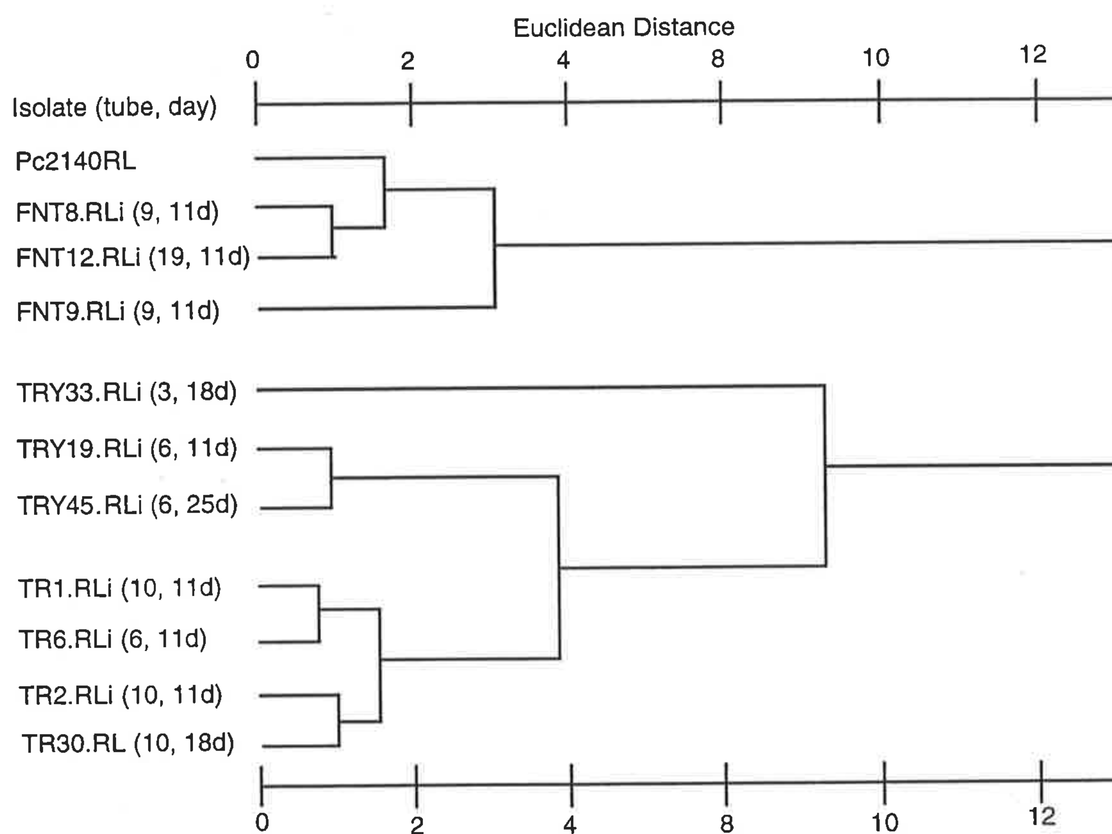


Fig. 4.5. Comparison by GC-FAME profiles of variant isolates with the same colony morphology. Variants colony types were isolated from replicate Pc2140RL cultures after 11, 18 or 25 days in static A-DB medium. Culture tube and number of days growth in A-DB is shown in brackets.

Euclidean distance (ED) between FNT8.RLi and FNT9.RLi (isolated from the same culture at the same time) was greater (3 ED units) than between FNT8.RLi and FNT12.RLi isolated from different cultures, and between these variants and the parent type (ED <2; Fig. 4.5). All four TR variants were grouped together at less than 2 ED units, and diverged from the ancestral Pc2140RL at 12 ED units (Fig. 4.5). The three TRY colony types shown in Fig. 4.5 were originally selected as single colonies of the TR type, but differed from the TR types in that they produced a diffusible yellow pigment (YDP) when streaked onto TZCA medium. These types were also different from the other TR types (lacking YDP) in their GC-FAME profiles. TRY19.RLi and TRY45.RLi were isolated from culture tube 6 at 11 and 25 days respectively and were different by 1 ED unit, and 4 ED units from TR6.RLi isolated from the same culture tube (Fig. 4.5). TRY33.RLi was isolated from a separate culture (tube 3) to the other TRY variants and diverged by 9 ED units from the other TRY, and TR, variants (Fig. 4.5).

4.3.5. Production of further variants from variant colony types

Variants derived from Pc2140RL, TR1.RLi, FNT8.RLi and FNTO22.RLi, were isolated, purified and assessed for their ability to produce new colony type variants when inoculated into a medium at a similar state to the medium they were isolated from originally. All three primary variants produced further variants with different colony morphology to the "parental" variant. Colony types are described in Table 2.1. A total of 115 isolates which varied in colony morphology from the "parental" variant were isolated. All isolates were rifampicin resistant and formed blue colonies on X-Gal medium. The appearance of new variant types in cultures was in a manner similar to the production of variant types from the wild type Pc2140R described previously. That is, new variants appeared in replicate cultures at different times and different new variants appeared in different replicate cultures. All cultures had produced further variant colony types by 17 days. The number of new colony types produced by the three primary variants (TR1.RLi, FNT8.RLi or FNTO22.RLi) was less than produced by the wild type Pc2140RL.

TR1.RLi produced three different colony types: a red opaque type with rough texture (CR), a small TR type (PPr) and a spreading TR type (TRS). FNT8.RLi produced three different colony types: a translucent orange type (FNTO), a larger translucent orange type (FNTOL) and a domed mucoid type (DM). FNTO22.RLi produced three different colony types: a FNTO type but yellow colour, a larger FNTO type (FNTOL) and a TR type. A comparison of GC-FAME profiles of the parental variants and two descendants from TR1.RLi and FNTO22.RLi, and three from FNT8.RLi is shown in Fig. 4.6. Descendants from TR1.RLi (TRS63.RLi and CR65.RLi) both clustered close to the parent TR1.RLi (Fig. 4.6). Variants from FNT8.RLi and FNTO22.RLi diverged from the parental variant type. Similar colony types (FNTO and FNTOL) derived from different parents

(FNT8.RLi or FNTO22.RLi) were indicated to be substantially different by their GC-FAME profiles (Fig. 4.6).

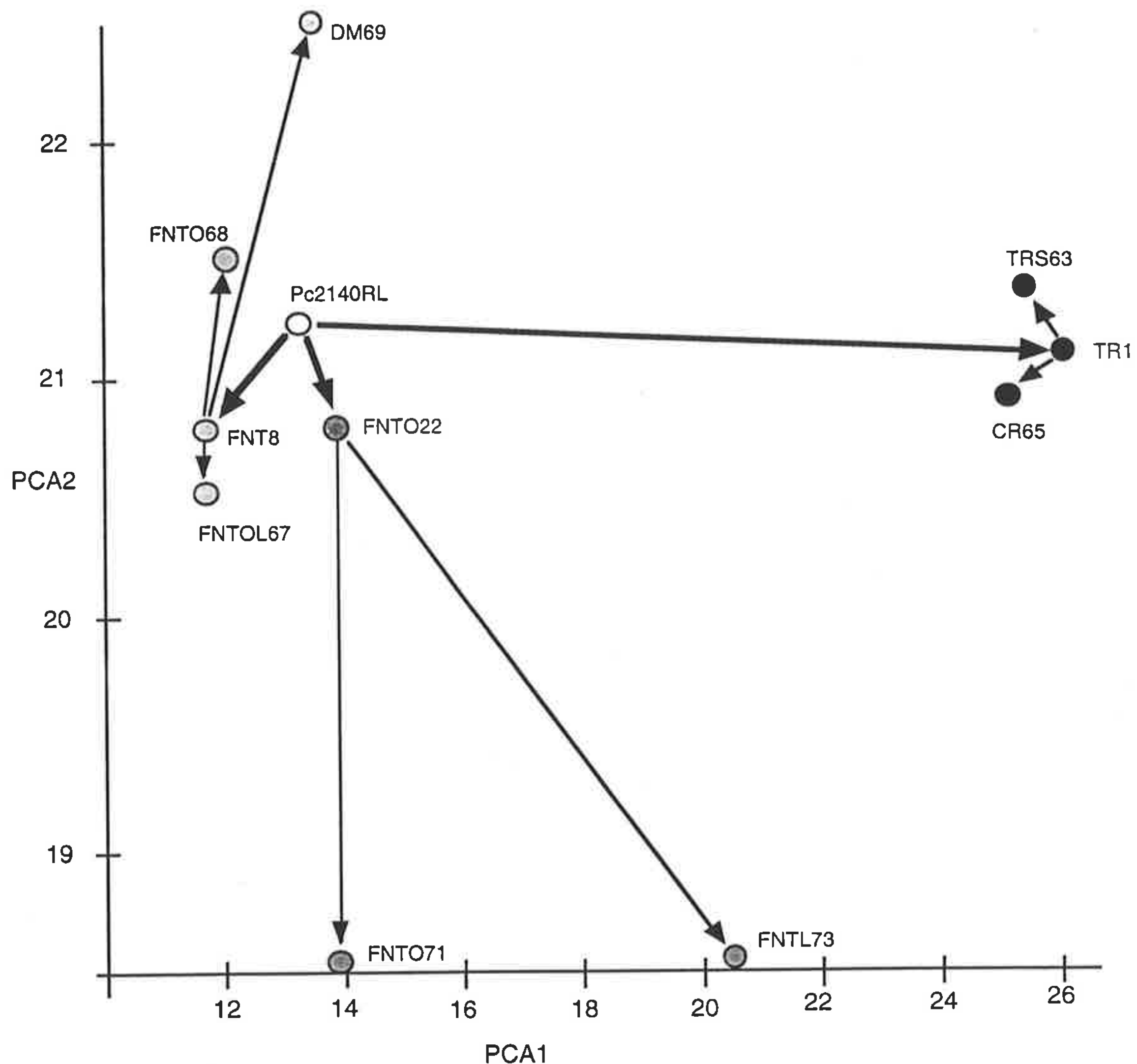


Fig. 4.6. Evolution of three variant colony types derived from Pc2140RL as represented by PCA of GC-FAME profiles. Three primary variants (TR1, FNT8, FNTO22) were isolated from Pc2140RL cultures after 11 days growth in static A-DB medium. Secondary variants were isolated from primary variants after culture in static A-DB medium. TRS63 and CR65 were isolated from TR1 cultures, FNTOL67, FNTO68 and DM69 from FNT8 cultures, and FNTO71 and FNTL73 from FNTO22 cultures. Production of primary variants is shown in dark arrows, secondary variants in light arrows.

4.3.6. Production of variants on common laboratory media

Common laboratory agar media were assessed for their influence in the production of variant colony types from Pc2140RL. The number of Pc2140RL cultures containing translucent (TR)

colony types on the different media at 16 and 31 days is shown in Table 4.1. LB/10 and TSA/10 were the only complex media where the TR variants (non inhibitory to Ggt) were not detected over this time. TR colony types were detected on all other media by 16 days. TR variants were detected as early as 6 days on LB and TSA. Full strength media generally produced more variants more frequently than 1/10 strength media, but *Pseudomonas* minimal medium (MM) also produced a high frequency of variants (Table 4.1). The TR colony type was the only distinctly different colony type detected on these media. Other subtly different colony types were sometimes also present. These varied from the wild type Pc2140RL by being slightly smaller in size and slightly less corrugated. None of the colony types from A-DB cultures (except for the TR type) were detected on the media used here.

Table 4.1. Number of *P. corrugata* 2140RL cultures (maximum 4) containing TR colony types after growth on different media for 16 and 31 days. There were four replicate patch cultures on each medium. Mean total population to the nearest power of 10 of each bacterial patch (cfu/patch) is shown for each sample time. Media are described in Section 2.2.2. Media are nutrient agar (NA), Luria Bertani agar (LB), tryptic soy agar (TSA) at full and 1/10 strength (/10), minimal media (MM) and modified nutrient agar (MNA).

	1 day	16 days		31 days	
Media	cfu/patch	No. cultures with TR types	cfu/patch	No. cultures with TR types	cfu/patch
NA	10^9	1	10^8	0	10^7
NA/10	10^8	1	10^7	0	10^6
LB	10^9	1	10^8	3	10^7
LB/10	10^8	0	10^7	0	10^7
TSA	10^9	4	10^8	3	10^7
TSA/10	10^8	0	10^7	0	10^7
MM	10^8	2	10^9	4	10^7
MNA	10^9	3	10^8	3	10^7

4.3.7. Production of variants from other *Pseudomonas* isolates

To compare the production of variant colony types by Pc2140 strains with other bacterial strains, three *Pseudomonas* strains and a *Burkholderia cepacia* strain were assessed for their ability to produce variant colony types when incubated in static A-DB medium. All four isolates produced variant colony types when incubated in A-DB medium and sampled at 20 days (Table 4.2). The *Pseudomonas* strains produced between 2 and 6 new colony types and *B. cepacia* produced one variant colony type (Table 4.2). Another three bacterial strains which have been noted to produce colony morphology variants in this laboratory are also shown in Table 4.2.

Table 4.2. Production of variants from a range of soil and rhizosphere bacteria. Variant colony types were isolated from static A-DB cultures after 20 days, from storage cultures (stab cultures) and from stationary phase cultures on tryptic soy agar (TSA) and modified nutrient agar (MNA).

Isolate	Source of isolate	No. of variants	Source of variants	Comments
P. fluorescens 2-79	D. Weller, USA.	2	A-DB, 20 days	[a]PGPR (Cook *et al.*, 1995)
P. fluorescens Pf-5	C. Howell, USA.	6	A-DB, 20 days	[a]PGPR (Corbell *et al.*, 1994)
P. putida 879	M. Ryder, Aust.	4	A-DB, 20 days	PGPR (Ryder, *pers comm.*)
Burkholderia cepacia Type strain	J. Balandreau, France	1	A-DB, 20 days	PGPR
P. aureofaciens 3732	D. Kluepfel, USA.	1	Storage cultures	PGPR
P. fluorescens P303	Y. Peng, China	1	MNA	PGPR (Peng *et al.*, 1996)
Bacillus subtilis B908	W. Tang, China	1	TSA	PGPR (Tang *et al.*, 1994)

[a] Reported to produce *lemA* type mutants.

4.4. Discussion

Pc2140R and Pc2140RL both produced multiple new colony types in stationary phase unshaken A-DB cultures. All variant colony types isolated from these cultures were rifampicin resistant, and variants from Pc2140RL all had constitutively functioning *lacZY* genes, indicating the variant colony types were derived from the initially pure parent strain, Pc2140R or Pc2140RL. This was further confirmed by comparison of genetic profiles (see Chapter 7). These new colony types appear to be the result of genetic changes, and not a regulatory response to culture conditions, as the new colony types maintained their different colony morphologies on the same medium (TZCA), and when subcultured onto TZCA. The large number (11) of different variant colony types were derived from Pc2140 cultures in unshaken A-DB, whereas only the TR type was detected on most media. This may be due to (1) the more heterogenous environment of an unshaken culture and (2) the stressful conditions imposed by the culture media. Rainey and Travisano (1998) discuss how the more heterogenous environment of an unshaken broth medium provides more niches (ie. the more aerobic top, and more anaerobic bottom, of the culture) than a homogenous shaken medium to explain the greater diversity of *P. fluorescens* SBW25 in unshaken KB broth cultures. Shaken cultures of strain SBW25 in KB did not produce new phenotypes, unshaken cultures produced two major types and a number of minor colony types. A-DB, using an ammonium nitrogen source, also provides a stressful environment for stationary phase cultures of Pc2140 as no viable cells were recovered from this medium after seven days when aerated by shaking (Barnett, 1994). Cultures of Pc2140 in this same medium with a nitrate nitrogen source (N-DB) only produced the TR type variant, whether shaken or unshaken (Barnett, 1994). As well, on the solid media assessed here only the TR type was detected in Pc2140RL cultures (Table 4.1). In this work,

the aim was to see whether PGPR produce a range of variant phenotypes, but in bacterial strains such as PGPR, and other microbial inoculants isolated for a particular function, it is important to maintain the wild type phenotype. From these results it appears that 1/10 strength complex media such as LB or TSA are better suited for routine culture of pseudomonads compared to full strength media (Table 4.1), at least during stationary phase conditions. The appearance of new phenotypes appears restricted to stationary phase cultures of Pc2140, as variants were previously not detected in log phase cultures (Barnett, 1994). Rawjeshwari *et al.* (1997) compared stationary and log phase cultures of *Xanthomonas oryzae pv. oryzae* and found that spontaneous variants were only present in stationary phase culture where they out-compete the parent type.

Pc2140 appears to be suited for use as a model PGPR to study phenotype plasticity as the diversification of Pc2140 during *in vitro* culture is similar to that of other PGPR pseudomonads studied here (Table 4.1) and to pseudomonad strains and other genera described in the literature. Studies on the sugar beet phylloplane isolate, *P. fluorescens* SBW25 (Rainey *et al.*, 1993; Rainey and Travisano, 1998), provides the closest example to Pc2140. Pc2140 produced new phenotypes in unshaken broth culture in an apparently random manner, similar to strain SBW25, as evidenced by the clear differences in the range and type of new colony types in replicate cultures of Pc2140 (Figs. 4.2 and 4.3). Variant colony types contributed to greater than 70 percent of the total population in some cultures, but were not present in others. This indicates that the appearance of variants in Pc2140 cultures is dependant on stochastic, or probabilistic, events within the constraints of the Pc2140 genome and culture conditions (Rainey *et al.*, 1993). The stochastic nature of the evolution of replicate bacterial populations is also evident in the work of Lenski and Bennett (1993), and Lenski and Travisano (1994), where replicate populations of *E. coli* diverged in their fitness after adaptation to different culture conditions, and in the random nature in the appearance of variant phenotypes from *Deleya aesta* (MacLeod *et al.*, 1997).

Analysis of the GC-FAME profiles of variant types from Pc2140 indicated that the variant colony types were consistently and substantially altered in their GC-FAME profiles (Fig. 4.4). Variation in FAME profiles between the wild type and variant colony types has also been noted in *P. corrugata* strains from Spain (Siverio *et al.*, 1996), and provides a relatively rapid means of characterising and comparing variant types in addition to colony morphology changes. Some variants in this study diverged from the parent type by up to 12 Euclidian Distance (ED) units from the parent isolate. This is greater than the suggested difference between species (10 ED). The relevance of differences in GC-FAME profiles to taxonomic identification by GC-FAME is discussed in detail elsewhere (Chapter 6). Differences in GC-FAME profiles are assumed to reflect underlying genomic differences as all isolates are cultured under the same conditions prior to extraction and analysis of fatty acids. This allows a greater range of variant types to be

compared than if the actual mutations giving rise to the new phenotypes had to be identified before comparisons could be made.

The same phenotype can arise independently in replicate cultures of Pc2140, as evidenced by variants with the same colony morphology and GC-FAME profiles appearing in replicate cultures of a common ancestor (Fig. 4.5). Flores *et al.* (1988) found that identical genomic rearrangements occurred in spontaneous mutants derived independently in replicate cultures of a *Rhizobium* strain. However different mutations in the same gene can also give rise to the same phenotype in *E. coli* (Schaaper *et al.*, 1986), as can mutations in different genes (eg. *lemA* or *gacA* mutants of *P. fluorescens* strains, Gaffney *et al.*, 1994). The TRY variants from Pc2140 have the same colony morphology as the TR variants on TZCA but can be differentiated from the TR types by producing a yellow diffusible pigment when a single isolates is streaked onto TZCA medium. The TRY types are also clearly different in GC-FAME profiles to the TR types from the same culture (ie. TRY19.RLi and TR6.RLi isolated from culture tube 6 at 11 days, Fig. 4.5), and can also vary from TRY isolates arising independently in another culture (ie. TRY19.RLi and TRY33.RLi, Fig. 4.5). MacLeod *et al.* (1997) found that in the 3 colony types produced by *Deleya aesta* 134 on low Na^+ medium there were a total of 7 different phenotypes and supports the suggestion of Rainey *et al.* (1993) that there may be many more phenotypes present than are detected.

The range of variant phenotypes produced by Pc2140 is also increased by the production of yet further variant colony types from an initial set of variants (Fig. 4.6). These new variants can each produce further new colony types. In addition, similar colony types can be produced from different variant ancestors. Similar colony types from different parents, however, can have divergent FAME profiles (ie FNTO colony types, Fig. 4.6). Rainey *et al.* (1993), MacLeod *et al.* (1997) and Rajeshwari *et al.* (1997) also reported *in vitro* variants producing further variants. Lenski (1998b) provided evidence that maladaptive pleiotropic effects in phage T4 resistant mutants of *E. coli* can be compensated for by further adaptation. Helling *et al.* (1987), reported that the prescence of minor (infrequent) variant phenotypes types from *E. coli* can depend on the prescence of major (frequent) phenotypes. However, this occurred in glucose limited cultures where the selection pressure was for carbon source utilisation and there is no evidence that selection for variants from Pc2140 is dependent on the presence of other variant types. The range of new phenotypes from Pc2140 that can be detected appears finite, even though the same phenotype can be repeatedly produced in replicate cultures, suggesting only a limited set of mutations are occurring which give rise to a favourable phenotype in A-DB culture. This is supported by the reduced number of new phenotypes produced by variant colony types, after purification and reinoculating into A-DB, compared to the number of types produced by the wild type Pc2140. Even though bacteria can rapidly diversify, Hartl *et al.* (1985) and Hall (1995), point out some of the limitations to adaptation in a particular environment. As the variant phenotypes

have a fitness advantage in stationary phase A-DB over the parent isolate (a prerequisite for them to multiply to detectable levels), they are closer to an optimal phenotype for this medium compared to the parent isolate, and so there will be a decreased probability that a new phenotype from a variant will be more fit than its parent.

4.5. Conclusions

Pc2140 produces multiple new variant phenotypes *in vitro* in a manner similar to other *Pseudomonas* isolates. Variant phenotypes from Pc2140 are readily detectable on TZCA medium. Variant phenotype can have divergent GC-FAME-profiles and this can be used to confirm the identity of the wild type isolates and compare variant colony types. The TR type variants, which do not control take-all disease, appeared in Pc2140 cultures on most media within 16 days. However, variants were not detected in Pc2140RL cultures grown on LB/10 and NA/10 up to 31 days and these would therefore be the more suitable media to use if cultures were to be left in stationary phase conditions (eg. during storage and transport of cultures). The spectrum of variants detected depends on the culture media and conditions. Only the TR type is recovered from Pc2140 cultures on agar media and a wide spectrum of variant types (11) are recovered from cultures grown in static A-DB. The pattern of appearance of variants in replicate cultures is consistent with the hypothesis that the underlying mutations which give rise to the new phenotypes are random. The same variant phenotype can arise independently in replicate cultures, and variants can give rise to further new colony type variants. Variants with the same colony morphology on TZCA do not necessarily have an identical phenotype (eg. TRY variants Fig. 4.5).

These results highlight the rapid diversification of bacterial isolates such as Pc2140 in laboratory culture and are of direct relevance to other pseudomonad strains isolated as biological control agents where diversification of these other strains may be similar to that observed for Pc2140 (ie. rapid and common on most media). The full extent of phenotype change may be hidden due to the difficulty in detecting phenotype variants on most media. Pc2140 provides a good model organism for studying the diversification of pseudomonad PGPR as the distinctive colony morphology of the wild type Pc2140 on TZCA allows variants with altered colony morphology to be readily detected.

Chapter 5. Production and Selection for Spontaneous Phenotype Variants of *Pseudomonas corrugata* strain 2140R on Root Lesions and Healthy Roots of Wheat

5.1. Introduction

It is hypothesised in this work that root lesions select for disease suppressive biota. The selection pressures exerted on the entire root-soil biotic community would also apply to individual strains of bacteria and select for any new variant phenotypes which have an ecological advantage. Root lesions then may be a niche which selects for phenotype variants with increased disease control. In Chapter 3 it was concluded that root lesions are a distinct niche which preferentially selects for pseudomonads, and it has been reported that populations of a PGPR, *P. fluorescens* 2-79, were increased on take-all diseased roots. There have been no reports however, on the impact of take-all root lesions on populations of an introduced PGPR. It is also not known if the substantial changes in phenotype observed in variants from PGPR isolates cultured *in vitro* also occur in the root-soil environment (Chapter 4), or whether diseased root lesions preferentially select for different variant phenotypes compared to healthy roots.

Although phenotype conversion is a common phenomenon *in vitro*, phenotype plasticity in the environment is less well studied. Avirulent plant and animal pathogens produced spontaneously *in vitro* have been reported to revert to the virulent wild type on or in the host organism (Rainey *et al.*, 1993), but there are no reports of *in vitro* variants from PGPR isolates reverting to the wild type either *in vitro* or *in vivo* (ie. in the root-soil environment). Thompson *et al.* (1995) have also shown that *P. fluorescens* SBW25 can diverge in GC-FAME profiles after culture on sugar beet leaves, however differences were relatively small compared to the divergence in GC-FAME profiles in Pc2140 variants (Chapter 4) and the effect of divergence on plant growth promotion or disease control by SBW25 was not reported.

The lack of reports on phenotype conversion in the root-soil environment may be partly due to the difficulty in detection of variant phenotypes on most laboratory media. Colony type variants were also not detected on TZCA in Pc2140R cultures reisolated from wheat roots after 4-6 weeks growth (Barnett, 1994). It may be that the short incubation time did not allow variants to multiply enough to reach detectable levels, and/or the location in the root-soil environment where variant types are selected for may not have been accurately targeted. If selection for variant types occurs at a specific site in the root-soil environment then variant types could be difficult to detect unless bacteria were specifically isolated from this site. The root-soil interface is a heterogenous environment, and different bacterial groups are selected for depending on their location in this environment (Liljeroth *et al.*, 1991; Lemanceau *et al.*, 1995). It is likely then, that

selection for disease antagonist also varies depending on the location of bacteria in the root-soil environment.

Culturing of a PGPR, such as Pc2140, on artificial media appears to select for phenotype variants which have lost the ability to produce metabolites with antimicrobial properties (Barnett, 1994). The production of these metabolites has no selective advantage on artificial media, and they may become toxic to the bacterium after prolonged periods (Barnett, 1994; Rich *et al.*, 1994). These same metabolites must however confer some advantage to the bacterium in the environment for their production to be maintained in the population. The pressures in the root-soil environment which select for variants with antimicrobial properties are probably not homogeneous, and a previous experiment showed that when the wild type (OP) Pc2140R was inoculated into take-all diseased wheat-soil pot microcosms in a 1:1 ratio with an *in vitro* TR variant, the TR variant which lost metabolite production associated with disease control was favoured in the bulk soil (ratio 35:63 OP:TR after 5 weeks), but had no growth advantage on the wheat rhizoplane (1:1 OP:TR, Barnett, 1994). The influence of root lesions was not considered in this experiment.

Bacteria readily produce new phenotypes *in vitro*, and in PGPR isolates new phenotypes produced *in vitro* have lost the ability to control disease. It is not known if PGPR isolates produce variant phenotypes in the root-soil environment, or if they do, what impact this has on disease control. In this work it is hypothesised that root lesions select for disease antagonists. Selection may also be for variant phenotypes that arise which are increased in their ability to control disease. Culture of PGPR isolates under conditions which select for variants with improved disease control ability may provide a method of obtaining better adapted PGPR strains with increased ability to control disease.

5.1.1. Aims

To evaluate colonisation, by Pc2140R, of (1) root lesions on take-all diseased wheat, (2) non-lesioned sections of diseased roots and (3) healthy roots not exposed to disease.

To inoculate Pc2140R in a number of successive cycles in wheat-soil microcosms for an extended period to determine if new phenotypes are produced.

To compare populations of Pc2140R cycled on healthy wheat roots and on root lesions caused by Ggt with populations with the ancestral Pc2140R with respect to *in vitro* inhibition of microorganisms, control of take-all disease and GC-FAME profiles.

5.2. Materials and Methods

The hypothesis that root lesions select for variant phenotypes with increased ability to control disease was tested by culturing rifampicin resistant Pc2140R on healthy and diseased roots of wheat and comparing populations for the presence of variant phenotypes by plating onto TZCA. Pc2140R was selectively re-isolated from Kapunda soil-sand mix by plating onto media containing rifampicin (100 µg/ml). Reisolates from evolved populations were compared to the ancestral or parent strain stored at ultra cold temperatures (Lenski and Bennett, 1993; Lenski and Travisano, 1994)

5.2.1. Colonisation of take-all diseased and healthy wheat roots by Pc2140R

Colonisation of wheat roots by Pc2140R was assessed in Kapunda soil pot microcosms (described in Section 2.2.8) after six weeks growth. Plants were grown without added Ggt (healthy roots, 5 replicate pots) and with added Ggt (120 Ggt-infected ryegrass propagules per kg soil-sand mix, Section 2.2.5.; diseased roots, 10 replicate pots). Microcosms were inoculated with 8 ml of Pc2140R bacterial suspension (10^9 cfu/ml) at seeding (Section 2.2.9).

In a separate experiment, root colonisation was assessed from 14 week old wheat plants grown in 2 L non-draining pots containing 2.7 kg Kapunda soil-sand mix, 4 pots without added Ggt and 4 pots with added Ggt. These 2 L pot microcosms were prepared in the same manner as the standard pot microcosm except that six wheat plants were grown and inoculated with 55 ml of bacterial suspension (10^9 cfu/ml). Twice the rate of Ggt inoculum was added to the larger pots, ie. 240 propagules of Ggt-infested ryegrass per kg soil:sand mix.

Populations of Pc2140R were determined after 6 or 14 weeks from healthy, lesioned and non-lesioned roots sections (Section 2.2.11) by plating 10 fold dilutions of macerated root extracts onto TZCA containing cycloheximide (75 µg/ml) and rifampicin (100 µg/ml, TZCArif).

To determine if bacteria resistant to rifampicin (100 µg/ml) were present in Kapunda soll and on wheat roots after growth in Kapunda soil, root (healthy, lesioned and non-lesioned) and soil (healthy and Ggt-infested) extracts from 6 week old wheat-Kapunda soil microcosms uninoculated with Pc2140 were serially diluted (one in ten) and plated onto TZCArif. There were five microcosms with and without added Ggt. These microcosms are described in Section 3.2.5.

5.2.2. Production of variant colony types from Pc2140R in wheat-soil microcosms

To determine if Pc2140R produced variant phenotypes on wheat roots, initially pure suspensions of Pc2140R with the wild type (OP) colony morphology were cultured on successive cycles of healthy or diseased wheat roots. Six wheat plants were grown for 10 to 14

weeks in 2L pot microcosms containing 2.7 kg of soil-sand mix, as described above, for 8 cycles of wheat (C1 to C8) for a total of 108 weeks. Plants were harvested and populations assessed at C1, 14 wk; C2, 24 wk; C3, 39 wk; C4, 53 wk; C5, 65 wk; C6, 83 wk, C7, 97 wk and C8, 108 wk. Four pot microcosms contained healthy (take-all free, no added Ggt) wheat plants (pots -1 to -4). Four pots contained take-all diseased wheat plants (pots +1 to +4) with Ggt added at 240 propagules (0.46 g) Ggt infested ryegrass per kg soil:sand mix, to provide a highly diseased environment.

In the first cycle of wheat (C1) plants were inoculated with pure Pc2140R (OP colony type) suspensions. The ancestral Pc2140R, stored at -70°C, was streaked onto 4 Petri dishes of TZCArif. After 48 h, 5 single colonies from each Petri dish (total 20 colonies) were streaked onto NA plates (1 colony per plate) and incubated for 48 h. Bacteria were harvested in 3 ml SDW and cultures pooled. The resultant bacterial suspension was diluted to A_{550}=1 (10^9 cfu/ml) and 55 ml applied to soil surfaces at planting as described in Section 2.2.9.

At the end of each cycle of wheat (10-14 wk) the Pc2140R root populations from each pot were reisolated and inoculated into the next cycle of wheat. Populations from each pot were kept separate throughout the experiment, and reinoculated into the same pot with a fresh soil-sand mix and pregerminated wheat seeds. After 10 to 14 weeks growth the wheat roots from each pot were washed in tap water to remove adhering soil and pooled. Pc2140R populations were reisolated from healthy roots (pots -1 to -4) and diseased roots (pots +1 to +4) as described in Section 2.2.11. Diseased roots were divided into lesioned and non lesioned sections of root, and only the Pc2140R population from root lesions were used to inoculate the next cycle of wheat in the diseased microcosms. Macerated root extracts were serially diluted and plated onto TZCArif for estimation of bacterial populations and the presence (number and proportion) of variant colony types at the end of each cycle. Individual Pc2140R colonies on TZCArif dilution plates used estimate bacterial populations were purified and stored at -70°C as described in Section 2.2.4. These will be referred to as Pc2140R reisolates.

The Pc2140R inoculum for the next cycle of wheat was prepared by plating undiluted root extracts (100 µl) from healthy and lesioned root sections onto NA containing cycloheximide (75 µg/ml) and rifampicin (100 µg/ml), (NArif, 4 or 7 plates per population from lesioned and healthy roots respectively). Cultures were incubated for 48 h, harvested in 3 ml SDW, diluted to A_{550} = 1 (10^9 cfu/ml) and inoculated into a fresh microcosm. This procedure was repeated at the end of each cycle for a total of 8 cycles of wheat (C1 to C8) for a total time of 108 weeks.

Samples of the Pc2140R populations used as inoculum were stored at -70°C. The cells from the undiluted bacterial inoculum harvested from NArif plates (2 x 1 ml aliquots), were pelleted by centrifugation (12,000 x g for 5 min) and resuspended in 15% glycerol before freezing.

5.2.3. Control of take-all by mixed Pc2140R populations after three cycles of wheat

The suppression of take-all disease by mixed populations of Pc2140R was assessed after three successive cycles on wheat (C3) in standard wheat-Kapunda soil 300 ml pot microcosms as described in Section 2.2.8. Populations of Pc2140R, from healthy roots and root lesions after C3, were recovered from -70°C storage by streaking directly onto NA for preparation of bacterial inoculum to avoid the bias incurred by selecting single colonies. Populations from each of the 8 microcosms were kept separate. Each population was inoculated into healthy (no added Ggt) and diseased (added Ggt) pot microcosms, 2 replications of each. The experiment was set up as a 2 (+/- Ggt) x 4 (control with no bacterial inoculum, and inoculated with the ancestral Pc2140R, and populations from healthy roots or diseased root lesions) factorial, RCBD with 8 replications. The 8 replications for the reisolated populations consisted of the 2 replicate pots of each of the four populations from either healthy roots or root lesions. Plants were harvested after 5 weeks and assessed for plant growth parameters (Section 2.2.12).

5.2.4. Control of take-all by Pc2140R wild type and variant colony types from one microcosm after three cycles of wheat

The Pc2140R population from pot +3, C3, produced the lowest percent root lesions on wheat roots in the first assessment of take-all control by mixed populations from C3 (described above, results Section 5.3.3). The Pc2140R population from this pot contained a mixture of the wild type, opaque corrugated colony type (OP) and an opaque, red-centred, convex colony type (RC). The effect of this mixed population from pot +3, C3, on take-all was repeated in a standard pot assay and compared to control of take-all achieved by each of the two colony types. The Pc2140R population was separated into wild type (OP) and variant (RC) colony types by spreading 10 fold dilutions of the population from pot +3, C3, stored at -70°C onto TZCA. Well separated single colonies (80) of each colony type were streaked onto NA, incubated for 48 hours and prepared for inoculation into pots as before (Section 2.2.9.). Reisolates of each colony type were pooled after harvesting from NA plates. Wheat plants were inoculated with the ancestral Pc2140R, the mixed Pc2140R population from pot +3, C3, and Pc2140R reisolates with the wild type (OP) or variant (RC) colony types. Uninoculated controls (+/- Ggt) were included and plants grown for 5 weeks. Plant growth parameters (Section 2.2.12) were analysed as ANOVA, RCBD with 8 replications of each treatment.

5.2.5. Control of take-all by mixed Pc2140R populations after eight cycles of wheat

The mixed Pc2140R populations from each of the eight microcosms were assessed for control of take-all disease after eight successive cycles of wheat (C8) in a standard pot assay. Mixed populations were recovered from -70°C storage by streaking directly onto NA, as described

previously, for inoculation into pots (Section 5.2.3). The mixed Pc2140R populations from each microcosm were kept separate and compared to the ancestral Pc2140R isolate and uninoculated controls (+/- Ggt). Plants were grown for 6 weeks, 8 eight replicate pots for each population and controls. Plant growth parameters (Section 2.2.12) were analysed as ANOVA, RCBD.

5.2.6. Control of take-all by individual Pc2140R reisolates recovered after eight cycles of wheat

After eight cycles of wheat (C8), six Pc2140R reisolates from each of the eight microcosms were assessed for control of take-all disease in a standard pot assay. Four experiments were carried out consisting of six reisolates from both a healthy and a diseased pot, the ancestral Pc2140R and +/- Ggt uninoculated controls. Paired healthy and diseased pots for the four experiments were pots +1 and -1; pots +2 and -2; pots +3 and -3; and pots +4 and -4. From each of the Pc2140R populations from C8, three reisolates with the wild type (OP) colony morphology and three with a red centred (RC) colony morphology were assessed, except for pot +1 and pot +3. From pot +1, one wild type (OP) colony and five RC colonies were assessed. From the pot +3 population, one wild type (OP) colony , three opaque colonies with a red star shaped centre (OPS) and two RC colony types were assessed. The Pc2140R reisolates were isolated on TZCArif at the end of the eighth cycle (C8) of wheat, purified and stored at -70°C (Section 2.2.4). For preparation of the bacterial inoculum, reisolates were streaked from -70°C onto TZCA for 48 hr to check for purity of cultures. For each reisolate, single colonies (4) of the appropriate colony type were streaked onto NA, one colony per plate, harvested after 48 h and inoculated into pots as before (Section 2.2.9). Plants were grown for 4 weeks and plant growth parameters analysed as ANOVA, RCBD with 8 replications.

5.2.7. Comparison of Pc2140R wild type and variant colony types from pot +3 after five cycles of wheat by GC-FAME and inhibition profiles

In order to determine the variation between similar colony types from the same population, five reisolates of each colony type (wild type, OP; or red centred variant, RC) from pot +3 (C5, 65 wk) were compared by GC-FAME profile and inhibition against seven test microorganisms (Table 2.2). The population from pot +3 was selected as this population had the most beneficial impact on plant growth when the mixed population was assessed at the end of C3. GC-FAME extraction and analysis is described in Section 2.2.13. *In vitro* inhibition tests are described in Section 2.2.14. Two replicate GC-FAME analysis and inhibition assays were carried out for each Pc2140R reisolate.

5.2.8. Comparison by *in vitro* inhibition of Pc2140R wild type and variant colony types from eight populations after six cycles of wheat

In order to determine the relationship between the same colony types derived from independently evolved mixed populations of Pc2140R, reisolates of each colony type from each pot were assessed for their ability to inhibit four test microorganisms. Five Pc2140R reisolates of each colony type (OP or RC) from each of the eight microcosms were assayed for *in vitro* inhibition of Ggt, *R. solani*, *P. irregulare* and *B. cereus* as described in Section 2.2.14. Five reisolates of the same colony type from each pot were spotted (10 µl, 10^9 cfu/ml overnight NB culture) onto the same petri dish for each assay. Each assay was replicated three times.

5.2.9. GC-FAME and *in vitro* inhibition profiles of Pc2140R colony types reisolated from roots after eight cycles of wheat

Each of the Pc2140R reisolates assessed for control of take-all at the end of eight successive cycles of wheat (Section 5.2.6) were assessed for *in vitro* inhibition of Ggt, *R. solani* and *P. irregulare* and for GC-FAME profiles. These were compared to the ancestral Pc2140R. GC-FAME analysis is described in Section 2.2.13. *In vitro* inhibition assays are described in Section 2.2.14. Two replicates of each reisolate were carried out.

5.3. Results

5.3.1. Colonisation of take-all diseased and healthy wheat roots by Pc2140R

When Pc2140R was added to wheat-soil pot microcosms as a soil drench, and populations on macerated roots measured after six or ten weeks, there were significantly higher populations of the introduced bacteria on the black necrotic root lesions compared to non-lesioned sections from the same diseased roots ($P<0.05$, Fig. 5.1). Populations from non-lesioned sections of diseased roots and healthy roots were similar and were not significantly different (Fig. 5.1). Populations were 2.5×10^3 cfu/cm on healthy and non-lesioned roots and increased 400 fold to 10^6 cfu/cm on root lesions at 6 wk. Pc2140R populations were 30 fold greater (4×10^4 cfu/cm root) on root lesions at 14 weeks compared to healthy and non-lesioned sections of wheat roots (Fig. 5.1). These results were consistent when Pc2140R populations on wheat roots were assessed at the end of each cycle of wheat (data not shown). There were no bacteria resistant to rifampicin (100 µg/ml) recovered from roots or soil on TZCArif from microcosms that had not been inoculated with Pc2140R.

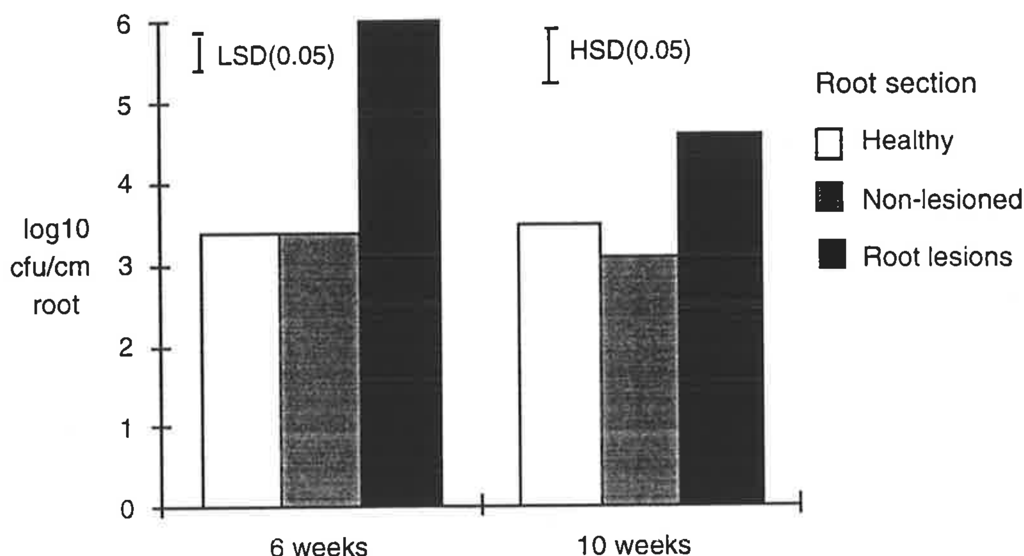


Fig. 5.1. Root colonisation by Pc2140R on healthy wheat roots, and on root lesions and non-lesioned sections of diseased roots. Populations of Pc2140R on roots were estimated on TZCArif after 6 weeks (n=5 healthy roots; n=10 diseased roots) and 10 weeks (n=4) of plant growth.

5.3.2. Production of variant colony types from Pc2140R in wheat-soil microcosms.

When Pc2140R was cultured and cycled on wheat roots, mixed populations were detected after one growth cycle of wheat (14 wk). Six out of eight populations contained a detectable phenotype variant at 1-10% of the Pc2140R population. All eight pots contained mixed populations after the second cycle (24 wk) and the proportion of variants increased with subsequent cycles of wheat (Fig. 5.2). After five cycles (65 wk) a red-centred, cream opaque colony variant (RC) was the only variant type detected, making up 10 to 50 percent of the Pc2140R population. This increased to 20 to almost 100% of the population after eight cycles (108 weeks) on wheat roots (Fig. 5.2). The progress of change in the population from pot +1 at C2, C5 and C8 is shown in Fig. 5.3. In this pot, wild type colonies of Pc2140R were less than 1% of the population after 108 wk. One population, (pot +3), produced a RC colony type and another colony type similar to the wild type colony morphology, but less corrugated with a star shaped red centre (OPS; Fig. 5.4). These OPS colony types were first noticed after C7 (97 wk) and may have been present earlier but not distinguished from the OP colony types due to their similar appearance on TZCA. The OPS types eventually dominated the Pc2140R population in pot +3 (Figs. 5.2 and 5.4).

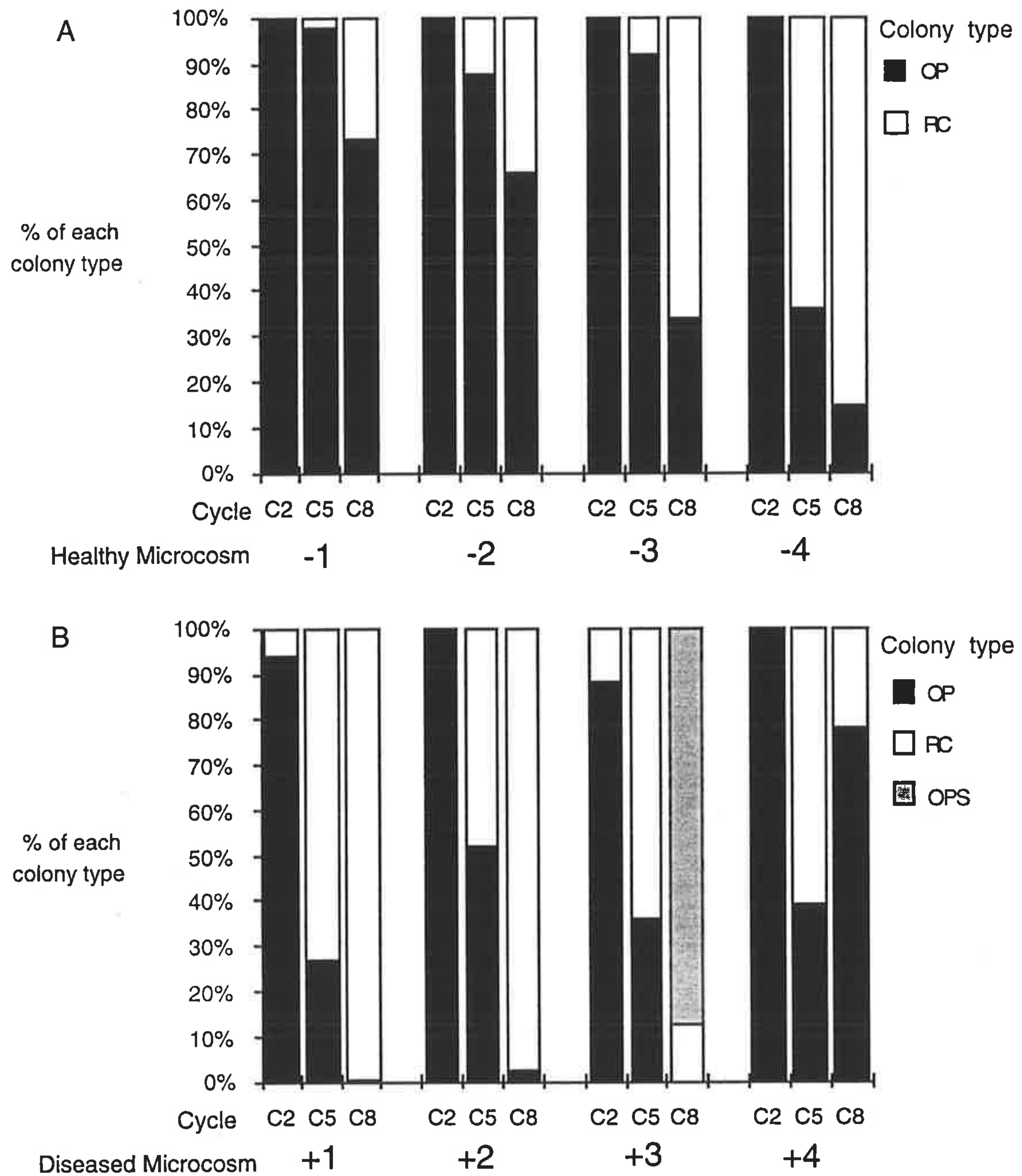
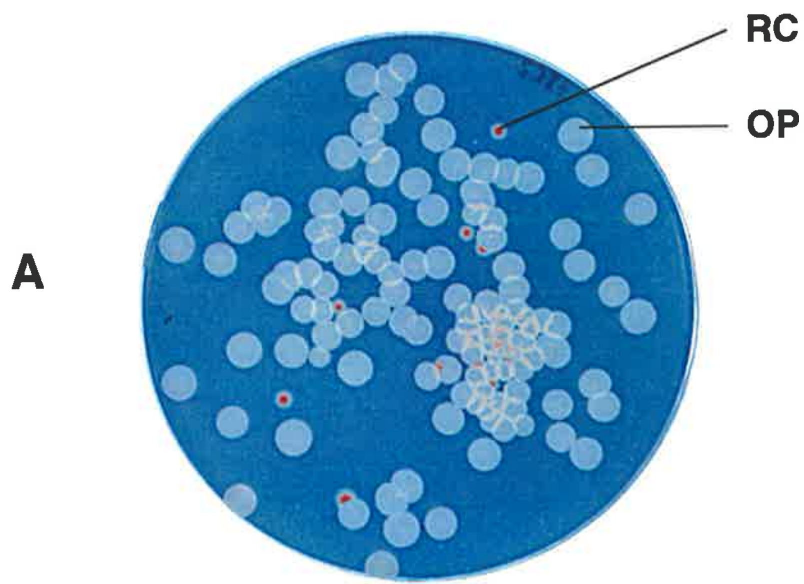
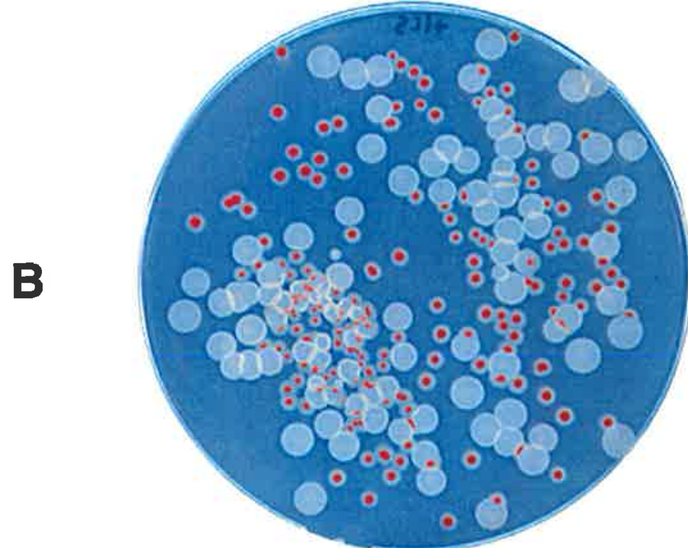


Fig. 5.2. Proportion of different colony types in Pc2140R populations cultured on healthy roots (A) and diseased root lesions (B) of wheat after two (C2, 24 wk), five (C5, 65 wk) and eight (C8, 108 wk) cycles of wheat. Pc2140R was cultured in healthy (no added Ggt) pot microcosms (A) and diseased (added Ggt) pot microcosms (B). Populations are expressed as the percentage of each colony type to the total Pc2140R population. Colony types on TZCA are: opaque corrugated wild type (OP); Red centred variants (RC) and opaque colony types with a red star shaped centre (OPS).

Fig. 5.3. Changes in the Pc2140R population cultured on diseased root lesions in pot +1 after two (A; C2, 24 wk), five (B; C5, 65 wk) and eight (C; C8, 108 wk) cycles of wheat. Populations at each time are shown after 72 h growth on TZCA in 9 cm Petri dishes at 25°C. Wild type (OP) and RC colony types are indicated.


A
RC
OP


B

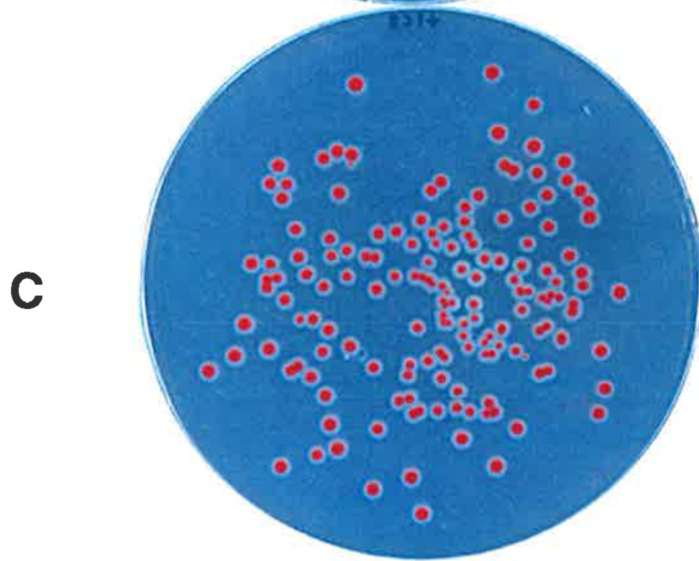
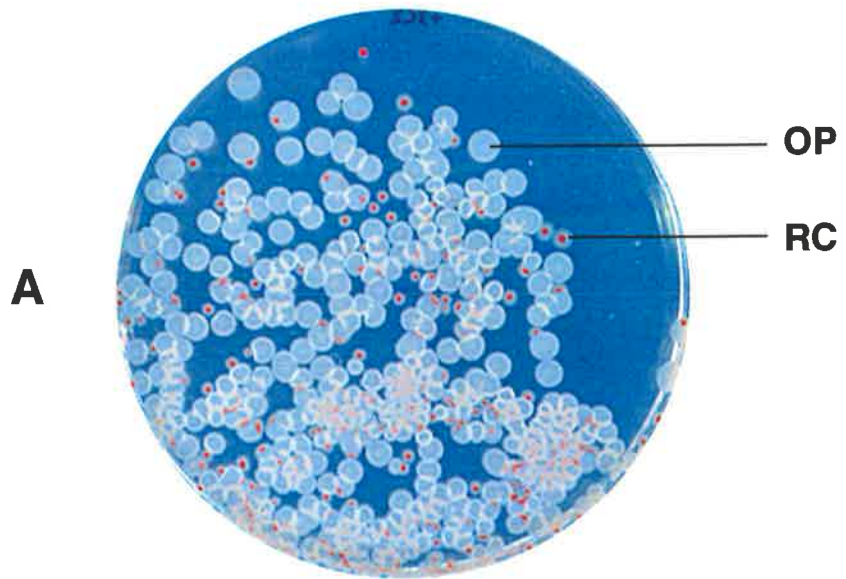
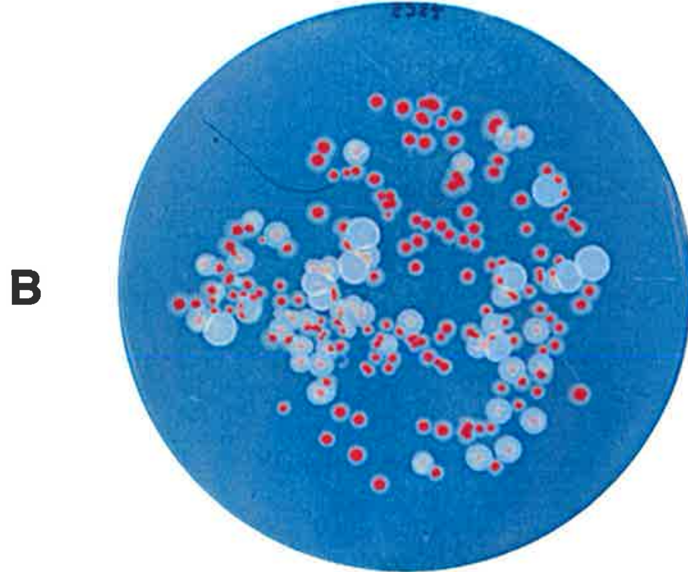
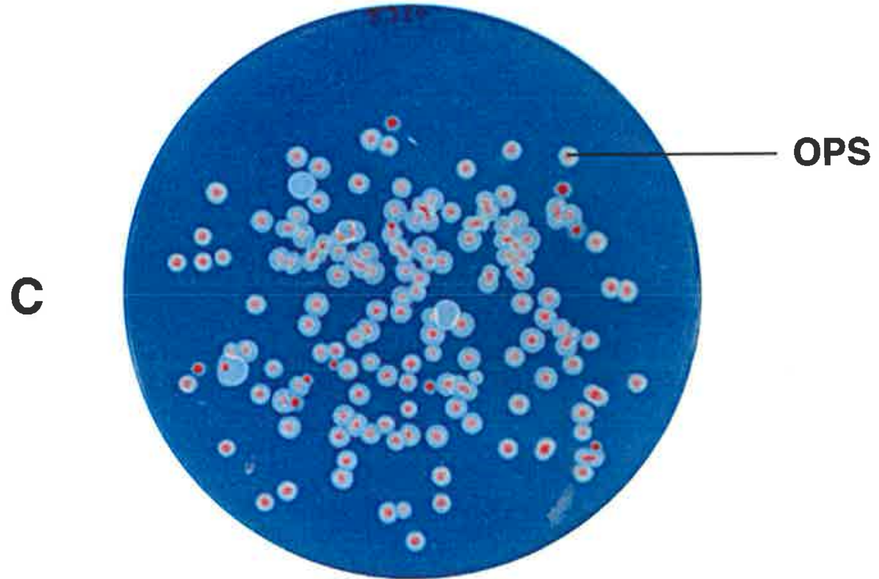

C

Fig. 5.4. Changes in the Pc2140R population cultured on diseased root lesions in pot +3 after two cycles (A, 24 wk), five cycles (B, 65 wk) and eight cycles (C, 108 wk) of wheat. Populations at each time are shown after 72 h growth on TZCA in 9 cm Petri dishes at 25°C. Wild type (OP), RC and OPS colony types are indicated.


A
OP
RC


B


C
OPS

5.3.3. Control of take-all by mixed Pc2140R populations after three cycles of wheat

The mixed Pc2140R populations from healthy and diseased microcosms after C3 (39 wk) were assessed for control of take-all disease in a standard pot assay. The ancestral Pc2140R and mixed populations from healthy and diseased microcosms significantly ($P<0.05$) increased shoot dry weight on plants inoculated with Ggt (Fig. 5.5). The ancestral Pc2140R and mixed populations from C3 decreased root disease severity, but this was only significantly different from control plants ($P<0.05$) when plants were inoculated with populations cycled on root lesions (Fig. 5.5). The effect of mixed populations on plant growth and root lesions was not significantly ($P>0.05$) different to the ancestral Pc2140R. Plants inoculated with the population from pot +3 had the greatest reduction in take-all severity. The ancestral Pc2140R and mixed populations also increased shoot dry weight 3-10% in plants not inoculated with Ggt, but this was not significant ($P=0.082$).

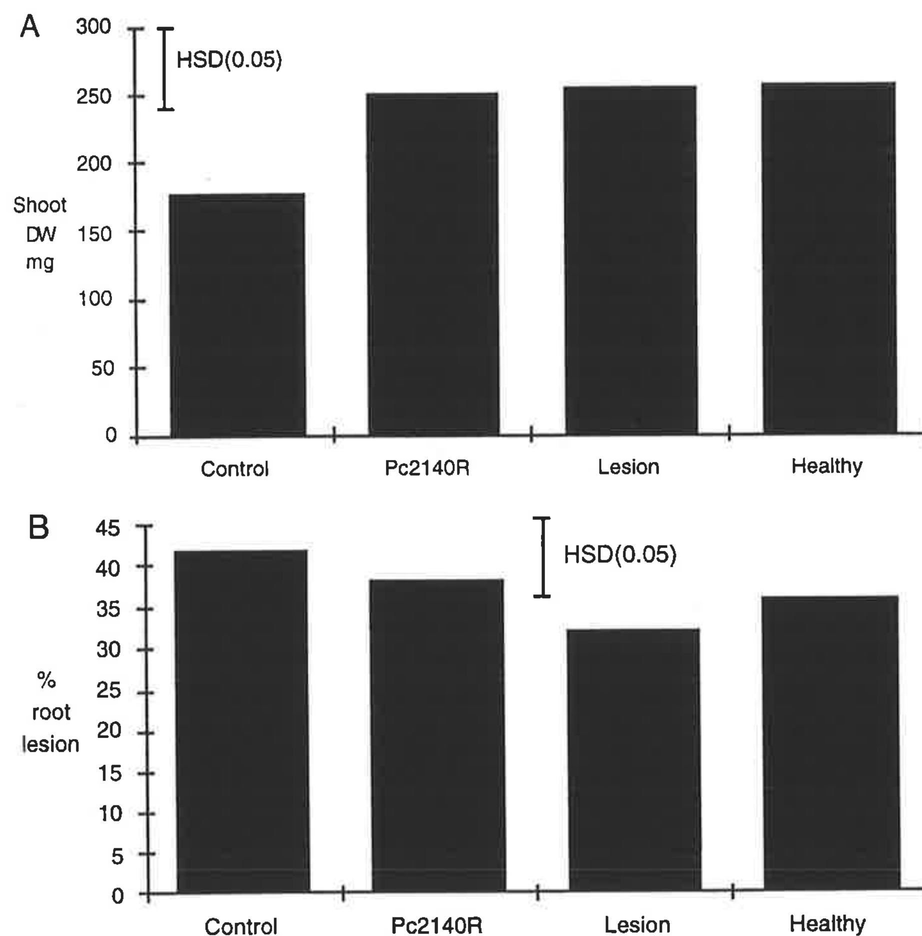


Fig. 5.5. Shoot dry weight (DW) of wheat (A) and average percent root lesions (B) on wheat roots of take-all infected wheat inoculated with the ancestral Pc2140R and mixed Pc2140R populations from the third cycle (C3, 39 wk) of wheat. Treatments are: Control, no added bacteria; and plants inoculated with the ancestral Pc2140R (Pc2140R); populations from diseased root lesions (Lesion); and populations from healthy roots (Healthy). All treatments were inoculated with Ggt, n=8.

5.3.4. Control of take-all by Pc2140R wild type and variant colony types from one microcosm after three cycles of wheat

As the mixed Pc2140R population from pot +3 after C3 (39 wk) produced the greatest reduction in disease in the previous experiment (Section 5.3.3), the wild type (OP) and variant (RC) colony types from this population were separated and assessed separately for their ability to control take-all, and compared to the ancestral Pc2140R strain. Shoot dry weight was increased and take-all severity was significantly reduced by all bacterial treatments compared to control diseased plants ($P<0.001$, Fig. 5.6). When the treatments inoculated with bacteria were analysed separately from uninoculated controls, the mixed population and the wild type and variant colony types from pot +3 significantly ($P<0.05$) increased shoot dry weight of plants compared to the ancestral Pc2140R, but there was no difference in the ability of these populations to reduce root disease compared to the ancestral Pc2140R (Fig. 5.6).

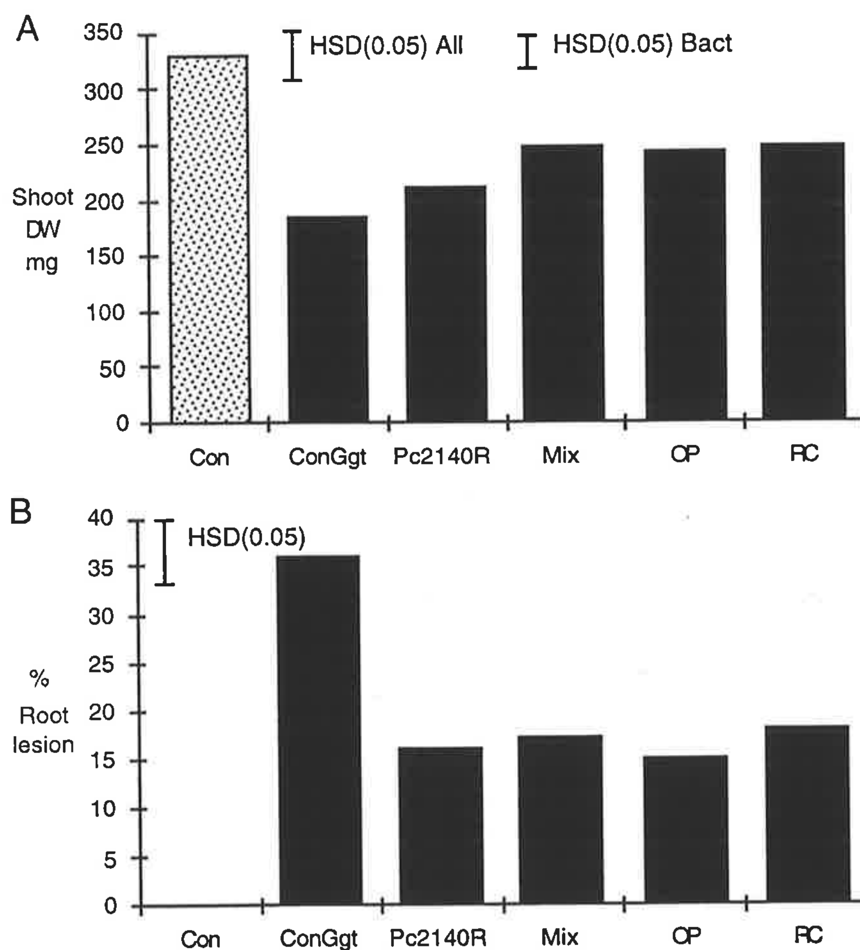


Fig. 5.6. Shoot dry weight (DW) of wheat (A) and average percent root lesions (B) on wheat roots inoculated with wild type and variant colony types from pot +3 after three cycles of wheat (C3, 39 wk). Treatments are: control, no added Ggt or bacteria (Con); control with added Ggt but no bacterial inoculum (ConGgt); and diseased plants inoculated with the ancestral Pc2140R (Pc2140R); the mixed Pc2140R population from pot +3, C3 (Mix); wild type colonies from pot +3, C3 (OP); and red centred variant colony types from pot +3, C3 (RC). HSD in A shown for all treatments (All) and for treatments inoculated with bacteria (Bact), n=8.

5.3.5. Control of take-all by mixed Pc2140R populations after eight cycles of wheat

After eight cycles of wheat (C8, 108 wk) the mixed populations from each pot microcosm were assessed for their ability to control take-all. Shoot weight and disease severity on Ggt-infected plants inoculated with mixed Pc2140R populations were similar to plants inoculated with the ancestral Pc2140R and not significantly different (P>0.05). The plants inoculated with the mixed population from pot -4 had a slightly higher shoot dry weight (2% increase) compared to plants inoculated with the ancestral Pc2140R. The populations from other pots produced a slightly lower shoot dry weight (2-4% reduction) and increased disease severity (4-16% increase) compared to the ancestral Pc2140R inoculated plants.

5.3.6. Control of take-all by individual Pc2140R reisolates recovered after eight cycles of wheat

After eight cycles of wheat (C8, 108 wk), six Pc2140R reisolates (consisting of wild type and variant colony types) from each pot microcosm were assessed for their ability to control take-all disease. These were assessed in four experiments, each consisting of six reisolates both from a healthy and a diseased microcosm (ie. pots +1 and -1; pots +2 and -2; pots +3 and -3; pots + and -4). None of the reisolates from C8 significantly (P>0.05) altered shoot weight or disease severity in the presence of Ggt when compared to the ancestral Pc2140R strain (Figs. 5.7 and 5.8). Reisolates with the wild type colony morphology were generally the same or slightly increased in their ability to control take-all, and reisolates with a variant colony type were generally the same or decreased in their ability to control take-all disease compared to the ancestral Pc2140R (Figs. 5.7 and 5.8). Some reisolates however, were significantly (P<0.05) different from other reisolates. For example, OP3 from pot-1 significantly reduced disease severity compared to plants inoculated with RC1 from pot-1 and RC1, RC2 and RC3 from pot +1 (Fig. 5.8A). Diseased plants inoculated with OP3 (pot +2) had increased shoot growth compared to the RC isolates (RC1, RC2 and RC3) from the same population (pot +2, Fig. 5.7B). With reisolates from pot-3, OP1 increased shoot weight compared to RC1 and RC3 (Fig. 5.7C). In one case, two OP colony types were significantly different (P=0.05) from each other in their effect on shoot growth. Plants inoculated with OP1 from pot +4 were increased in shoot growth compared to OP2 from pot +4 (Fig. 5.7D). Whether the reisolates were recovered from populations cultured on healthy or diseased roots had no effect on the range of ability of the reisolates to control take-all (Figs. 5.7 and 5.8).

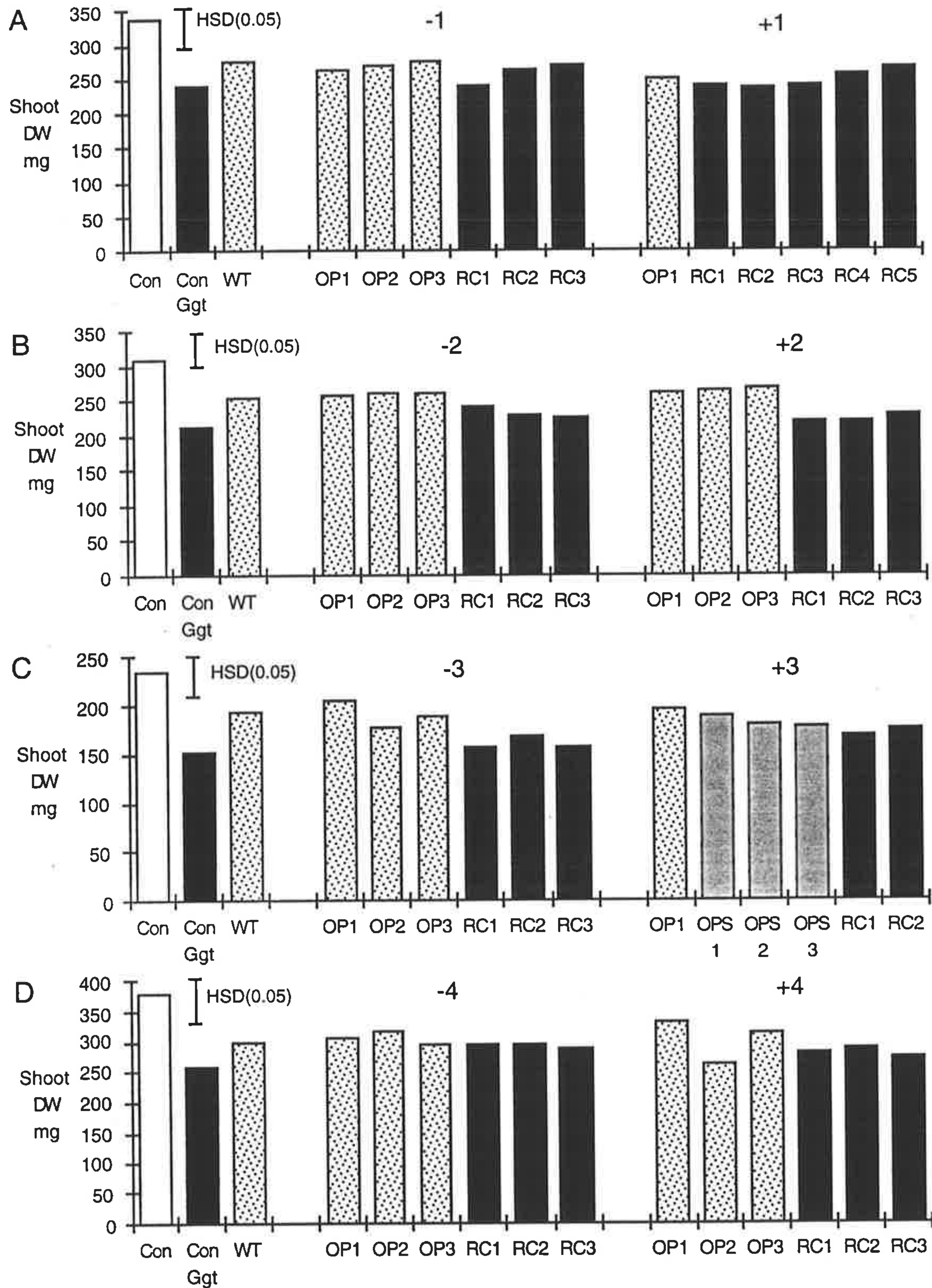


Fig. 5.7. Shoot dry weight (DW) of wheat plants inoculated with individual Pc2140R reisolates with the wild type (OP) and variant (RC and OPS) colony types recovered after eight cycles (C8, 108 wk) of wheat. Take-all diseased plants were inoculated with reisolates in four assays consisting of six reisolates from: pots -1 and +1 (A); pots -2 and +2 (B); pots -3 and +3 (C); and pots -4 and +4 (D). In each assay control plants were included, which were: no added Ggt or bacteria (Con); added Ggt but no added bacteria (ConGgt); and added Ggt inoculated with the ancestral wild type Pc2140R (WT). Columns with the same shading indicate reisolates with the same colony morphology on TZCA. n=8

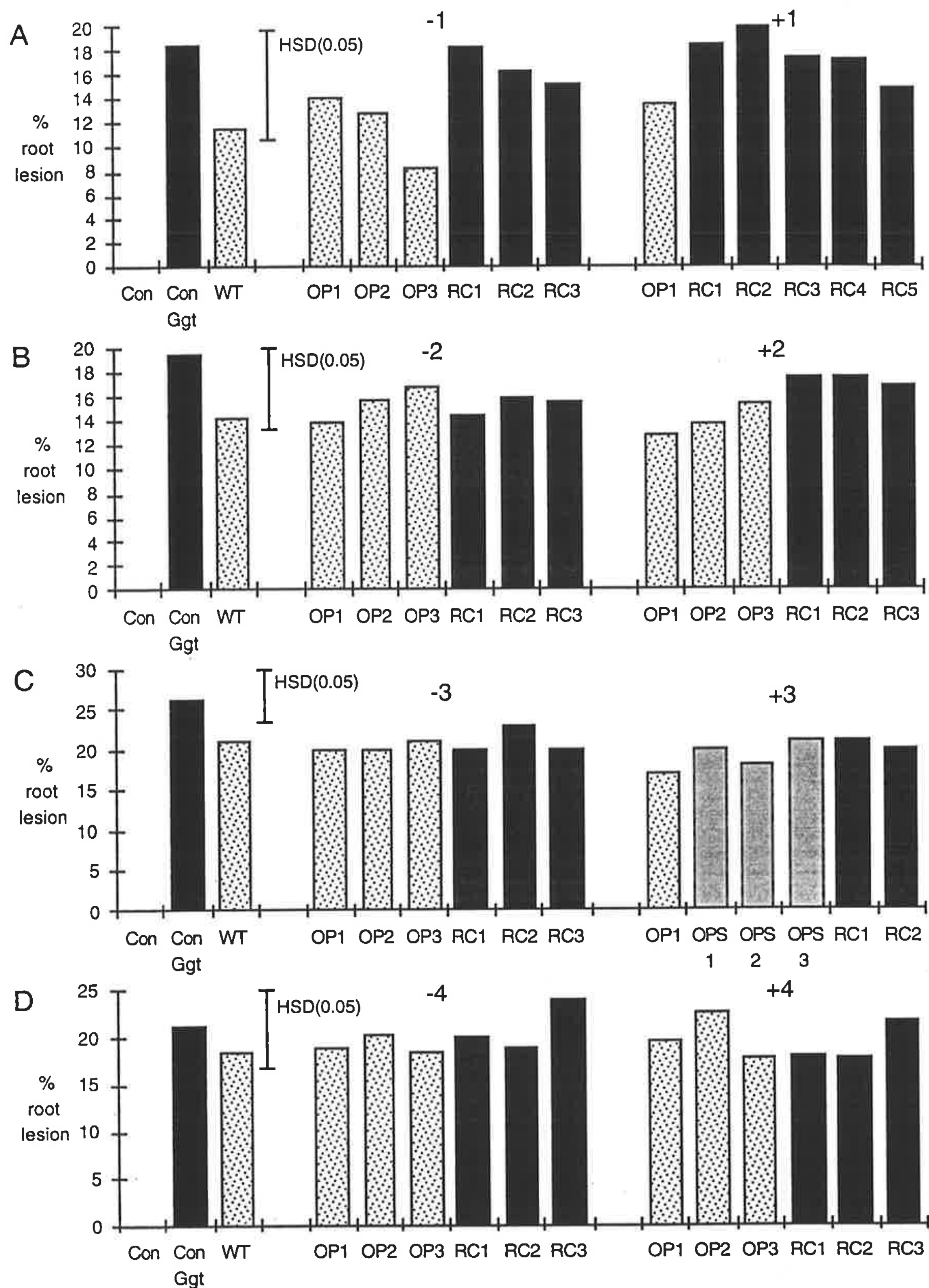


Fig. 5.8. Percentage of root length occupied by lesions on wheat seedlings inoculated with individual Pc2140R reisolates with the wild type (OP) and variant (RC and OPS) colony types recovered after eight cycles (C8, 108 wk) of wheat. Take-all diseased plants were inoculated with reisolates in four assays consisting of six reisolates from: pots -1 and +1 (A); pots -2 and +2 (B); pots -3 and +3 (C); and pots -4 and +4 (D). In each assay control plants were included, which were: no added Ggt or bacteria (Con); added Ggt but no added bacteria (ConGgt); and added Ggt inoculated with the ancestral wild type Pc2140R (WT). Columns with the same shading indicate reisolates with the same colony morphology on TZCA. n=8.

5.3.7. Comparison of Pc2140R wild type and variant colony types from pot +3 after five cycles of wheat by GC-FAME and inhibition profiles

To determine the variation between reisolates with the same colony morphology from the same Pc2140R population, five reisolates with the wild type (OP) and five with the variant (RC) colony types from pot +3 after five cycles of wheat (C5, 65 wk) were assessed for their *in vitro* inhibition of microorganisms and GC-FAME profiles. Reisolates with the same colony morphology were similar to each other in both *in vitro* inhibition characteristics (Table 5.1) and GC-FAME profiles (Fig. 5.9). Reisolates after C5 with a different colony morphology (OP or RC) were different from each other and both colony types diverged from the ancestral Pc2140R strain (Table 5.1 and Fig. 5.9). Where inhibition of microorganisms by reisolates differed from the ancestral Pc2140R, inhibition was reduced (Table 5.1).

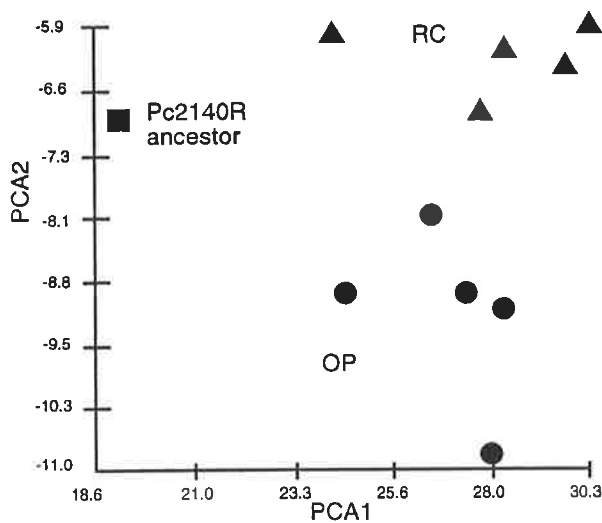


Fig. 5.9. Comparison of GC-FAME profiles of Pc2140R reisolates from pot +3 after five cycles of wheat by principal component analyses (PCA). Five reisolates with the wild type (OP) and five variant colony types (RC) were recovered from pot +3 after C5 (65 wk) and subject to GC-FAME analyses, two replicate extractions for each reisolate. Mean GC-FAME profiles were compared to the ancestral Pc2140R (mean of eight replicates) by PCA using principal components one and two. OP colonies are shown as circles, RC types as triangles.

Table 5.1. Inhibition of 7 microorganisms by the ancestral Pc2140R, and wild type (OP) and variant (RC) colony types reisolated from pot +3 after 5 cycles (65 wk) on diseased wheat roots. Five reisolates of each colony type were assessed, with 2 replications for each reisolate. Symbols: - indicates no inhibition; + to ++++ indicates increasing level of inhibition. Each reisolate with the same colony morphology had the same inhibition characteristics, except where shown.

Test organism	Pc2140R Ancestor	OP colony types	RC colony types
Ggt-8	+++	+	+++
R. solani AG-8	++++	++	+++ or ++++
P. irregulare BH25	+++	+++	++
T. koningii 7a	+++	++ or +++	++ or +++
Rp. oryzae fu1	++	+++	+++
P. putida K4	+	+	- or +
B. cereus bu3	+++	++	+

5.3.8. Comparison by *in vitro* inhibition of Pc2140R wild type and variant colony types from eight populations after six cycles of wheat

To determine the relationship between reisolates with the same colony morphology derived independently from replicate populations, five reisolates of each colony type (OP and RC) from each population after C6 (83 wk) were assessed for their ability to inhibit Ggt, *R. solani*, *P. irregulare* and *B. cereus*. There was no difference in inhibition of microorganisms by reisolates with the same colony type from the same population. Reisolates with a wild type (OP) colony morphology gave similar inhibition profiles to the ancestral Pc2140R, except for OP reisolates from pots +3 and -3 which were slightly reduced in inhibition of *R. solani* (Table 5.2). Reisolates with the RC colony type from different populations were different from each other, and the ancestral Pc2140R, in their inhibition profiles (Table 5.2). Inhibition of microorganisms by all RC colony types was reduced compared to the ancestral Pc2140R (Table 5.2).

Table 5.2. Inhibition of soil microorganisms by Pc2140R reisolates with the wild type (OP) and variant (RC) colony types reisolated from different pot microcosms after 6 cycles (83 wk) on wheat roots. Five reisolates of each colony type were assessed from each population, 3 replications for each reisolate. Reisolates with the same colony morphology from the same pot microcosm had the same inhibition characteristics: - indicates no inhibition, + to ++++ indicates increasing level of inhibition.

Pot	Colony type	Ggt-8	*R. solani* AG-8	*P. irregulare* BH25	*B. cereus* bu3
	Pc2140R	+++	++++	+++	+++
-1	OP	+++	++++	+++	+++
-2	OP	+++	++++	+++	+++
-3	OP	+++	+++	+++	+++
-4	OP	+++	++++	+++	+++
+1	OP	+++	++++	+++	+++
+2	OP	+++	++++	+++	+++
+3	OP	+++	+++	+++	+++
+4	OP	+++	++++	+++	+++
-1	RC	+	-	++	++
-2	RC	+	-	++	+
-3	RC	+++	+++	+++	+++
-4	RC	+++	+++	++	++
+1	RC	+	-	++	+
+2	RC	+	-	++	+
+3	RC	+++	++++	++	+
+4	RC	+	-	+	+

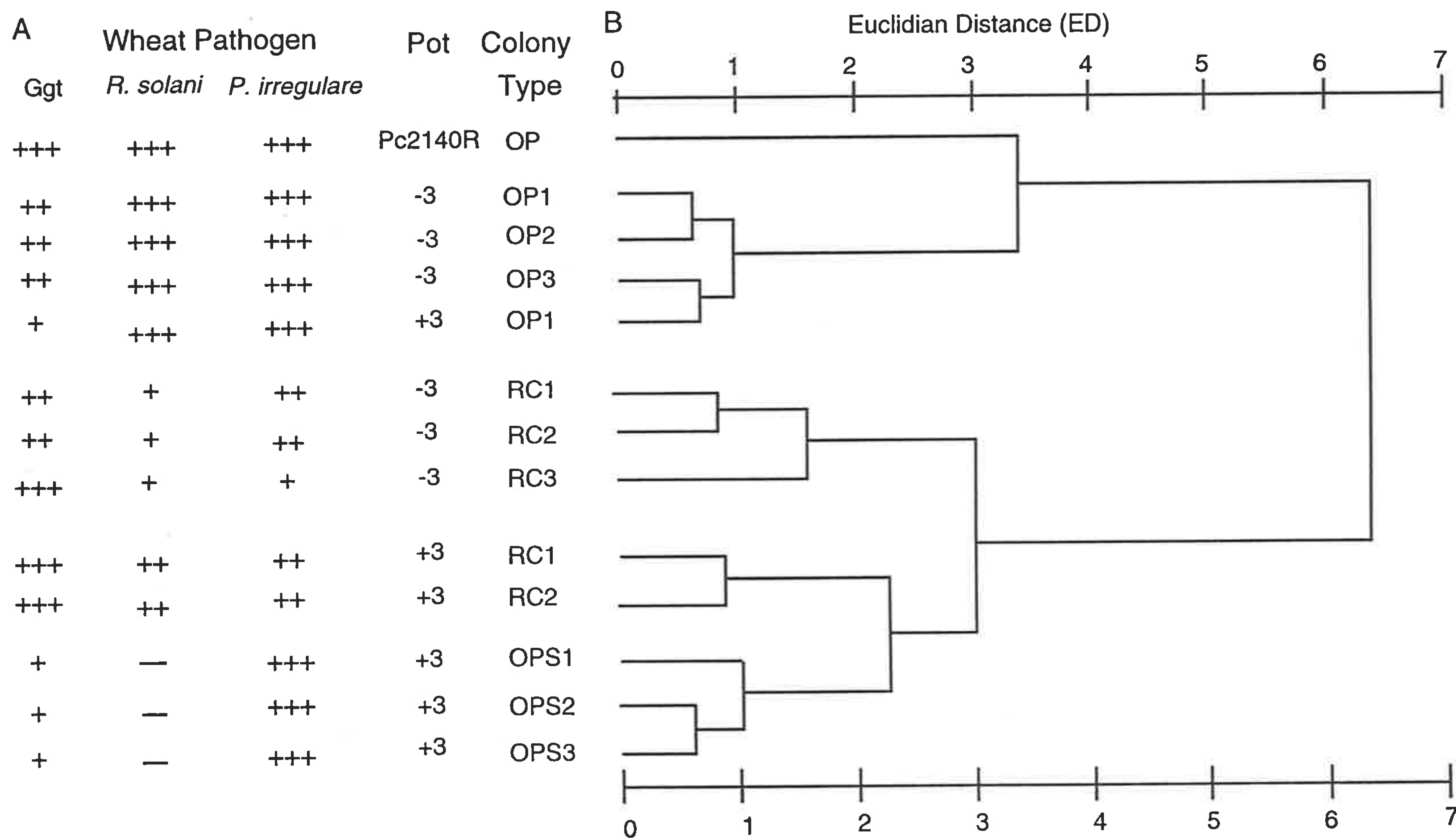


Fig. 5.10. Inhibition of wheat pathogens (A) and cluster analyses by GC-FAME profiles (B) by Pc2140R re-isolates with the wild type and variant colony morphologies recovered from a healthy (pot -3) and a diseased (pot +3) microcosm after eight cycles of wheat (C8, 108 weeks). Six reisolates from each microcosm were assessed for *in vitro* inhibition of Ggt, *R. solani* and *P. irregular* and for GC-FAME profiles which were compared to the ancestral Pc2140R. Inhibition is indicated by - no inhibition, + to +++ increasing inhibition. Hierarchical cluster analysis of mean GC-FAME profiles by UPGMA.

5.3.9. GC-FAME and *in vitro* inhibition profiles of Pc2140R colony types reisolated from roots after eight cycles of wheat

The six reisolates from each population that were assessed for their ability to control take-all disease after C8 (108 wk) were compared with each other and with the ancestral Pc2140R for their ability to inhibit Ggt, *R. solani* and *P. irregulare,* and by their GC-FAME profiles. Reisolates with the wild type colony morphology were similar to the ancestral Pc2140R in their ability to inhibit *R. solani* and *P. irregulare*, but generally reduced in their ability to inhibit Ggt *in vitro*, and were also divergent in their GC-FAME profiles (ca. 3 ED) compared to the ancestral Pc2140R. Variant colony types were reduced in their ability to inhibit the wheat pathogens compared to the ancestral Pc2140R, and diverged from the ancestral Pc2140R in their GC-FAME profiles at a ED >6. Reisolates with the same colony morphology from the same population were similar to each other, and different to reisolates from a different population. The inhibition profiles and relationship between GC-FAME profiles of the six reisolates from pots +3 and -3 are shown in Fig. 5.10. Similar results were observed for reisolates from the other populations (ie. pots -1, +1, -2, +2, -4 and +4).

5.4. Discussion

In the Introduction (Chapter 1) it was hypothesised that root lesions caused by Ggt would be a specialised niche which selects for disease antagonists, including selection of spontaneous phenotype variants arising which have increased antagonism towards take-all disease. Root lesions clearly support higher populations of bacteria and preferentially select for pseudomonads (Chapter 3). Work in this chapter demonstrated that root lesions also supported much higher populations of Pc2140R compared to non-lesioned sections of the same root (Fig. 5.1). The 30 to 400 fold difference in populations of Pc2140R between root lesions and non-lesioned sections of roots found here was much greater than the difference between populations of another PGPR, *P. fluorescens* 2-79, on wheat roots grown with and without Ggt, but in this case, the impact of root lesions was not assessed (Weller, 1983; Mazzola and Cook, 1991). The results reported here support the hypothesis that root lesions are a distinct niche, but there is little support from this section of work for the idea that variant phenotypes derived from populations cultured on root lesions are any different in their ability to control take-all disease from variants derived from populations cultured on healthy roots. This is in contrast to the situation observed *in vitro* where variant colony types commonly lose the ability to control disease.

When an initially pure culture of the wild type Pc2140R was inoculated into eight separate wheat-soil pot microcosms and cultured on successive generations of healthy or diseased wheat roots,

phenotype variants were produced in all eight Pc2140R populations. The time to the first appearance of these new variant phenotypes differed between different populations (ie. pots +1 and +3 contained RC colony types at 6% to 13% of the population, respectively, after 24 weeks, C2, but were not detected in pots -1 and -2 until 65 weeks, C5; Fig. 5.2). Although one variant colony type (RC) was produced in all populations, the inhibition characteristics and GC-FAME profiles of RC colony types from replicate populations were different. This indicates that new variant phenotypes were produced in a random manner and each population evolved separately. Research on *in vitro* cultures has also shown that replicate cultures diverge from each other in response to long term culture (Dykhuizen, 1990; Korona *et al.*, 1994; Lenski and Travisano, 1994). Lenski and Travisano (1994) suggest that independent populations reach "different fitness peaks of unequal height in the adaptive landscape". In the case of Pc2140R it is easy to see how populations could diverge in fitness as replicate populations appeared to evolve independently. This is evidenced by the time difference (up to 50 weeks) between populations of Pc2140R before variant types were detected, and different phenotypes becoming dominant in replicate populations (eg. pots +1 and +3; Figs. 5.3 and 5.4). These results are also in agreement with the Wright (1980) model of bacterial evolution, with frequency dependent selection being important with many different optimal genotypes.

Diversification of bacteria on, or in, artificial media in the laboratory has been shown to be greater in a heterogenous environment compared to homogenous environment (Korona *et al.*, 1994; Rainey and Travisano, 1998). It should reasonably be expected then that diversification in the complex and very heterogenous root-soil environment will be greater than in the relatively simple environment of artificial media and laboratory conditions. Based on the colony morphology of variants, it at first appeared that the diversification, or range of phenotypes produced, by Pc2140R on wheat roots was less than when Pc2140R was cultured in liquid medium *in vitro* (static A-DB, Chapter 4), as only the RC colony type was clearly different from the wild type (OP) colony morphology. On closer analysis, the range of variant phenotypes was in fact greater than it appeared at first because independently derived RC colony types from different populations cultured on wheat roots were not the same in their inhibition and GC-FAME profiles. In addition, the OPS colony types that were produced in only one population (pot +3) were similar to the wild type OP colony morphology but clearly different in their GC-FAME and inhibition characteristics (Fig. 5.10). Interestingly, some reisolates with identical colony morphology to that of the wild type also appeared to diverge from the ancestral Pc2140R in their GC-FAME profiles (Figs. 5.9 and 5.10).

Once variant phenotypes appeared in Pc2140R populations on wheat roots the proportion of variants increased with time, eventually dominating the population (Figs. 5.2, 5.3, and 5.4). This also occurs when spontaneous mutants are produced in laboratory cultures (Foster, 1993; Lenski and Mittler, 1993). That the variant types eventually dominate the population indicates that the Pc2140R variants have a greater fitness relative to the parent type in that situation, ie. on

wheat roots grown in Kapunda soil. Competition studies in which the Pc2140R ancestor and variant colony types were co-inoculated onto wheat roots could be carried out to confirm this. If the variant phenotypes produced by Pc2140R are more fit for colonisation of plant roots compared to reisolates with the wild type colony morphology, this increase in fitness was not reflected in increased disease control because the RC types were generally reduced in their ability to control take-all. Disease antagonism and root colonisation might then involve different mechanisms as suggested by Bull *et al.* (1991).

When mixed populations containing different colony types from either root lesions or healthy roots were compared to the ancestral Pc2140R for control of take-all, the populations cycled on wheat roots performed better (ie. increased shoot dry weight and reduced root lesions) than the ancestral Pc2140R when mean values were compared (Figs. 5.5 and 5.6). This difference however was small, and shoot dry weight was only significantly greater than that seen with the ancestral strain when the population from pot +3 was inoculated onto plant roots (Fig. 5.6A). In this case seedling shoot weight was increased 15% when plants were inoculated with the mixed population from pot +3 or with the wild type (OP) or RC colony types compared to the ancestral type.

When individual reisolates were assessed for their ability to control take-all after 108 weeks, changes in disease control compared to the ancestral type were not significant, but differences between some OP and some RC reisolates were significant (Figs. 5.7 and 5.8). This indicates that Pc2140R isolates within a population can diverge with respect to disease control. Importantly the variant colony types produced *in vivo* do not completely lose their ability to control disease in contrast to the TR colony types produced *in vitro* which have complete lose of disease control ability. The slight increase in disease control by reisolates with the wild type colony morphology may provide a mechanism for increasing the efficacy of PGPR isolates by inoculating them in soil and reisolating better performing strains.

It is possible, but unlikely, that the divergence of wild type Pc2140R colony types reisolated after culture in soil from the wild type Pc2140R ancestor may be due to changes in the ancestral Pc2140R stored at -70°C. Other researchers have also adopted this strategy to compare evolved populations to the ancestral population stored at ultra cold temperatures (Lenski and Bennett, 1993; Lenski and Travisano, 1994) . This method also appears to be suitable for comparison of Pc2140R reisolates as the GC-FAME profile of the ancestral Pc2140R strain stored in 15% glycerol at -70°C remained the same (ED <2) over the same period of time as this experiment (data not shown).

5.5. Conclusions

Pc2140R diversifies during culture on wheat roots in field soil to produce new variant phenotypes, and these phenotypes are different to those produced *in vitro.* Variant phenotypes with altered colony morphology were generally slightly less effective in controlling take-all disease compared to the ancestral Pc2140R. It is important to note that reisolates from wheat-soil microcosms with the wild type colony morphology also differed from the ancestral Pc2140R in GC-FAME profiles and that they were generally slightly increased in their ability to control take-all disease. Populations of Pc2140R were much higher on black necrotic root lesions produced after infection by Ggt compared to populations of Pc2140R on healthy roots and non-lesioned sections of diseased roots. There was no difference between populations cycled on root lesions and healthy roots with respect to (1) the production (ie. frequency and colony types produced) of new variant phenotypes or (2) the ability of new phenotypes produced on healthy or diseased roots to control take-all disease.

Chapter 6. Phenotypic Characterisation, Identification and Hierarchical Clustering of a Range of *Pseudomonas corrugata* strain 2140 Variants

6.1. Introduction

The rRNA homology group I of the genus *Pseudomonas* (Palleroni *et al.*, 1973) contains many well known and important species, including plant and animal pathogens, plant growth promoting rhizobacteria and bioremediation agents. Pseudomonads are also implicated in the suppression of take-all and other soil-borne diseases (Chapter 1). To study the ecology of this group in the environment, methods are needed that enable bacterial isolates to be identified and similar isolates to be grouped together. These are prerequisites for comment on the population structure and comparison of spatial and temporal differences between populations sampled from the environment where the true genetic relationships between isolates is unknown. Analysis of phenotypic characteristics such as gas chromatography of cellular fatty acid methyl esters (GC-FAME; Stead, 1992; Rainey *et al.*, 1994; Siverio *et al.*, 1996) and carbon source utilisation (Rainey *et al.*, 1994; Lemanceau *et al.*, 1995; Latour *et al.*, 1996; Frey *et al.*, 1997) have become increasingly important as rapid tools for identification and characterisation of bacterial isolates. Bacterial isolates, including Pc2140, can change phenotype (Chapters 4 and 5), but there is little information on the phenotype range that might be possible from the same basic genotype.

Studies have demonstrated bacterial plasticity, ie. that bacteria readily produce new phenotypes in the laboratory (Terzaghi and O'Hara, 1990; Rainey *et al.*, 1993). The spontaneous production of new phenotypes is of importance in *Pseudomonas* spp. isolated as antagonists of soil-borne plant pathogens because the common variant phenotypes were shown to undergo pleiotropic changes in phenotype, including loss of ability to control plant diseases. For example, loss of disease control ability in isolates of *P. fluorescens* was due to deleterious mutations in *lemA-gacA* type genes. These genes regulate the production of multiple secondary metabolites which contribute to disease suppression in the wild type isolate (Laville *et al.*, 1992; Corbell *et al.*, 1994; Gaffney *et al.*, 1994; Cook *et al.*, 1995). Genes homologous to *lemA/gacA* are also involved in phenotype conversion from virulence to avirulence in the plant pathogenic pseudomonads *P. syringae* (Hrabak and Willis, 1992; Rich *et al.*, 1994), *P. aeruginosa* (Rahme *et al.*, 1995) and in the closely related *Ralstonia (Pseudomonas) solanacearum* (Brumbly and Denny, 1990). Phenotype conversion has also been reported in a wide variety of other Gram negative bacteria, as well as Gram positive bacteria and actinomycetes (Section 1.4). The appearance of mutants with a pleiotropic change in phenotypic characteristics appears common,

but it is not known what impact phenotype conversion has on the phenotypic characterisation and grouping of isolates derived from environmental samples.

Work from previous Chapters (4 and 5) has shown that Pc2140R diversifies *in vitro* and on plant roots to produce new phenotypes altered in colony morphology, GC-FAME profiles and their ability to inhibit soil microorganisms and control disease. This shows that an initially homogenous population containing only one phenotype, can become a more complex heterogenous population containing more than one phenotype. In studies characterising bacterial populations from the environment mixed populations are isolated and characterised in an attempt to understand the population structure, however the true relationship between isolates is unknown. Populations of Pc2140 containing variant phenotypes can also be viewed as mixed populations, and importantly, in a mixed Pc2140 population we know that the variant phenotypes are derived directly, or indirectly via an intermediate variant phenotype, from a known common ancestor.

6.1.1. Aims

The work described in this chapter aimed to phenotypically characterise an artificial "mixed" population of 14 variants derived from a common ancestor (Pc2140R or Pc2140RL) by GC-FAME (biochemical analysis), BIOLOG (metabolic profile) and *in vitro* inhibition of seven soil microorganisms (ecological function). This would determine the range of phenotypes that may be produced by a single isolate, and the effect of phenotype change on: (1) the taxonomic identification of variant phenotypes; and (2) cluster analyses, or grouping of like isolates, based on these above characteristics. This will demonstrate the usefulness (or otherwise) of GC-FAME and BIOLOG for population studies.

6.2. Materials and Methods

6.2.1. Source of Pc2140 variant phenotypes

Variant phenotypes were produced by Pc2140R and Pc2140RL during culture in a static ammonium defined broth medium (A-DB; Chapter 4), and by Pc2140R during culture on wheat roots (Chapter 5). Based on colony morphology and initial GC-FAME analysis, 14 variants which represented a range of phenotypes produced from Pc2140R or Pc2140RL were selected for characterisation and for comparison to the ancestral strains. The source of variants is given in Table 6.1.

All the variant types used here were confirmed to be derived from Pc2140. Variant phenotypes from Pc2140RL were all positive for constitutive expression of the *lacZY* genes, as indicated by the formation of blue colonies on X-Gal medium containing glucose as the sole carbon source

(Drahos *et al.*, 1986; Ryder *et al.*, 1994; Chapter 4). Variant phenotypes from Pc2140R were shown to have the same genetic fingerprint as determined by macrorestriction fragment profiles and profiles of repetitive sequences amplified by PCR (Chapter 7).

Table 6.1. Source of variant colony types derived from Pc2140R and Pc2140RL.

Isolate	Source	
Pc2140R	rifampicin resistant Pc2140 (ancestor)	Chapter 2
TR4.Ri	A-DB; 25 day	Chapter 4
PPr6.Ri	A-DB; 25 day	Chapter 4
FNT8.Ri	A-DB; 25 day	Chapter 4
RC1.Rs	non-lesioned section of diseased roots; C1, 14 wk; pot +2	Chapter 5
RC4.Rs	healthy wheat roots; C3, 39 wk; pot -4	Chapter 5
RC5.Rs	root lesions; C3, 39 wk; pot +1	Chapter 5
RC6.Rs	diseased wheat roots; C3, 39 wk; pot +3	Chapter 5
DM1.Rs	non-lesioned section of diseased roots; C3, 39 wk; pot +3	Chapter 5
Pc2140RL	rifampicin resistant and *lacZY* marked Pc2140 (ancestor)	Chapter 2
TR1.RLi	A-DB; 11 day	Chapter 4
FNT8.RLi	A-DB; 11 day	Chapter 4
TRY19.RLi	A-DB; 11 day	Chapter 4
FNTO22.RLi	A-DB; 11 day	Chapter 4
TRY33.RLi	A-DB; 11 day	Chapter 4
LF48.RLi	A-DB; 11 day	Chapter 4

6.2.2. Phenotypic characterisation of Pc2140R, Pc2140RL and variant phenotypes

6.2.2.1. GC-FAME

GC-FAME profiles were determined using the Microbial Identification System (MIS) version 4 (MIDI Inc. Newark, Delaware). Fatty acids were extracted and prepared according to the MIS procedure described in Section 2.2.13. Fatty acid peaks were identified by the MIS system, and compared to the Sherlock TSBA aerobe library version 3.8 for taxonomic identification. A total of three independent replicates were performed for each variant isolate, and there were 8 and 4 replications for the parental isolates, Pc2140R and Pc2140RL respectively. A new separate library (2140V) was generated which included individual library entries of the mean GC-FAME profiles of Pc2140R, Pc2140RL and each of the colony morphology variants. The Sherlock Library Generation Software was used to cluster Pc2140R, three variants (TR4.Ri, PPr6.Ri and FNT8.Ri) and the Sherlock aerobe TSBA library entries for *P. fluorescens* and *P. putida* biovars, *P. corrugata*, *P. chlororaphis*, *P. savastanoi* pv. *nerium*. and *P. syringae* biovars *pisi* and *tomato*. The unweighted pair-group method using arithmetic averages (UPGMA) was used for

hierarchical cluster analysis and generation of a dendrogram. Fatty acid assignment of peaks in the gas chromatographs are given in Stead *et al.* (1992) and Siverio *et al.* (1996).

6.2.2.2. BIOLOG

Isolates were assayed for carbon source utilisation using the BIOLOG GN (Gram negative) MicroPlate^{TM} system (BIOLOG Inc., Hayward, CA) as per the manufacturers instructions. Each 96 well BIOLOG MicroPlate^{TM} contains 95 carbon sources plus a control well with no carbon source. Bacteria were subcultured twice on Tryptic Soy Agar (TSA; Tryptic soy broth (Difco) 30 g/l; Bacto-Agar (Difco) 15 g/l) for 24 h at 28°C. Bacteria were harvested by rolling a sterile cotton bud over the bacteria and resuspending the cells in sterile saline solution (0.85% NaCl). The bacterial suspension was diluted (A_{550nm}=0.3) and each well of the BIOLOG MicroPlate^{TM} inoculated with 150 μl of suspension at a cell density of $3x10^{8}$ cfu/ml. Two independent BIOLOG assays were performed for each of the Pc2140 parent strains and variant colony types. Tetrazolium dye reduction, indicating carbon source utilisation, was recorded after 24 h at 28°C. Dye reduction was scored as; - no colour; / extremely faint colouring (indeterminate); +, significant blue colour with the bottom of the well still visible; and ++ dark blue colour, bottom of well no longer visible. Isolates were identified using the associated MicroLog 2 computer software (BIOLOG Inc., Hayward, CA). For isolate identification by MicroLog 2 , dye reduction was entered as negative (-), indeterminate (/) or positive (+ or ++).

6.2.2.3. *In vitro* inhibition

The wild type *P. corrugata* 2140 inhibits a number of fungi and bacteria *in vitro*, and this character was altered in some variants (Chapter 5). *In vitro* inhibition by the parent and variant Pc2140 isolates was assayed against five soil fungi and two soil bacteria. These microorganisms included the wheat pathogens Ggt-8, *Rhizoctonia solani* AG-8 and *Pythium irregulare* BH25, a biological control fungus *Trichoderma koningii* 7a and a rhizosphere fungus *Rhizopus oryzae* fu1 and a Gram positive and Gram negative bacterium, *Bacillus cereus* bu3 and *Pseudomonas putida* K4 respectively. The source of these microorganisms is given in Chapter 2, Table 2.2.

Inhibition of each test organism was conducted on solid agar medium in 9 cm Petri dishes as described in Section 2.2.14. The medium used for each test microorganism was selected on the basis that the test organism was inhibited by the wild type *P. corrugata* 2140 strains but not by one or more colony morphology variants. All tests were done in duplicate and incubated at 25°C.

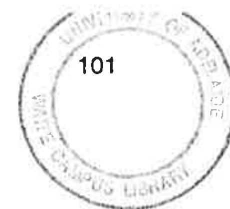
Inhibition of the test organisms by the wild type and variant Pc2140 was scored as; - no inhibition (test organism grew over bacterial patch); or + inhibition, with increasing + signs indicating increasing inhibition, as described in Chapter 2, Table 2.3.

6.2.3. Hierarchical clustering of Pc2140R, Pc2140RL and variant phenotypes

Hierarchical cluster analysis was carried out on the three data sets, GC-FAME, BIOLOG and inhibition profiles for Pc2140R, Pc2140RL and 14 variant colony types using the same clustering algorithm. The GC-FAME profiles for each isolate from the new 2140V GC-FAME library (Section 6.2.2.1) were analysed using the percent area for each peak, including unnamed peaks and summed features. Trace amounts were treated as zero. Carbon source utilisation after 24 h incubation, as measured by dye reduction, was used as the BIOLOG data. Of the 95 carbon sources, only 22 were analysed. These 22 were chosen because there were differences between at least two isolates in dye reduction which ranged from negative (-) or indeterminate (/) to positive (+ or ++). Lactose and lactulose were not included in the analysis as these are positive only for the *lacZY* marked Pc2140RL and its descendant variants. When carbon source use varied between replicate BIOLOG analysis, the value closest to the parent strain was used. Dye reduction results were transformed to numerical values for analysis; 0 (-); 0.1 (/); 0.5 (+) and 1.0 (++). The inhibition scores against each of the seven test organisms were transformed to numerical values, 0 (-), 1 (+) to 4 (++++), and scaled to fall between 0 and 1.0 (inhibition score/maximum inhibition). Cluster analyses were performed with GENSTAT 5 version 3.1 using the same similarity matrices and average linkage clustering method. For quantitative variables, the test parameter city-block was used (Gower, 1985). The commands for hierarchical cluster analysis by GENSTAT 5 are given in the Appendix. Hierarchical clustering was represented by dendrograms.

6.2.4. Comparison of cluster analysis using Procrustes comparison

Principal Component Analysis (PCA), using the first two principal components, for each of the three data sets were compared to each of the other profiles using Procrustes rotation. Procrustes rotation uses the fact that the relative distance between values from PCA are not altered by a change in the origin, scaling or rotating the principal component plots (Gower, 1975; Gower, 1985a and b) to compare the results of principal component analysis using different data. In paired comparisons of each combination of the three data sets (GC-FAME, BIOLOG and inhibition profiles), principle component plots (PCP) of the first two principal components were scaled and rotated to match one principle component plot onto the other by minimising the sum of squares using GENSTAT 5 version 3.1. The commands for Procrustes comparison of each paired combination of the three data set by GENSTAT 5 are given in the Appendix. The goodness of fit was measured by the residual sum of squares and given as the fitted configuration between 0 and 1.0.



6.3. Results

6.3.1. Phenotypic characterisation of Pc2140R, Pc2140RL and variant phenotypes

Variant colony types were phenotypically characterised using GC-FAME (biochemical characterisation), BIOLOG (substrate utilisation) and pattern of inhibition against a set of soil microorganisms (ecological characterisation or biocontrol potential). The GC-FAME, BIOLOG and inhibition profiles of Pc2140R, Pc2140RL (*lacZY* marked) and the 14 variant colony types are shown in Table 6.2. Variant colony types differed from the parent Pc2140R isolates in all three characteristics measured, and there were large differences between colony type variants. GC-FAME profiles varied slightly between replicate extractions and the mean percentage and standard deviation of each fatty acid present in the GC eluate for each isolate in the new 2140V library is given in Table 6.2. The predominant fatty acid was 16:0 (26.5-32.2% of the profile) and the most variable fatty acids were 12:0 (2.0-4.0%), 12:1 3OH (0.2-2%) and 17:0 cyclo (0.9-8.9%) (Table 6.2). Some fatty acids were not detected in some variants but were consistently present in other variants at a low percentage in the profile. These included for example, 10:0 (range 0-1.0%), unknowns eluting at 12.486, 13.961 and 14.503 minutes (0-0.5%), 16:0 3OH (0-0.3%) and 19:0 cyclo w8c (0-1.1%) (Table 6.2).

Carbon source utilisation by the parent and variant Pc2140 isolates was assessed using the BIOLOG system, with tetrazolium dye reduction to indicate carbon source use. Two replicate analysis were carried out for each isolate and the indicated carbon source use was generally consistent between replicates samples, however carbon source use sometimes varied between replicates, depending on the carbon source and Pc2140 isolate. When the indicated carbon source utilisation varied between replicate samples, this variation was generally between no dye reduction and indeterminate dye reduction and both values are shown in Table 6.2. The difference in carbon source use between replicate samples was much less than between different isolates. Of the 95 carbon sources there were differences (negative or indeterminate to positive) between at least two isolates in dye reduction in 22 carbon sources (Table 6.2). Carbon source use which did not vary substantially between isolates is shown in Table 6.3. Lactose and lactulose use was positive for Pc2140RL and all descendant variants, and negative for Pc2140R and all descendant variants. Interestingly, dye reduction by Pc2140RL of the 22 variable carbon sources was also positive (+) for many of the carbon sources which were apparently not used by Pc2140R. For example alaninamide, L-histidine and hydroxy L-proline were indicated to be used by Pc2140RL but not by Pc2140R. Dye reduction in the variable carbon sources was generally increased in the variant types compared to their parent, except for the FNT variants. In the variants FNT8.Ri and FNT8.RLi dye reduction was reduced or lost for a number of carbon sources compared to the parent Pc2140 strain (eg. D-mannitol and sucrose; Table 6.2).

Table 6.2. Fatty acid, BIOLOG and inhibition profiles of Pc2140R, Pc2140RL and their descendants. Profiles are described in text.

	% Fatty acid in profile (SD)						
Fatty acid	Pc2140R	TR4.Ri	PPr6.Ri	FNT8.Ri	RC1.Rs	RC4.Rs	RC5.Rs
10:0	tr[a]	0.0	0.0	tr	tr	0.4 (0.4)	0.3 (0.1)
10:0 3OH	5.1 (1.1)	3.1 (0.3)	3.1 (0.5)	4.2 (0.5)	3.5 (0.4)	5.0 (0.7)	3.7 (0.4)
12:0	2.2 (0.2)	3.0 (0.1)	2.6 (0.1)	4.1 (0.2)	2.5 (0.2)	2.6 (0.3)	3.0 (0.3)
unknown 12.486	tr	0	0	tr	0	tr	0
12:0 2OH	4.7 (0.3)	3.9 (0.2)	4.3 (0.2)	3.1 (0.1)	4.5 (0.2)	4.7 (0.2)	4.4 (0.3)
12:1 3OH	1.4 (0.4)	0.3 (0.0)	0.2 (0.0)	0.7 (0.1)	0.6 (0.1)	1.2 (0.4)	0.4 (0.2)
12:0 3OH	4.8 (0.6)	3.9 (0.3)	3.9 (0.5)	4.3 (0.3)	4.3 (0.4)	5.2 (0.4)	4.5 (0.3)
unknown 13.961	0.5 (0.0)	0.0	0	0.4 (0.0)	tr	tr	0
14:0	0.3 (0.0)	0.3(0.1)	0.5 (0.0)	0.4 (0.0)	0.3 (0.0)	0.3 (0.0)	0.4 (0.1)
unknown 14.503	0.3 (0.1)	0	0	0.3 (0.1)	tr	0.3 (0.1)	0
16:00	28.8 (1.1)	29.2 (0.6)	32.2 (0.8)	30.0 (1.0)	29.7 (0.6)	28.6 (0.6)	28.9 (0.6)
17:0 cyclo	8.8 (1.2)	1.4 (0.1)	2.0 (0.4)	10.3 (1.4)	4.1 (1.1)	5.8 (0.8)	2.3 (0.7)
16:0 3OH	tr	0	0	0.3 (0.0)	tr	tr	0
18:0	0.7 (0.1)	0.6 (0.1)	0.5 (0.0)	0.6 (0.1)	0.7 (0.1)	0.7 (0.0)	0.6 (0.0)
19:0 cyclo w8c	0.9 (0.2)	0	0	1.0 (0.1)	0.3 (0.1)	0.5 (0.1)	tr
summed feature 3	0.9 (0.2)	0	0	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	tr
summed feature 4	23.6 (1.6)	32.4 (0.9)	35.0 (0.3)	24.6 (1.0)	30.2 (1.6)	27.7 (1.3)	33.1 (0.7)
summed feature 7	16.4 (1.0)	21.7 (0.9)	15.8 (0.5)	15.0 (0.3)	18.1 (0.4)	16.0 (0.8)	18.1 (1.1)

	BIOLOG dye reduction at 24hr						
BIOLOG Carbon	Pc2140R	TR4.Ri	PPr6.Ri	FNT8.Ri	RC1.Rs	RC4.Rs	RC5.Rs
N-acetyl-D-glucosamine	-	+	++	-	+	+	+
D-arabitol	+	+	+	-	+	+	+
D-mannitol	++	++	++	-	++	++	++
D-psicose	-	\	\	-	- \	\	+
sucrose	++	++	++	-	++	++	++
mono-methyl succinate	- \	+	+	\	+	+	+
formic acid	-	+	+	- +	+	+	+
D-glucosaminic acid	\	++	+	-	+	+	+
succinamic acid	-	\ +	- \	-	\	\	+
alaninamide	-	+	\	-	\	+	+
L-histidine	-	++	++	-	+	+	+
hydroxy L-proline	-	+	+	-	+	+	+
L-leucine	\	+	+	\	+	+	+
L-ornithine	-	\ +	-	-	\	\	\
D-serine	-	+	- +	-	-	\	\
L-serine	+	+	+	-	+	\	+
L-threonine	-	+	\	-	-	\	+
D,L-carnitine	+	++	++	\ +	+	+	++
uridine	-	\	\	-	+	+	+
putrescine	-	\	-	-	-	- \	\
2,3-butanediol	-	- \	-	-	-	\	\
DLa-glycerol phosphate	-	\ +	+	-	+	\	+

	In vitro inhibition						
Test organism	Pc2140R	TR4.Ri	PPr6.Ri	FNT8.Ri	RC1.Rs	RC4.Rs	RC5.Rs
Ggt-8	++++	-	+++	+++	++++	++++	-
R. solani AG-8	+++	-	+	+	+	++	+
P. irregulare BH25	+++	-	+	+++	+	+	+
T. koningii 7a	+++	-	++	++	+++	+++	++
Rp. oryzae fu1	+++	+	+++	+++	+++	+++	+++
P. putida K4	+	-	+	+	+	+	-
B. cereus bu3	+++	-	++	++	+++	++	+

[a]trace amounts

Table 6.2. *-Continued.*

% Fatty acid in profile (SD)

RC6.Rs	DM1.Rs	Pc2140RL	TR1.RLi	FNT8.RLi	TRY19.RLi	FNTO22.RLi	TRY33.RLi	LF48.RLi
0.5 (0.1)	1.0 (0.9)	tr	0	tr	0	tr	tr	tr
4.4 (0.3)	7.1 (1.5)	4.8 (0.2)	3.5 (0.2)	4.7 (0.1)	3.6 (0.0)	4.9 (0.1)	4.2 (0.1	4.7 (0.3)
2.9 (0.4)	2.6 (0.3)	2.3 (0.4)	3.2 (0.1)	2.0 (0.1)	2.8 (0.1)	2.2 (0.1)	2.4 (0.1)	3.0 (0.3)
0.2 (0.0)	0.5 (0.1)	0.3 (0.1)	0	tr	0	0.3 (0.1)	tr	tr
4.5 (0.1)	5.2 (0.4)	4.8 (0.2)	4.3 (0.1)	5.1 (0.1)	4.5 (0.1)	4.8 (0.2)	4.9 (0.1)	4.9 (0.2)
1.0 (0.3)	2.2 (0.7)	1.1 (0.2)	0	0.9 (0.0)	tr	1.2 (0.1)	0.7 (0.0)	0.9 (0.1)
4.8 (0.3)	5.9 (0.7)	5.1 (0.3)	4.5 (0.1)	4.5 (0.1)	4.5 (0.0)	5.1 (0.1)	4.7 (0.1)	5.2 (0.3)
0	0	0	0	tr	0	tr	0.4 (0.1)	0
0.3 (0.0)	tr	0.3 (0.0)	0.3 (0.0)	0.4 (0.0)	0.3 (0.1)	0.3 (0.0)	tr	tr
0	0.5 (0.1)	tr	0	tr	0	0.3 (0.0)	0.34 (0.0)	0
28.1 (0.9)	26.4 (1.0)	28.9 (0.7)	27.8 (0.5)	28.9 (0.2)	28.7 (0.4)	28.3 (0.2)	26.5 (0.3)	27.6 (0.4)
3.5 (0.3)	7.5 (2.1)	8.9 (2.0)	0.9 (0.1)	9.9 (0.5)	2.4 (0.2)	8.4 (0.7)	5.6 (0.2)	5.8 (0.5)
0	0	tr	0	tr	0	tr	0.3 (0.1)	0
0.6 (0.0)	0.7 (0.1)	0.8 (0.0)	0.7 (0.0)	0.7 (0.0)	0.7 (0.1)	0.7 (0.1)	0.8 (0.0)	0.8 (0.0)
0.3 (0.0)	0.9 (0.3)	1.1 (0.3)	0	1.2 (0.1)	tr	1.1 (0.1)	0.8 (0.1)	0.7 (0.1)
0.4 (0.1)	0.9 (0.2)	0.6 (0.1)	0	0.7 (0.1)	0	0.8 0.1)	0.9 (0.2)	0.5 (0.1)
30.4 (0.4)	23.3 (3.3)	23.2 (2.1)	31.3 (0.5)	22.1 (0.2)	31.4 (0.3)	23.4 (0.6)	25.8 (0.1)	26.3 (0.8)
18.1 (0.3)	15.2 (2.0)	17.6 (1.2)	23.7 (0.2)	17.8 (0.3)	20.8 (0.4)	17.8 (0.6)	21.3 (0.2)	19.1 (0.6)

BIOLOG dye reduction at 24 hr

RC6.Rs	DM1.Rs	Pc2140RL	TR1.RLi	FNT8.RLi	TRY19.RLi	FNTO22.RLi	TRY33.RLi	LF48.RLi
+	\	+	+	-	+	+	+	+
+	+	+	+	\	+	\	-	+
++	++	++	++	+	++	++	++	+
- \	- \	\	+	\	\	\	\	\
++	++	++	++	+	++	+	++	+
+	\ +	+	+	+	++	+	+	+
\	+	+	+	+	+	+	+	+
+	+	+	+	\	+	+	+	+
\	- \	- \	+	-	++	- \	- \	-
+	- \	+	+	\	+	\	\	\
+	+	+	+	+	++	+	+	+
+	+	+	+	\	++	+	+	+
+	+	+	+	\	+	+	+	+
\	- \	\	\	-	+	-	-	- \
\	- \	-	\	- \	+	-	-	-
+	- \	+	+	\	+	\	+	+
\	-	\	- \	-	+	-	-	\
+	+	+	- \	\	+	+	+	+
\	- \	\	\	- \	\	-	-	\
- \	-	-	-	-	+	-	-	-
-	- \	-	+	- \	-	- \	-	-
\	\	+	+	+	+	+	+	+

In vitro inhibition

RC6.Rs	DM1.Rs	Pc2140RL	TR1.RLi	FNT8.RLi	TRY19.RLi	FNTO22.RLi	TRY33.RLi	LF48.RLi
+++	++++	++++	-	+++	++	++++	++++	-
++	++++	+++	-	+++	++	+++	+++	+
+	+++	+++	-	+++	+	++	+++	+++
+++	++	+++	-	+++	++	++	++	++
++	++	+++	-	+++	+++	+++	+++	+++
-	+	+	-	+	+	+	+	+
-	+++	+++	-	++	+	+++	++	+++

Table 6.3. BIOLOG dye reduction after 24 h at 28°C for carbon sources that did not vary between Pc2140 and variant isolates. Dye reduction was: negative, no colour change; indeterminate, possible faint colour change; and positive, distinct change in colour from clear to blue.

Dye reduction	Carbon source
Negative	α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, cellobiose, i-erythritol, L-fucose, gentiobiose, ß-methyl-D-glucoside, D-raffinose, L-rhamnose, D-sorbitol, xylitol, D-galacturonic acid, ρ-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, sebacic acid, thymidine, glucose-1-phosphate
Negative to indeterminate	maltose, D-melibiose, turanose, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid , glucuronamide, glycyl-L-aspartic acid, L-phenylalanine, phenyl ethylamine, 2-amino ethanol, glucose-6-phosphate
Positive	tween 40, tween 80, acetic acid, D-fructose, D-galactose, α-D-glucose, m-inositol, D-mannose, D-trehalose, methyl-pyruvate, D-galactonic acid lactone, D-gluconic acid, ß-hydroxybutyric acid, α-keto glutaric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, succinic acid, bromo succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-aspartic acid, glycyl-L-glutamic acid, L-proline, l-pyroglutamic acid, γ-amino butyric acid, urocanic acid, glycerol, *cis*-aconitic acid, citric acid, D-saccharic acid, L-asparagine, L-glutamic acid, inosine.

The parent Pc2140 and variant colony types were assessed for their ability to inhibit a range of soil microorganisms *in vitro*. Inhibition against each test organism by Pc2140 isolates could be grouped into distinct classes which were consistent between replicate treatments (Table 6.2). Inhibition of the test microorganisms by both parental isolates, Pc2140R and Pc2140RL, was the same, and both exhibited the maximum inhibition against all test organisms, except for *R. solani* AG-8 where inhibition by DM1.Rs was greater (Table 6.2). All variant isolates showed reduced inhibition against one or more of the test organisms, depending on the variant and test organism. The TR types had the greatest loss of inhibition (Table 6.2).

6.3.2. Taxonomic identification of Pc2140 and variants by GC-FAME and BIOLOG

Taxonomic identification by GC-FAME and BIOLOG methods was taken as the best match (highest similarity) to the standard entries in the GC-FAME or BIOLOG libraries using the associated software for each method. All isolates were identified as belonging to the genus *Pseudomonas*, but species identification varied within each method, and between the two methods. Pc2140RL and its descendants were all identified as *P. putida* bv A (0.73-0.93 similarity) by GC-FAME and *P. corrugata* (0.46-0.72 similarity) by BIOLOG (Table 6.4). Identification by GC-FAME was most varied for Pc2140R and its variants. Pc2140R was identified as *P. putida* bv A (0.65-0.85) or *P. chlororaphis* (0.61-0.68 similarity), and most variants derived

from Pc2140R were identified as different species to Pc2140R (Table 6.4). Replicate extractions (GC-FAME) of the same variant sometimes gave a different species name, but when individual replicates of an isolate (parent or variant types) were compared to the new 2140V library, all were identified as being closest to library entry for that particular isolate, with greater than 0.95 similarity (data not shown).

Table 6.4. Taxonomic classification of Pc2140R, Pc2140RL and variant phenotypes by GC-FAME and BIOLOG taxonomic identification systems. The identification (name) given by each system has the greatest similarity to each replicate analysis of the Pc2140 isolate. The similarity of the Pc2140 isolate to the closest match in the GC-FAME or BIOLOG data base is given for each replicate analysis. For GC-FAME there were 3 replicate extractions and analysis for each variant, 8 replicates for Pc2140R, 4 replicates for Pc2140RL. There were 2 replicate BIOLOG analysis of each isolate.

Isolate	GC-FAME Name (no. reps)	Similarity	BIOLOG Name	Similarity
Pc2140R	*P. putida* bv A (6)	0.65 - 0.85	*P. cichorii*	0.57
	P. chlororaphis (2)	0.61 - 0.68	*P. corrugata*	0.58
FNT8.Ri	*P. putida* bv B (3)	0.76 - 0.82	*P. cichorii*	0.43 - 0.56
PPr6.Ri	*P. fluorescens* bv B (3)	0.92 - 0.98	*P. corrugata*	0.82 - 0.85
TR4.Ri	*P. putida* bv A (2)	0.89 - 0.90	*P. corrugata*	0.82 - 0.83
	P. fluorescens bv A (1)	0.9		
RC1.Rs	*P. putida* bv A (3)	0.84 - 0.90	*P. corrugata*	0.64 - 0.86
RC4.Rs	*P. chlororaphis* (2)	0.75 - 0.76	*P. corrugata*	0.87
	P. putida bv A (1)	0.84		
RC5.Rs	*P. fluorescens* bv B (2)	0.84 - 0.85	*P. corrugata*	0.87 - 0.90
	P. fluorescens bv A (1)	0.95		
RC6.Rs	*P. corrugata* (2)	0.79 - 0.80	*P. corrugata*	0.70 - 0.84
	P. putida bv A (1)	0.89		
DM1.Rs	*P. chlororaphis* (2)	0.48 - 0.58	*P. corrugata*	0.74- -0.90
	P. corrugata (1)	0.76		
Pc2140RL	*P. putida* bv A (4)	0.77 - 0.90	*P. corrugata*	0.66 - 0.72
TR1.RLi	*P. putida* bv A (3)	0.79 - 0.82	*P. corrugata*	0.61 - 0.65
FNT8.RLi	*P. putida* bv A (3)	0.83 - 0.85	*P. corrugata*	0.46 - 0.70
FNTO22.RLi	*P. putida* bv A (3)	0.79- 0.85	*P. corrugata*	0.67 - 0.70
TRY33.RLi	*P. putida* bv A (3)	0.73 - 0.80	*P. corrugata*	0.65
TRY19.RLi	*P. putida* bv A (3)	0.90 - 0.93	*P. corrugata*	0.64 - 0.67
LF48.RLi	*P. putida* bv A (3)	0.75 - 0.88	*P. corrugata*	0.67 - 0.69

Using the BIOLOG method for taxonomic identification, Pc2140R was named as *P. cichorii* (0.57 similarity) or *P. corrugata* (0.58 similarity), FNT8.Ri as *P. cichorii* (0.43-0.56 similarity) and other Pc2140R descendants as *P. corrugata* (0.64-0.90 similarity; Table 6.4). Cluster analysis performed by GC-FAME and BIOLOG software in both cases separated some variant isolates at a distance greater than that between *Pseudomonas* reference species in their respective libraries (Fig. 6.1). GC-FAME analysis calculated that half the variants, mainly the TR and RC types, were

an Euclidean Distance (ED) of >11 from the wild type ancestors. Hierarchical clustering by the MIDI GC-FAME MIS software of Pc2140R and three variants, FNT8.Ri, TR4.Ri and PPr6.Ri, as well as the library entries for a number of *Pseudomonas* spp. is shown in Fig. 6.1.

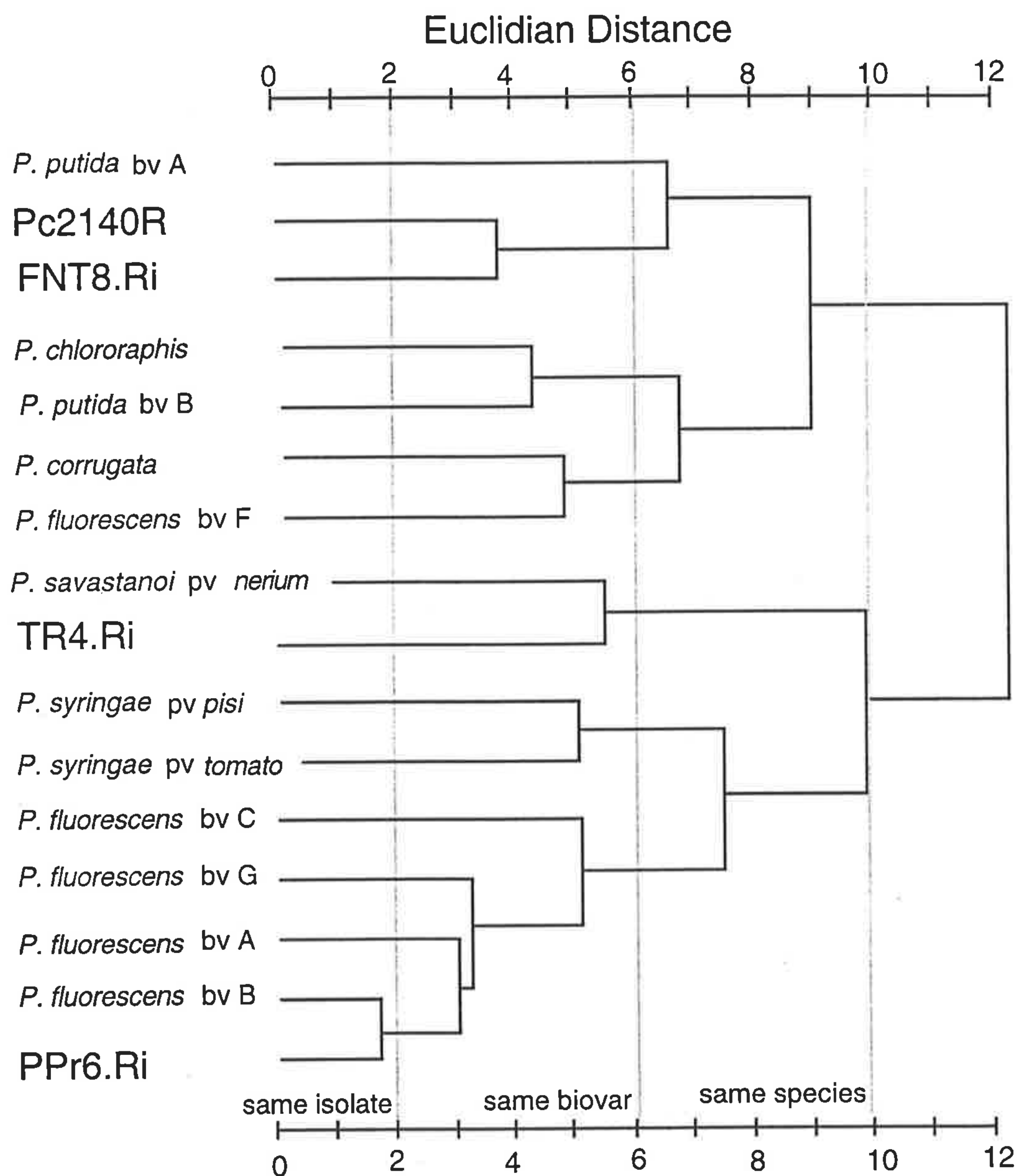


Fig. 6.1. Comparison of Pc2140R and 3 variant phenotypes (TR4.Ri, FNT8.Ri and PPr6.Ri) to *Pseudomonas* spp. in the MIDI GC-FAME MIS Sherlock TSBA aerobe library version 3.8. The suggested cut off distances given by the MIS system for the same isolate, biovar and species are shown.

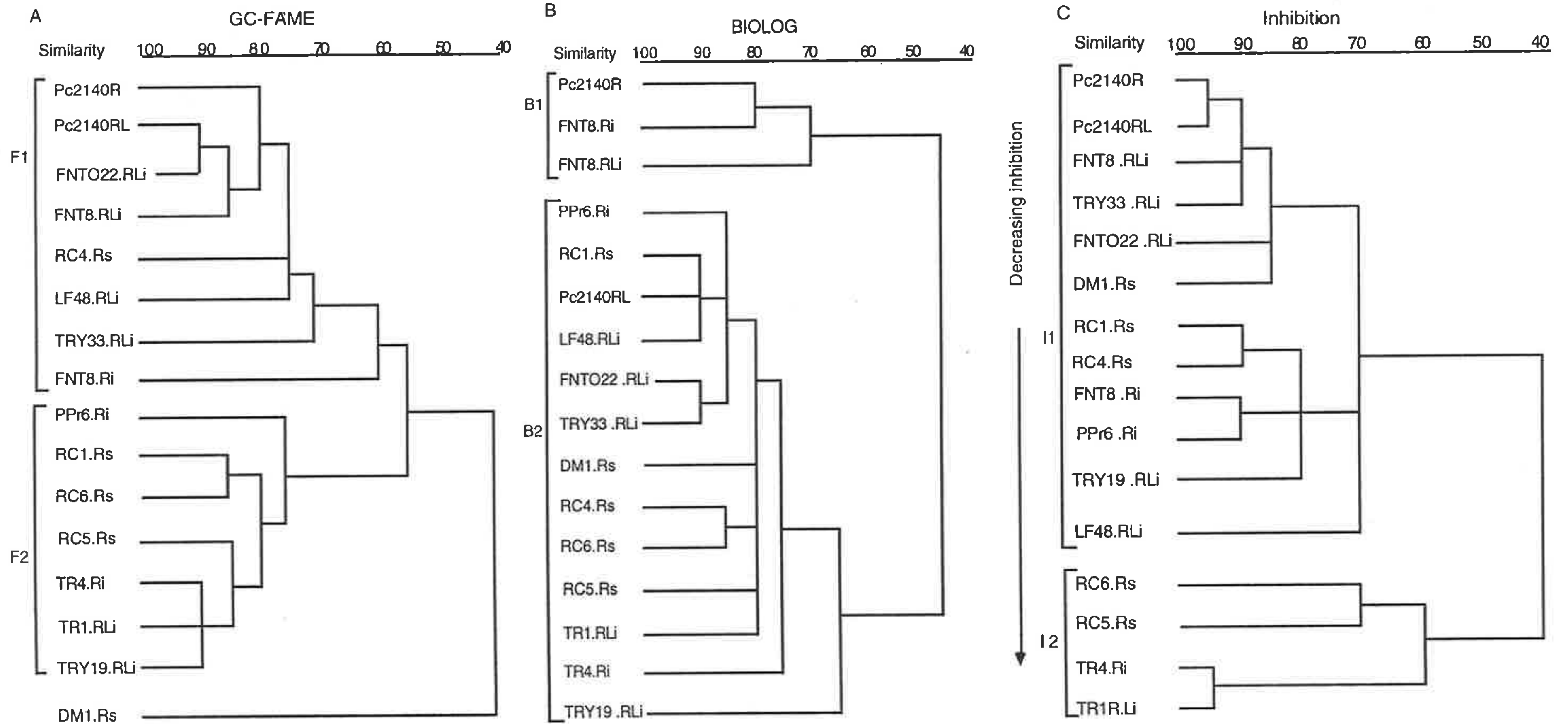


Fig. 6.2. Hierarchical cluster analysis of Pc2140R, Pc2140RL and descendant variants by GC-FAME (A), BIOLOG (B) and *in vitro* inhibition (C) profiles. The two major clusters for each characteristic is given by F1, F2 (GC-FAME); B1, B2 (BIOLOG); and I1, I2 (inhibition).

6.3.3. Hierarchical clustering of Pc2140R Pc2140RL and variant phenotypes

Variant colony types were clustered according their GC-FAME, BIOLOG and inhibition profiles by the same hierarchical clustering method. Analysis of each profile produced a different clustering of isolates, as represented by dendrograms in Fig. 6.2. Pc2140 isolates could be grouped into two main clusters at the 40-55% similarity level. Cluster one by GC-FAME, BIOLOG and inhibition (clusters F1, B1 and I1 respectively; Fig. 6.2), contained Pc2140R, FNT8.Ri, FNT8.RLi and (except for BIOLOG) Pc2140RL. Cluster 2 (F2, B2 and I2 by GC-FAME, BIOLOG and inhibition respectively) contained the TR colony types TR4.Ri and TR1.RLi which exhibited the greatest phenotype change (Fig. 6.2). By GC-FAME, clusters F1 and F2 had 55% similarity with nearly equal numbers of isolates, and DM1.Rs showed a 40% similarity to both of these clusters. Cluster B1 (BIOLOG) contained only three isolates with 70% similarity which were separated from the rest of the isolates in B2 at the 45% similarity level. In contrast, cluster I1 (inhibition) contained 12 isolates with 70% similarity, and 40% similarity to I2 with the remaining four variant isolates (Fig. 6.2).

6.3.4. Comparison of cluster analysis using Procrustes comparison

The principal component analysis of each profile was compared to the others using Procrustes rotation, based on the first two principal components of each data set. The result confirmed the visual observation that there were substantial differences between the three data sets in the distribution of isolates. The similarity of PCA for each profile compared to the others after Procrustes rotation is shown in Table 6.5. Identical PCA would have given a fitted configuration of 1.0. The highest similarity was between GC-FAME and inhibition profiles of 50%, and the least similarity was 22% between inhibition and BIOLOG profiles (Table 6.5).

Table 6.5. Procruste's paired comparisons of GC-FAME, BIOLOG and inhibition profiles by PCA. Identical PCA of paired profiles would give a fitted configuration = 1.0.

Comparison	GC-FAME-BIOLOG	GC-FAME-Inhibition	Inhibition-BIOLOG
Fitted Configuration	0.4467	0.5048	0.2231
Residual	0.5533	0.4952	0.7769
Fixed Configuration	1.0000	1.0000	1.0000

6.4. Discussion

Terzaghi and O'Hara (1990) and Rainey *et al.* (1993) reviewed microbial plasticity, and the relevance this may have to the ecology and diversification of the organisms concerned, but to date there is little information on the range of phenotypes that may be produced by a single isolate and how this relates to the phenotypic characterisation and grouping of isolates from environmental samples. Pc 2140 readily produces new phenotypes, *in vitro* and in wheat-soil microcosms, which are detectable by altered colony morphology on TZCA (Chapters 4 and 5). This diversification of Pc2140 provided us with a unique opportunity to characterise a mixed population derived from a known parent in order to determine the potential range of phenotypes produced from one isolate and their properties. Colony morphology variants derived from Pc2140R or Pc2140RL were characterised by biochemical analysis, (GC-FAME profile), their carbon source utilisation profile or metabolic fingerprint (BIOLOG), and *in vitro* inhibition of a set of seven soil microorganisms to reflect ecological function. The results presented here show that very closely related variant isolates can vary markedly in all three characteristics measured (Table 6.2).

Both the GC-FAME and BIOLOG taxonomic identification systems classified some variants of Pc2140R as different biovars or even species of *Pseudomonas*, with a different identification given for each system (Table 6.4). The wild type Pc2140 was initially (1988) identified as *P. corrugata* by an earlier version of the MIDI MIS library software, but is now identified as *P. putida* bv A using the Sherlock aerobe TSBA library version 3.8. Different taxonomic identification of isolates by GC-FAME and carbon source utilization have recently been reported by other workers (Rainey *et al.*, 1994; Tonso *et al.*, 1995), and raises concern as to the reliability of identification of bacteria using these methods. Results produced by the GC-FAME MIS comparing the ancestral wild type Pc2140R and three variants to related *Pseudomonas* spp., clearly shows that the difference between GC-FAME profiles of Pc2140R isolates was greater than that between reference entries for species within the fluorescent pseudomonad group. (Fig. 6.1). That isolates known to be closely related are classified as different species is clearly wrong if the isolates only differ by one or few mutational events, and suggests that the GC-FAME system is unable to reliably differentiate the (fluorescent) pseudomonads below the genus level.

Siverio *et al.* (1996) have also reported that colony variants from *P. corrugata* strains had overlapping GC-FAME profiles with other *Pseudomonas* spp.. The TR colony variants from Pc2140 have similar changes in GC-FAME profiles when compared to the wild type as the *P. corrugata* strains of Siverio *et al.* (1996) when variant smooth colonies were compared to the wrinkled wild type colonies. For example, the percentage of the fatty acids 10:0 3OH, 12:0 2OH, 12:0 3OH, and 17:0 cyclo were reduced in the TR variants in this work, and by smooth colony variants in Siverio *et al.* (1996), compared to the parent wild type strains. The percentage of the

fatty acids 12:0 and 16:0 were increased in both the TR types from Pc2140 and smooth variants described by Siverio *et al.* (1996), compared to the parent isolates. Holloway *et al.* (1997) also found that fatty acid profiling could not differentiate *P. syringae* pathovars. Sutra *et al.* (1997) found that *P. corrugata* DNA hybridisation groups could be differentiated on the basis of four fatty acids, however in this current study, three of these four fatty acids, 16:0 3OH and unknowns with retention times of 13.961 and 14.503 minutes, were variable between Pc2140 and variant isolates, and the other fatty acid (14:0 3OH) was not present (Table 6.2). Siverio *et al.* (1996) suggested that GC-FAME analysis should be done shortly after isolation to avoid confounding results with variant phenotypes, but did not consider the possibility that phenotype conversion may occur prior to, or upon, isolation. The divergence of GC-FAME profiles in spontaneous Pc2140 variants was much greater than previously reported for a genetically modified *P. fluorescens* isolate which differed up to 1.71 ED in GC-FAME profile when reisolated after 220 days from sugar beet leaves in the field (Thompson *et al.*, 1995), compared to distance of 11 ED between some Pc2140 variants (Fig. 6.1).

Taxonomic identification using BIOLOG was more consistent in the naming of isolates, but presented a few problems. After 24 hours incubation 22 carbon sources indicated a difference for at least one variant of Pc2140, with either a gain or loss of dye reduction compared to the wild type (Table 6.2). Reduction of the tetrazolium dye from colourless to purple is used in the BIOLOG system to indicate carbon source utilisation. Colony morphology variants of *P. corrugata* 2140 strains are initially detected on a medium which contains glucose and a similar tetrazolium dye which is reduced to the red coloured formazan (Kelman, 1954). All variants grow on this medium but differ in ability to reduce the tetrazolium dye. After 48 hours on TZCA, the wild type Pc2140 produces a cream coloured colony (no obvious reduction of tetrazolium dye) while the TR type colonies are red due to reduction of the tetrazolium dye. This raises the question as to whether the BIOLOG system accurately reflects carbon source utilisation, or a combination of carbon source usage and changes in ability to reduce tetrazolium. This could be tested by growing variants in minimal medium plus individual carbon sources which show differences in dye reduction.

Spontaneous change in carbon source utilisation may help explain the high degree of variability in strains within *Pseudomonas* rRNA homology group I (Palleroni *et al.*, 1973) as noted in diagnostic guides for the identification of bacteria using carbon source utilisation (Stanier *et al.*, 1966; Fahy and Lloyd, 1983; Palleroni, 1984; Hildebrand *et al.*, 1988). Gain in substrate utilisation via mutational activation of cryptic genes appears widespread in bacterial populations (Young, 1989), and Leblond *et al.* (1990) hypothesised that spontaneous mutants of *Streptomyces ambofaciens* DSM40697 were hyper variable, ie. they had an increased mutation rate. Combined with the highly selective conditions of a single carbon source, cryptic genes and hyper variable mutants may further complicate interpretation of dye reduction in the BIOLOG plates. Pc2140RL utilised more carbon sources than Pc2140R, excluding lactose and lactulose

for which differences were expected and observed (Table 6.2). This may be due to insertion of the *lacZY* genes in Pc2140RL either aiding uptake or utilisation of other substrates besides lactose and lactulose, or increasing dye reduction by an unknown mechanism.

As strain Pc2140 is a potential biocontrol agent, it was desirable to characterise the inhibition potential of variant types as an indication of altered ecological function in the new colony types. Pathogen inhibition is important in the control of plant diseases by *P. corrugata* 2140 (Ross, 1996) and other *Pseudomonas* spp. where spontaneous pleiotropic mutants have lost the ability to control disease. Loss of disease control in these other isolates is due to mutation in either a gene coding the sensor (*lemA* type) or transcriptional regulator (*gacA* type) component of a two component regulatory system which controls production of multiple metabolites associated with disease control (Laville *et al.*, 1992; Corbell *et al.*, 1994; Gaffney *et al.*, 1994; Cook *et al.*, 1995). These appear analogous to the TR variant types reported here which have lost the ability to inhibit all test microorganisms (Table 6.2), and are usually the only Pc2140 variant phenotypes found on common laboratory media (Chapter 4). The partial loss of inhibition in other variants may be due to mutations in structural genes for synthesis of inhibitory metabolites, or other regulatory elements.

Cluster analysis of GC-FAME, BIOLOG and inhibition profiles produced different clustering of isolates, with Procrustes analysis indicating a low similarity between clustering by the different profiles (Fig. 6.2, Table 6.5). This is in contrast to Rainey *et al.* (1994) and Tonso *et al.* (1995) who found that clustering of isolates based on different phenotypic properties were similar. Comparisons between phenotypic and genetic characterisations of isolates have also been shown to be comparable (Rainey *et al.*, 1994; Lemanceau *et al.*, 1995; Tonso *et al.*, 1995 and Frey *et al.*, 1997). However, some isolates within the same genomic group can have different phenotypic characteristics (Rainey *et al.*, 1994), and similar phenotypes can have different genetic profiles (Latour *et al.*, 1996). In the case of Pc2140R, all the variants discussed here have the same genetic fingerprint (Chapter 7), but different phenotypic characteristics. Due to selection pressures in the environment there may be only a limited number of phenotypes which persist and become dominant for each genotype. This is supported by the fact that when initially homogenous populations of Pc2140R were cultured on wheat roots, populations became mixed, and then tended to become dominated by the new variant phenotype (Chapter 5). This indicates only one phenotype dominates within each population of genetically similar bacteria.

Clustering of Pc2140 variants by *in vitro* inhibition differed from that obtained using GC-FAME and BIOLOG data. This is consistent with other work where *in vitro* inhibition of Ggt (Smiley, 1979; Charigkapakorn and Sivasithamparam, 1987) or *in vivo* antagonism towards take-all disease (Sarniguet *et al.*, 1992b) varied within sets of fluorescent pseudomonad isolates that were grouped together according to physiological and biochemical tests. This indicates that potential disease antagonists within the fluorescent pseudomonad group cannot be selected by

using GC-FAME profiles or carbon source utilisation for clustering of potential isolates. The sensitivity of the GC-FAME method, however, makes it a valuable tool for identifying a known wild type strain in mixed cultures.

6.5. Conclusions

Pc2140 produces variant phenotypes during *in vitro* and *in vivo* culture. Although these variant types are very closely related genetically, as they are derived from a common ancestor, they vary considerably from the parent type, and from each other, in both taxonomically (fatty acid profile and carbon source use) and ecologically (inhibition of microorganisms) important characteristics. When a range of variant colony types were treated as a mixed population and characterised by different phenotypic characteristics (GC-FAME, BIOLOG and inhibition profiles), hierarchical clustering of variants, or the indicated similarity between variants, depended on the method of characterisation used. This diversity of phenotypes produced by a single isolate presents difficulties in interpreting the results of taxonomic identification and cluster analysis of unknown isolates from environmental populations based on GC-FAME and BIOLOG characteristics alone.

Chapter 7. Molecular Confirmation That Variant Phenotypes of *Pseudomonas corrugata* 2140R Are Derived From The Original Parent Strain

7.1. Introduction

In Chapters 4 and 5 a range of variant colony types were detected in initially pure cultures of Pc2140R and Pc2140RL. This occurred both *in vitro* and on wheat roots. Variant colony types were detected when populations were diluted and plated onto TZCA medium. These new colony types were not recovered from *in vitro* media unless inoculated with a Pc2140 strain, and were not detected as laboratory contaminants. As well, no bacteria resistant to rifampicin at 100 µg/ml were recovered from the Kapunda red-brown earth used in this study for wheat-soil microcosms unless the soil had been inoculated with Pc2140R (Section 5.3.1). All variant colony types isolated, including those described in this thesis, which were derived from Pc2140R or Pc2140RL cultures were resistant to rifampicin at 100 µg/ml, as are the parent strains. Pc2140RL is marked with the *lacZY* gene from *E. coli* and these genes are not present in *Pseudomonas* spp.. All variant colony types derived from Pc2140RL cultures tested positive for presence and activity of the *lacZY* genes as they formed blue colonies when plated onto X-Gal MM. Previously a TR and a TRY type variants have been shown to have the same *Eco*R1 restriction enzyme fingerprint of total DNA as its parent, Pc2140 (Ryder, unpublished data). Based on these facts it was determined that these variant colony types were derived from the parent, or ancestral Pc2140 strain with which the cultures had been inoculated. In order to further support this assertion it was desirable to compare genetic profiles of the variant colony types with that of the wild type Pc2140.

A number of methods have been used for comparing genetic profiles of bacteria isolated from the environment. Rainey *et al.* (1994) used pulse field gel electrophoresis (PFGE) to genetically characterise phyllosphere populations of pseudomonads by macrorestriction fragment fingerprints. Genetic profiles of enterobacterial repetitive intergeneric consensus sequences amplified by polymerase chain reaction (ERIC-PCR) have been shown to be the same only in isolates of the same strain (DeBruijn, 1992), and have been used to characterise pseudomonad populations from plant roots (Frey *et al.*, 1997). If variant phenotypes are derived from the parent Pc2140 strain, then the genetic profiles of variant isolates should be the same as the parent isolate. The aim of this section was to test whether variant colony types derived from Pc2140R cultures had the same genetic profile as the parent strain using PFGE and ERIC-PCR.

7.2. Materials and Methods

7.2.1. PFGE

Pc2140R and three variants (TR4.Ri, FNT8.Ri and PPr6.Ri) derived from A-DB cultures (Chapter 4) were compared by macrorestriction fragment profiles of total DNA. These characteristics of these variants are described in Chapter 6. Total DNA extracts were digested with the restriction enzymes *Xba*1 or *Spe*1 and separated by Clamped Homogeneous Electric Field-Pulse Field Gel Electrophoresis (CHEF-PFGE, Bio-Rad) based on the method of Rainey *et al.* (1994). *P. fluorescens* Pf-5 was included as a control.

7.2.1.1. DNA extraction

Total DNA was extracted by a method based on that of D. Drahos and G. Barry (Monsanto). Single colonies from TZCA were cultured overnight in 10 ml LB broth. Eight ml of overnight culture was centrifuged (12,350 x g) for 3 min and supernatant discarded. Cells were resuspended in 2.5 ml TEG buffer (Tris-HCl, 10 mM pH 8; EDTA 50 mM pH 8; 1% glucose). Cells were lysed by adding 300 μl 10% SDS, mixed by inversion and heated at 65-70°C for 10 min. DNA was extracted by adding 3 ml phenol/$CHCl_3$ (1:1 TE8 (Tris-HCl, 10 mM; EDTA 1 mM; pH 8) saturated phenol:chloroform), inverting 30 times to mix, and centrifuged at 12,350 x g for 10 min. The top phase containing DNA was transferred to a fresh tube and the phenol extraction repeated. Only the top 2 ml of the upper phase was transferred to a glass centrifuge tube after the second extraction. DNA was precipitated by adding 10 ml of 100% ethanol and inverting slowly (3 times) until a 2 ml clear zone containing DNA became evident at the bottom of the tube. The DNA preparation was chilled on ice for 10 min and the top phase removed. The DNA was washed in 10 ml of 70% ethanol by inverting slowly, letting the DNA precipitate, and removing and discarding the top phase. Precipitated DNA was transferred to a 1.5 ml centrifuge tube, 1 ml of 95% ethanol added and centrifuged at 12,350 x g for 4 min to pellet DNA. Supernatant was decanted and the DNA pellet was dried and resuspended in 50 μl of TE8 buffer (Tris-HCl, 10 mM; EDTA 1 mM; pH 8).

7.2.1.2. Restriction digests

For restriction enzyme digests, 15 μl of DNA suspension in TE8 buffer, containing 1.5-2.0 μg DNA, was transferred to a 1.5 ml centrifuge tube and 2 μl restriction enzyme buffer, 2 μl of restriction enzyme (*Xba*1 or *Spe*1, 10 U/μl, Boehringer Mannheim) and 1 μl of RNase (Boehringer Mannheim) added. This mixture was incubated at 37°C overnight. An extra 1 μl of restriction enzyme was added after 1 h of incubation to ensure complete digestion of DNA.

7.2.1.3. Electrophoresis conditions

PFGE conditions were based on those used by Rainey *et al.* (1994). DNA restriction fragments were separated in a 1.25 % agarose (high melting temperature, Sigma) gel made with 100 ml of 0.5x TBE buffer (Tris-borate, 0.089 M; boric acid, 0.089 M; EDTA pH 8, 0.002 M; Maniatis *et al.*, 1982). Gels were 12 x 14 x 0.5 cm with 10 wells of size 10 x 1 mm. Restriction digests (20 µl) were mixed with 2 µl loading buffer (40% sucrose in 0.5 x TBE) and loaded into wells. PFGE Marker I (concatemers of lambda c1857Sam7 genome, Boehringer Mannheim) was used as a molecular weight marker.

Gels were subject to electrophoresis using Bio-Rad Clamped Homogeneous Electric Field-Pulse Field Gel Electrophoresis (CHEF-PFGE) equipment at 200 V with variable amperage (Bio-Rad 200/2.0 constant voltage power supply). Ramped pulse times were; initial, 1 sec; final, 30 sec, ratio A:B of 1:1 (Bio-Rad Pulsewave 760 switcher) and run for 22 h in 2 L of 0.5x TBE buffer at 14°C.

Gels were stained in ethidium bromide for 30 min and destained in water for 2.5 h. DNA bands were visualised under ultraviolet light (310 nm) and photographed (Polaroid MP-4 Land Camera).

7.2.2. ERIC-PCR

Pc2140R and reisolates from long term wheat-soil microcosm (Chapter 5) were assessed for ERIC-PCR profiles. Reisolates were OP1 - OP5 and RC1 - RC5 from cycle 5, pot +3 (C5+3, Fig. 5.9), and 4 reisolates from the eighth cycle (C8), two from pot +3 (C8+3, OP1 and RC2) and two from pot-3 (C8-3, OP1 and RC2, Fig. 5.10). ERIC-PCR profiles were produced by the method used by Frey *et al.*, (1997). Lysed cells were subject to PCR in the presence of ERIC primers. To ensure that ERIC-PCR profiles were specific to Pc2140 isolates, three *P. corrugata* strains, 8270, 5819 and 2152, were included as controls. *P. corrugata* strain 2152 was isolated at the same time and from the same soil as Pc2140 (Ryder and Borrett, unpublished data). The ERIC consensus sequence and sequence of ERIC primers are as given in DeBruijn (1992). ERIC primers were produced by Bresatec.

ERIC consensus sequence (DeBruijn, 1992):

5' GTGAATCCCAGGAGTTACATAAGTAAGACTGGGGTGAGCG 3'

ERIC primers:

ERIC1R 3' CACTTAGGGTCCTCAATGTA 5'

ERIC2 5' AAGTAAGACTGGGGTGAGCG 3'

7.2.2.1. Preparation of cells

Pc2140R and variant colony types were subcultured onto TZCA from -70°C storage, incubated for 48 h, and 2 single colonies picked into 5 ml LB broth and cultured overnight on a rotary shaker. Aliquots (1 ml) of overnight culture were transferred to 1.5 ml centrifuge tubes and cells pelleted (12,000 x g, 10 min), and washed twice in 1 ml sterile nanopure (NP) water. Cells were resuspended in NP water to give A_{600}=1. Suspensions were stored at -20°C until use.

7.2.2.2. Preparation of PCR mix

PCR mixes 1 and 2 (Table 7.1) were mixed separately in a volume sufficient for all reactions to be undertaken at one time. Promega *Taq* polymerase and associated reagents were used for PCR. To 17.9 µl of mix 1 in a 0.5 ml PCR tube, 5 µl of bacterial suspension (A_{600}=1) was added and 1 drop of paraffin oil placed on top of the reaction mixture. Cells were lysed at 95°C for 10 min during PCR precycle. Mix 2 (2.1 µl) was then added under the paraffin oil layer for a total reaction mixture of 25 µl. A control PCR mixture containing 5 µl nanopure water instead of bacterial suspension was included with each PCR run.

Table 7.1. PCR reaction mixes 1 and 2.

Mix 1	Concentration	µl/reaction	Mix 2	Concentration	µl/reaction
NP water		9	NP water		1.7
Tp-Buffer	10x	2.5	*Taq*	10x	0.4
$MgCl_2$	25 mM	1.5	Polymerase		
Dimethyl Sulfoxide	100%	2.5	Total volume		2.1 µl
dNTPs	25 mM	0.4			
ERIC1R	50 pmole	1			
ERIC2	50 pmole	1			
Total volume		17.9 µl			

7.2.2.3. PCR conditions

The PCR reaction was carried out using a Perkin Elmer DNA Thermal Cycler. PCR conditions were as DeBruijn (1992), with 35 cycles of denaturation (94°C, 1 min), hybridisation (52°C, 1 min) and amplification (65°C, 8 min). At the end of 35 cycles the mixture was kept at 65°C for 16 min to finish amplification.

7.2.2.4. Electrophoresis of ERIC-PCR products

PCR products were separated on a 12 cm long, 15 cm wide 1.5% agarose gel (70 ml, Agarose MP, Boehringer Mannheim) in 0.5x TAE Buffer (Tris-acetate, 0.04 M; EDTA pH 8, 0.001 M;

Maniatis *et al.*, 1982) with 30 wells. Two µl of loading buffer (bromophenol blue, 0.25%; xylene cyanol, 0.25%; sucrose, 40% w/v; Maniatis *et al.*, 1982) was added to and mixed with each PCR reaction mixture under the oil layer, and 6 µl of reaction mixture added to each well. Genetic marker X (MX, Boehringer Mannheim) was added to each gel. Gels were run in 0.5x TAE buffer at 25 mA current for 2 h. Gels were stained for 1 h in ethidium bromide and destained for 10 min in water. PCR bands were visualised under ultra violet light and photographed.

7.3. Results

When the genetic profiles of variant colony types derived from Pc2140R cultures were compared by macrorestriction fragment profiles (PFGE, Fig. 7.1) or by repetitive DNA sequences (ERIC-PCR, Fig. 7.2), they were the same as those of the parent Pc2140R strain. A non Pc2140R strain, *P. fluorescens* Pf-5, exhibited different *Xba*1 and *Spe*1 profiles to the Pc2140R strains (Fig. 7.1). Other *P. corrugata* strains, 8270, 5819 and 2152 all had different ERIC-PCR profiles to the Pc2140R strains, and there were no DNA bands present in the PCR control (Fig. 7.2).

7.4. Discussion

Variant phenotypes that were isolated from *lacZY* marked Pc2140RL could be confirmed as being descendent from Pc2140RL as they all tested positive for the prescence of the *lacZY* genes. This was not possible with variants derived from Pc2140R cultures so it was desirable to further confirm that variants from Pc2140R cultures were derived from the parent isolate. It was expected that the variant phenotypes derived from Pc2140R cultures should all have the same genetic fingerprint as the parent strain as they are directly or indirectly descendant from the Pc2140R strain used to inoculate the cultures. This expectation was confirmed as the variant isolates tested here all exhibited the same genetic profile whether compared by macrorestriction fragment profiles (Fig. 7.1) or enterobacterial repetitive intergeneric consensus sequences (Fig. 7.2). In addition, a co-worker in this laboratory (Dr. Younes Alami) has also shown that the Pc2140R variants described in Chapter 6 also have the same ERIC-PCR profiles (Barnett *et al.*, 1999).

Rainey *et al.* (1994) used PFGE to characterise pseudomonads from sugar beet phyllosphere and commented on the usefulness of the procedure in detecting genomic rearrangements by treating macrorestriction fragment patterns of the entire genome as a highly polymorphic RFLP pattern. However, when the mutations responsible for spontaneous phenotype conversion (ie. pleiotropic changes in phenotype) in *Pseudomonas* strains have been determined, the genetic changes have been small. Mutations ranged from 2 to 5 bp insertions or deletions (Brumbley *et al.*, 1993; Gaffney *et al.*, 1994; Swords *et al.*, 1996) up to 5 kbp deletions (Laville *et al.*, 1992).

Fig. 7.1. Macrorestriction fragment profiles of Pc2140R and variant colony types after digestion with *Xba*1 and *Spe*1 restriction enzymes and separation by CHEF-PFGE.

Lane	Isolate	Restriction Enzyme
1	Pc2140R	*Xba*1
2	TR4.Ri	*Xba*1
3	PPr6.Ri	*Xba*1
4	FNT8.Ri	*Xba*1
5	*P. fluorescens* Pf-5	*Xba*1
6	Pc2140R	*Spe*1
7	TR4.Ri	*Spe*1
8	PPr6.Ri	*Spe*1
9	FNT8.Ri	*Spe*1
10	*P. fluorescens* Pf-5	*Spe*1

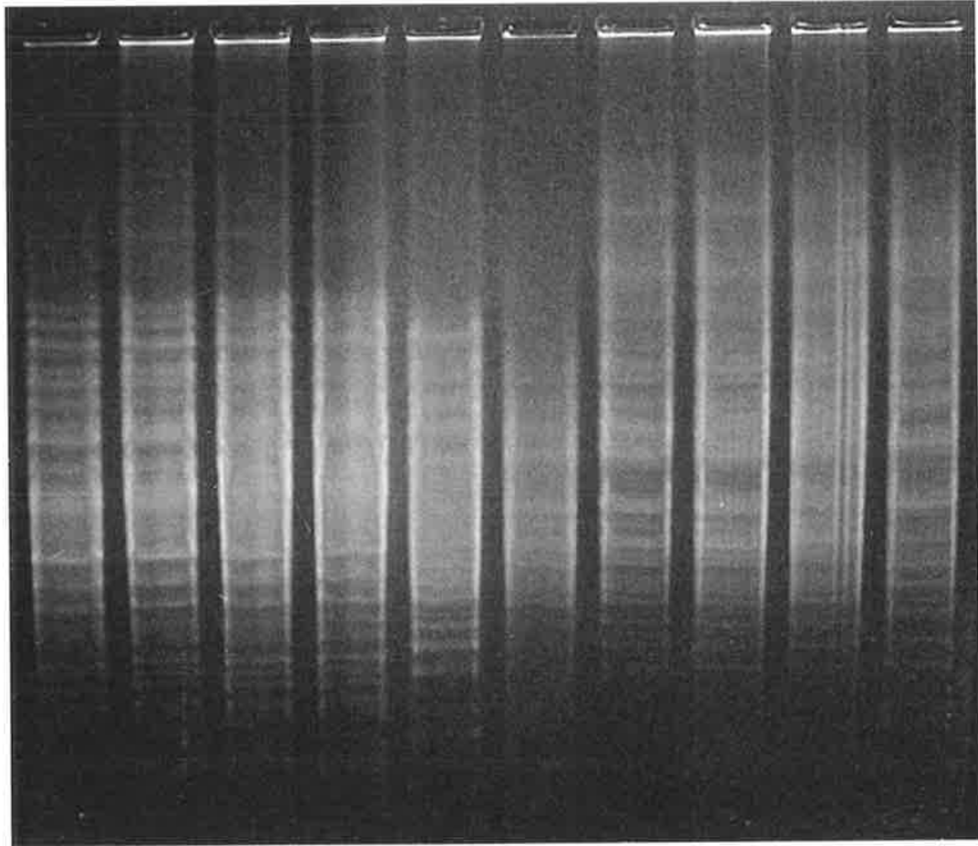
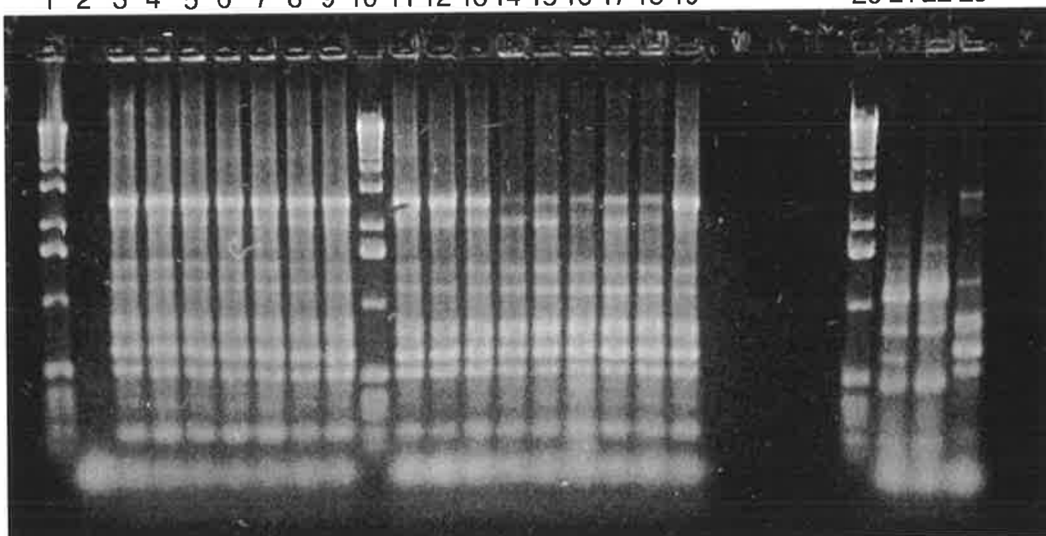

kbp
1
2
3
4
5
6
7
8
9
10
291
194
97

Fig. 7.2. ERIC-PCR profiles of Pc2140R and variant colony types.

Lane	Isolate
1	Marker X
2	PCR control
3	Pc2140R
4	OP1.Rs (C5, pot +3)
5	OP2.Rs (C5, pot +3)
6	OP3.Rs (C5, pot +3)
7	OP4.Rs (C5, pot +3)
8	OP5.Rs (C5, pot +3)
9	RC1.Rs (C5, pot +3)
10	Marker X
11	RC2.Rs (C5, pot +3)
12	RC3.Rs (C5, pot +3)
13	RC4.Rs (C5, pot +3)
14	RC5.Rs (C5, pot +3)
15	OP1.Rs (C8, pot -3)
16	RC2.Rs (C8, pot -3)
17	OP1.Rs (C8, pot +3)
18	RC1.Rs (C8, pot +3)
19	Pc2140R
20	Marker X
21	*P. corrugata* strain 8270
22	*P. corrugata* strain 5819
23	*P. corrugata* strain 2152


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
20 21 22 23

These small genomic changes would probably not be detected by PFGE with the restriction enzymes used here producing fragments in the 50 to 500 kbp range.

Repetitive DNA sequences occur in eubacteria and are useful for fingerprinting bacterial genomes by amplifying these sequences using the polymerase chain reaction (Versalovic *et al.*, 1991), and De Bruijn (1992) has shown that ERIC-PCR fingerprints are strain specific. These repetitive sequences however, are not known to occur in genes coding for known functions. The mutations responsible for the different variant phenotypes produced by Pc2140R must occur in genes coding some function to produce the observed phenotype changes. Changes to these genes would not be detected by primers designed to identify repetitive sequences outside these genes. It would not be expected then that ERIC-PCR fingerprint would vary between variant phenotypes and the parent Pc2140R.

Genetic profiles have been used to characterise bacterial populations, often with good correlation between clustering of isolates by genetic and phenotypic profiles, although this did not apply to all isolates within a sample (Rainey *et al.*, 1994; Lemanceau *et al.*, 1995; Tonso *et al.*, 1995; Frey *et al.*, 1997). Tonso *et al.* (1995) found that some isolates with the same repetitive extragenic palindrome PCR fingerprint were in a different BIOLOG cluster and had different colony morphology. Rainey *et al.* (1994) also reported that some isolates with identical macrorestriction fragment fingerprints were clustered differently when based on phenotypic characteristics. Rainey *et al.* (1994) suggested that this was an example of intraclonal polymorphism, or phenotype conversion, occurring in the environment. This suggestion is supported by the range of variant phenotypes produced by Pc2140R. These variant phenotypes all have the same genetic fingerprint and come from a known common ancestor, but have different phenotypic characteristics.

Chapter 8. General Discussion

Soil-borne diseases of plants are the result of complex interactions between the plant, pathogen and the abiotic and biotic components of the soil, and disease can be suppressed by soil microorganisms even in the presence of a virulent pathogen. One example of this is take-all decline (TAD) where take-all disease is suppressed after continuous monoculture of wheat. Disease suppression during TAD is due to soil microorganisms, and microorganisms (in particular fluorescent pseudomonads) which can reduce take-all disease have been isolated from suppressive soil (Raaijmakers and Weller, 1998). Two related strategies for controlling soil-borne pathogens are the *in situ* development of disease suppressive soil and, or combined with, the application of a biological control agent. Both these strategies, however, present some difficulties when applied to the field. The high levels of disease in the initial stages of TAD, and the long time till take-all is suppressed (up to 10 or more years) are major impediments to the *in situ* development of suppression. Inoculation with microorganisms suppressive to disease could be used to reduce the initial disease peak and shorten the time until suppression develops, however disease control by biological control agents has generally been inconsistent. Previous research has shown that the microflora, in particular the pseudomonad community, is altered in suppressive soils (Smiley, 1979; Weller *et al.*, 1988), indicating that a disease suppressive microbial community has been selected for. However, it is not known what factors in the root-soil environment, or which niches, select for disease antagonists, or whether single bacterial isolates are capable of changing phenotype in the root-soil environment and what impact this could have on disease control by the organism. If factors or conditions which select for disease antagonists were known, then we would be better able to direct the evolution of the soil microflora towards a suppressive microbial community.

Bacterial populations and communities evolve by selection for more favourable phenotypes for a particular environment. In the case of TAD soils, disease suppressive microorganisms have been selected for. This selection could be for existing isolates within the microbial community or for new phenotypes which arise in an existing population and are selected for. These two models are shown in Fig. 8.1. In the first model (Fig. 8.1A) selection between existing populations within the community would act by selecting for populations of disease antagonists which are present at low relative numbers and increase in relative number during TAD leading to a suppressive soil community. In the second model (Fig. 8.1B), an initially non-suppressive population produces mutants which are increased in their ability to suppress disease and these are selected for to produce a new suppressive population. The diversification of Pc2140 on wheat roots (Chapter 5) provides evidence that bacteria can produce new phenotypes in the environment and so selection within a population for new variants is a possibility. Selection pressures which select for

disease antagonists within a community would also select for new disease antagonists which arise by mutation. In this study it was hypothesised that root lesions are a niche which preferentially selects for disease antagonists. To test this hypothesis, the pseudomonad population was cycled on successive generations of diseased root lesions, and the ability of this population after each wheat cycle was compared to the pseudomonad population cycled on successive generations of healthy roots and non-lesioned sections of diseased roots.

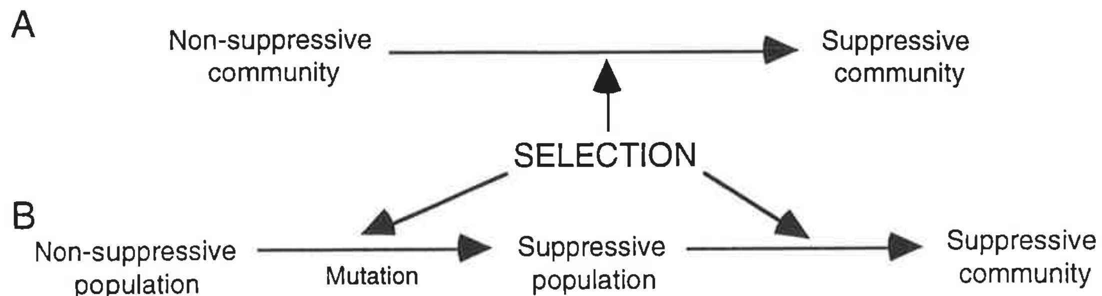


Fig. 8.1. Development of a disease suppressive microbial community by: (A) selection between existing populations; and by (B) selection within a population for spontaneous mutants with increased disease control ability.

8.1. Directed selection by root lesions

In Chapter 3, it was shown that diseased root lesions produced after infection by Ggt are a distinct niche, and that they select for an altered microflora compared to non-lesioned sections of the same diseased root system. When the population sizes of the total aerobic bacteria and pseudomonads were considered, populations on non-lesioned sections of disease roots were similar to healthy roots. When population sizes from these non-lesioned root sections were compared to root lesions there were many similarities to previous results comparing populations on healthy roots with diseased roots (Weller, 1983; Mazzola and Cook, 1991), and comparing populations in non-suppressive soils with suppressive TAD soil (Weller *et al.*, 1988; Andrade *et al.*, 1994a). In suppressive soil and on diseased roots and on root lesions there is an increase in the size of the TAB and pseudomonad population, and the proportion of fluorescent pseudomonads is increased (Fig. 8.2). This suggests that the same selection pressures are possibly operating within each system to bring about the same kinds of changes in the bacterial community.

It had been suggested that take-all disease selects for a suppressive microflora, and this is associated with an increase in bacterial populations and in the percentage of fluorescent pseudomonads (Fig. 8.2A; Shipton, 1972; Sarniguet *et al.*, 1992a). Take-all disease is caused by Ggt, and infection of wheat roots by Ggt produces the same population changes found in suppressive soils as evidenced by comparisons between healthy and diseased roots (Fig. 8.2B,

Weller, 1983; Charigkapakorn and Sivasithamparam, 1987; Sarniguet *et al.*, 1992a), and supports the suggestion that disease is the selection pressure producing the population changes during TAD. From this work (Chapter 3), it is clear that the differences in population sizes in comparisons between healthy and diseased roots is due to the effect of the root lesion niche (Fig. 8.2C). This supports the hypothesis that root lesions select for the observed population changes, but the results presented in this thesis show that lesions do not necessarily select for disease antagonistic pseudomonads.

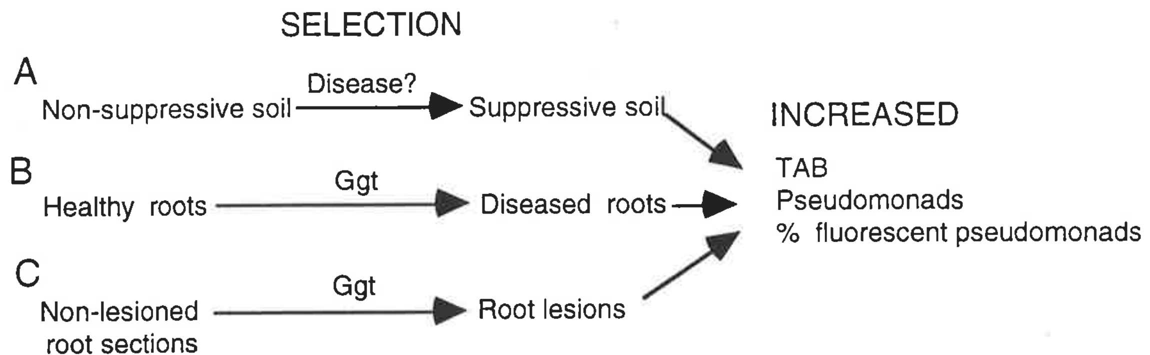


Fig. 8.2. Comparisons of bacterial populations between: (A) non-suppressive and suppressive soil; (B) healthy roots and diseased roots; and (C) root lesions and non-lesioned sections of diseased roots. The known or hypothesised selection pressure exerted on each system to cause the microbial changes is indicated.

The pseudomonad population was targeted in this study, as this group has been implicated in disease suppression during take-all decline, and a number of studies have shown that the relative number of fluorescent pseudomonads antagonistic to the pathogen, Ggt, or the disease, take-all are increased in suppressive soils (Weller, 1988; Andrade *et al.*, 1994a). It is less clear though whether antagonistic fluorescent pseudomonads are selected for by Ggt. The proportion of antagonistic fluorescent pseudomonads was found to be increased on take-all diseased roots compared to healthy roots when plants were fertilised with nitrate nitrogen (Sarniguet *et al.*, 1992a), and antagonistic fluorescent pseudomonads were increased on roots of turf grass infected with the closely related *Gaeumannomyces graminis* var. *avenae* (Sarniguet and Lucas, 1992). Charigkapakorn and Sivasithamparam (1987) however, did not find any increase in the proportion of antagonists on Ggt diseased roots compared to healthy roots. If the pseudomonad population is a major bacterial group contributing to TAD, and root lesions select for antagonistic pseudomonads, then cycling of the pseudomonad population on root lesions should select for an increased proportion of disease antagonistic pseudomonads, but this did not occur in the current work. It appears then that root lesions select for fluorescent pseudomonads, but not for pathogen or disease antagonistic pseudomonads.

8.2. The implications of targeting the fluorescent pseudomonad group in studies on take-all decline

The fluorescent pseudomonad group is often the target group in studies on take-all decline, and although the relative proportion of fluorescent pseudomonads is increased in suppressive compared to non-suppressive soil, it has not been shown that this is the group responsible for disease suppression in take-all decline soils. A summary of the changes which occurred in the pseudomonad population during this work, and the effect on take-all disease, when bacterial populations were cycled on wheat roots is shown in Fig. 8.3. Cycling of both the total aerobic bacterial population (Fig. 8.3A) and pseudomonads (Fig. 8.3B) on root lesions produced the same response in the pseudomonad population, ie. the proportion of fluorescent isolates was increased and Ggt-inhibitors decreased. The TAB population after cycling on wheat roots reduced the severity of take-all in contrast to the pseudomonad population which increased the effect of take-all disease. This suggests that the non-pseudomonad proportion of the TAB population was antagonistic to take-all. This was confirmed in a separate experiment where the non-pseudomonads (cca sensitive) and pseudomonads (cca resistant) were separated and tested for their ability to reduce take-all. The non-pseudomonads reduced take-all to the same extent as the total mixed population. The pseudomonads from the TAB also contributed to the reduction of disease by the mixed population, but were slightly less effective than the non-pseudomonads (Fig. 3.6). One explanation for this is that pseudomonad disease antagonists were selected for when other bacterial groups were present.

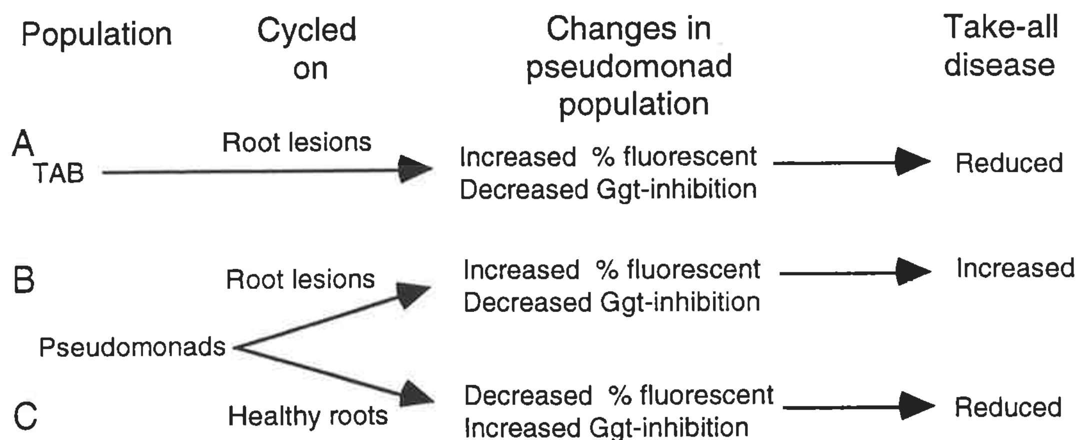


Fig 8.3. Changes in the pseudomonad population, and the effect on take-all disease, by bacterial populations cultured on successive generations of wheat roots in Kapunda soil. Populations are: (A) the total aerobic bacterial (TAB) population cycled on root lesions; and the pseudomonad population cycled on (B) root lesions; and (C) healthy roots.

In contrast to the pseudomonad population cycled on root lesions, the pseudomonad population cycled on healthy roots produced a positive response in both plant growth promotion and disease control (Fig. 8.3C). On healthy roots though, the proportion of fluorescent isolates

was decreased while Ggt-inhibitors increased. This indicated that non-fluorescent pseudomonads were responsible for the reduction in take-all. This result, combined with the observation that TAB from root lesions reducing disease, but not the pseudomonads from root lesions, suggests that the fluorescent pseudomonad group is not the correct target group to be studied in relation to take-all decline, at least in the soil used in this work. The results of studies which concentrate on the fluorescent pseudomonads may be biased and exclude important groups such as non-fluorescent pseudomonads. Non-fluorescent pseudomonads are increased in their proportion of the pseudomonad population on healthy roots, and pseudomonads from healthy roots reduce the severity of take-all. Pc2140 is also a non-fluorescent pseudomonad which can reduce take-all, but would have been excluded in population studies if only fluorescent pseudomonads were isolated.

8.3. Selection for variant phenotypes

Factors, conditions or niches in the root soil environment which select between populations for disease antagonists would also select for mutants with increased disease antagonism (Fig. 8.1B). Although there have been many studies on the diversification of bacteria in the laboratory, and Thompson *et al.* (1995) showed that *P. fluorescens* SBW25 diversified on the phylloplane, there was previously no information on the diversification of biological control strains in the root-soil environment, or the impact this has on disease control. Pc2140 diversifies *in vitro* in a manner similar to other pseudomonad strains (Chapter 4), and the distinctive colony morphology of Pc2140 enhances the use of this strain as a model pseudomonad biocontrol agent for studying phenotype plasticity.

The diversification of Pc2140 *in vitro* and on wheat roots is summarised in Fig. 8.4. The diversification of Pc2140 *in vitro* and *in vivo* was consistent with reports on the diversification of other bacterial strains *in vitro*. That is, (1) variants were produced in an apparently random manner, (2) multiple phenotypes were produced which could give rise to further variants, (3) the variant phenotypes which were selected for depended on the culture conditions, and (4) variant phenotypes eventually became the dominant phenotype in the Pc2140 population. It is difficult to extrapolate results from *in vitro* diversification to those that may occur in the environment. In this study the diversification of Pc2140 *in vitro* was compared to the diversification of Pc2140R on wheat roots, and there were many similarities between the two. Pc2140R populations on roots also produced multiple phenotypes in an apparently random manner, and these new variants became the dominant phenotype. This process though, occurred over a much longer time frame on wheat roots (years) compared to *in vitro* cultures (weeks). The similarities between *in vitro* and *in vivo* diversification of Pc2140 provides evidence that studying the evolution of bacteria on a Petri dish can reflect what may occur in the environment. It must be remembered though that different phenotypes will be selected for in the natural environment compared to

phenotypes selected for by laboratory conditions because Pc2140 produced a different set of variant phenotypes on wheat roots *in vivo* compared to the phenotypes produced in *in vitro* cultures.

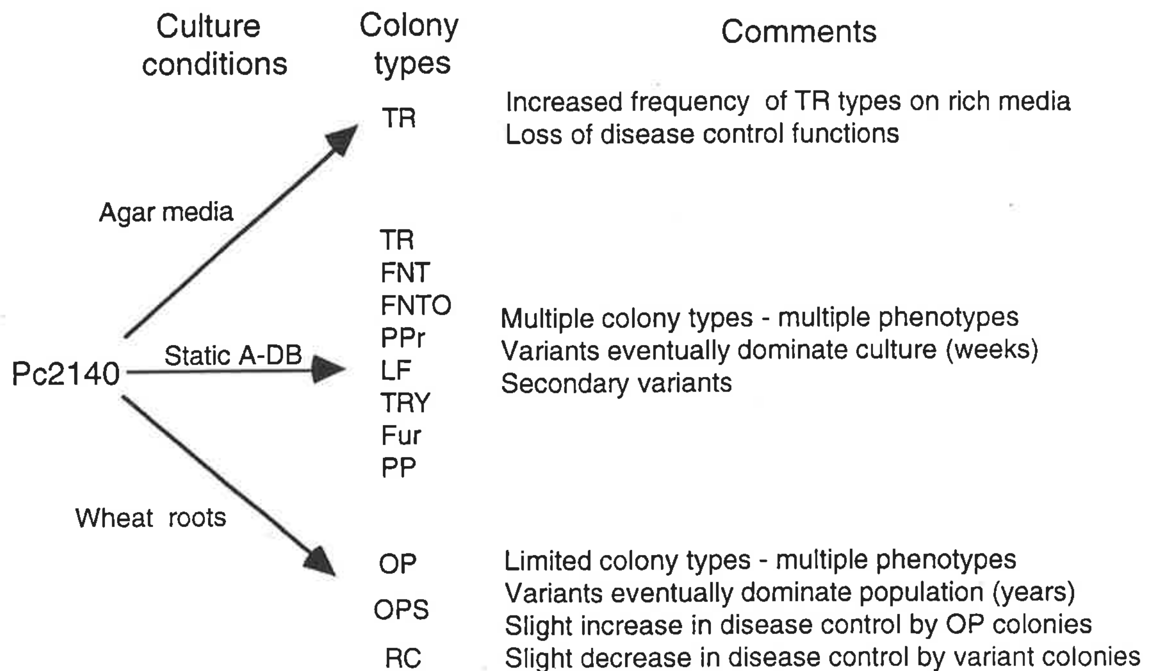


Fig. 8.4. Summary of the diversification of Pc2140 on agar media, in static ammonium defined broth (A-DA) and on wheat roots.

In this study, it was hypothesised that root lesions, if they selected for disease antagonists, would also select for variant phenotypes with increased ability to reduce take-all disease. Variant phenotypes were produced by Pc2140R on both healthy and diseased roots, but isolates with variant colony morphologies (mainly RC types produced on both healthy and lesioned roots) were generally decreased in their ability to control take-all compared to the ancestral Pc2140R. This suggests that selection for variant phenotypes on both healthy or lesioned roots under the conditions used in this study is not related to their ability to control take-all. For variant phenotypes to be selected for, they must have some advantage, or greater fitness compared to the parent type, such that they reach higher population numbers or have increased survival. It has been previously suggested that root colonisation and disease control may be dependent on different mechanisms (Bull *et al.*, 1991; Mazzola *et al.*, 1992), and this appears to be the case with Pc2140R cultured on wheat roots under these conditions.

Mechanisms for improved root colonisation or survival may be linked by regulatory networks to mechanisms for disease control. For example, exopolysaccharide (EPS) production by bacteria is important for colonisation of plant roots (Saile *et al.*, 1997) and survival (Griffiths *et al.*, 1998; Poplawsky and Chun, 1998), and EPS production is linked to virulence factors in plant

pathogens by common regulatory elements (Kao and Sequeira, 1991). Virulence and disease control factors can be regulated by homologous genes such as *lem*A and *gac*A (Rich *et al.*, 1994). Polysaccharide production by bacteria may also have a direct effect on disease control by induced resistance (Van Peer and Schippers, 1992), or indirectly by improving plant growth through increased soil aggregation on roots leading to better water relations between roots and soil (Amellal *et al.*, 1998). EPS production appears to be linked to factors involved in disease control in Pc2140, because the wild type (OP) colony type is noticeably more mucoid (increased polysaccharides) on TZCA than variant colony types such as the TR colony type, which does not control disease, and the RC colony type which has a reduced ability to control take-all compared to the wild type. Production of polysaccharides and metabolites involved with disease suppression may be linked by common regulatory genes, eg *lem*A-*gac*A type genes (Sacherer *et al.*, 1992; Gaffney *et al.*, 1994). EPS production improves bacterial resistant to osmotic stress (Wai *et al.*, 1998) and so mutants with improved EPS production, and thus survival, can be preferentially selected for by using desiccation as a selection pressure.

8.4. Phenotype conversion and microbial inoculants

Phenotype conversion is of particular importance in bacterial strains isolated for biological control of plant diseases as the common variant phenotypes produced *in vitro* have lost the ability to control disease. It is also important to maintain the desired phenotype in other bacteria that have been isolated for use as bioremediation agents or as microbial inoculants for other purposes. The occurrence of phenotype conversion in bacterial cultures appears to be severely underestimated by many researchers, and during the course of this study it became evident that most bacterial strains (both Gram positive and Gram negative strains) used at the CSIRO Land and Water laboratory produce variant phenotypes. This was most evident after transfer of strains between countries as stab cultures. The results presented in Chapter 4 indicate this to be an ideal situation for the production of *in vitro* variants. TZCA is a good medium for the detection of colony morphology variants (which indicates altered phenotype), particularly for *Pseudomonas* spp. or closely related species. The use of this medium to monitor phenotype conversion should be encouraged because variants can be easily differentiated from the wild type even though they may not be noticeably different on other media. TZCA however is not a suitable medium to differentiate variant and wild type for all bacterial strains because spontaneous variant phenotypes from *Bacillus* strains are only a little different from the wild type on TZCA, but easily distinguishable from the wild type on TSA medium. Further research still needs to be undertaken to identify, or develop (1) media on which variant phenotypes produced by other bacterial genera (non-pseudomonads) can be detected and (2) media which reduce the incidence of phenotype conversion. The sensitivity of GC-FAME in detecting changes in

phenotype is also useful for identifying or confirming an isolate in a mixed (or potentially mixed) population is in fact the wild type strain, and not a spontaneous phenotype variant.

8.5. Phenotype plasticity: implications for studies on bacterial populations

The fact that bacteria diversify so rapidly to produce a range of phenotypes also has implications for the interpretation of data derived from the phenotypic characterisation of isolates recovered from environmental samples. Previously, it has not been reported what range of phenotypes might be produced by a single isolate in the environment, except for the study of Thompson *et al.* (1995) where the diversification of GC-FAME profiles was assessed from a marked *P. fluorescens* strain reisolated after 220 days on sugar beet leaves. The GC-FAME profiles of the isolates in Thompson *et al.* (1995) diverged only a small amount (less than 2 ED) in contrast to the divergence of Pc2140 GC-FAME profiles in this study (up to 12 ED). This suggests that Thompson *et al.* (1995) were only measuring minor changes in the wild type strain, and had not recovered any isolates with gross phenotype changes (ie. colony morphology variants) because wild type (OP) colonies of Pc2140R reisolated after 108 weeks on wheat roots diverged in GC-FAME profiles from the ancestral Pc2140R at a ED <3, a similar level to the divergence found by Thompson *et al.* (1995). Pc2140R reisolates with variant colony morphologies reisolated from roots diverged from the ancestral strain at a ED of 6. Variants with gross phenotype changes may have been present in the Thompson *et al.* (1995) study, but only at low relative numbers. All populations of Pc2140 contained variant colony types after 168 days (24 weeks, C2), but these occurred at only at a low percentage, about 1% in most populations (Fig. 5.2). At this low level, variants could easily be missed unless they had a noticeably different phenotype (ie. altered colony morphology).

In ecological studies on bacterial populations in the environment, grouping of isolates based on phenotypic characteristics attempts to show relationships between the bacteria that have been isolated, and hopefully the groupings reflect the true relationships between unknown isolates. The results of this work show that an individual bacterial isolate (Pc2140) can diversify significantly in phenotypic characteristics, ie. biochemical (GC-FAME) and metabolic (BIOLOG) fingerprints, and ecological function (inhibition profile), to produce a range of phenotypes. These can be treated as a mixed population with the important distinction that the relationship between variants is known, ie. they have common ancestor, Pc2140. Variant phenotypes produced by Pc2140 diverged at the species level according to the microbial identification system using GC-FAME profiles, and diverged to a lesser extent using the BIOLOG taxonomic identification system. Researchers using phenotypic characteristics to identify and group similar isolates should be aware of this fact when interpreting the results of phenotypic analysis. These results also indicate that the relationship between isolates needs to be based on genetic data to

avoid confounding results that may arise due to the same basic genotype having a range of phenotypes which may overlap with other genotypes.

Rainey *et al.* (1994) also provided evidence of phenotype divergence in the environment, with isolates with different phenotypes having the same genetic fingerprint, but in the study of Rainey *et al.* (1994) the actual relationships between the isolates were unknown. Isolates with the same genetic profiles may have divergent phenotypes in environmental samples. It is possible that this may not be recognised or reported in ecological studies on environmental samples if only one phenotype is dominant in each niche for each genotype. In populations of Pc2140R on wheat roots, each replicate population tended to become dominated by one variant phenotype. Different replicate pot cultures however, became dominated by different variant phenotypes (Figs. 5.3 and 5.4).

8.6. Conclusions

The results of the research presented here show that the bacterial community on wheat roots can be partitioned by (1) their location in the root-soil environment (eg. healthy roots and root lesions) and (2) by biotic group (eg. TAB, pseudomonads, TB-T isolates). These groups can be selectively isolated and produce a response when inoculated onto wheat plants, with the response determined by bacterial group and their location on wheat roots. In selectively partitioning the root microflora by location and bacterial group, it can be seen that root lesions are a distinct niche which preferentially select for fluorescent pseudomonads. The hypothesis that root lesions select for a take-all suppressive pseudomonad community is not supported by the results presented here. Pseudomonads which have a positive effect on the growth of healthy and diseased wheat seedlings appear to be selected for by healthy roots. Root lesions appear to select for a component of the TAB population which is not isolated on TSAcca. Previous studies on the bacterial ecology associated with TAD have not considered variation in community structure within a root system due to infection with Ggt (ie. root lesions) and may have over emphasised the fluorescent pseudomonad group to the exclusion of other bacterial isolates. For accurately targeted studies on TAD there is a need to identify which groups contribute to disease suppression, and the location in the root-soil environment where disease suppressive bacteria are selected for during the development of TAD.

Pc2140 is a suitable model disease antagonist for studying phenotype plasticity because the behaviour of Pc2140 in producing variant phenotypes is similar to the other *Pseudomonas* strains used in this study, and to other bacterial strains used in studies on bacterial diversification. Pc2140 diversifies both *in vitro* and *in vivo* to produce multiple variant phenotypes which are altered in (1) ability to inhibit other microorganisms *in vitro*, including wheat pathogens; (2) ability to reduce take-all disease; (3) GC-FAME profiles; and (4) carbon

source utilisation. The types of variant phenotypes that are selected for depends on the culture conditions, with different variant phenotypes being selected for by *in vitro* and *in vivo* conditions. There was no apparent difference in selection for variant phenotypes between healthy roots and diseased root lesions, with variant phenotypes produced in both locations and all being generally slightly reduced in their ability to control take-all. The slight increase in disease control by some Pc2140 reisolates with the wild type colony morphology after culture on wheat roots may also provide a mechanism for improving PGPR strains.

Although take-all disease on wheat, caused by Ggt, and its control by Pc2140, was used was used as the model system in this work, the results presented here could also be applicable for other soil-borne diseases and bacteria isolated as microbial inoculants, and may have far reaching implications for the selection, storage and improvement of microbial inoculants for a variety of uses.

Appendix: GENSTAT 5 commands

Hierarchical cluster analysis

Hierarchical cluster analysis and comparison of principal component plots (Procrustes) described in Chapter 6 were carried out using GENSTAT 5 statistical package.

Phenotypic data was entered in files; fame2.dat (GC-FAME); blog3.dat (BIOLOG); invit2 (inhibition). Each file consisted of a 16 x n matrix, with 16 rows (Pc2140 isolates) and n columns (n=18, GC-FAME; n=22, BIOLOG; n=7, inhibition) containing the phenotype characters.

Heirarchical cluster analysis was used to group similar Pc2140 isolates by each characteristic, GC-FAME, BIOLOG or *in vitro* inhibition. Results are presented as groupings at 5% similarity and as a dendrogram.

GC-FAME

```
job   'fame2'
output  [w=160]1
units  [nvalues=16]
open    'fame2.dat';chan=2 ;width=199
read    [chan=2] Vset[1...18]
close   chan=2
fsimilarity[print=similarity;style=abbreviated;similarity=similars]\
 Vset[1...18]; TEST=cityblock
hcluster[print=amalgamations,dendrogram;method=average]similars
stop
```

BIOLOG

```
job   'blog3'
output  [w=160]1
units  [nvalues=16]
open    'blog3.dat';chan=2 ;width=199
read    [chan=2] Vset[1...22]
close   chan=2
fsimilarity[print=similarity;style=abbreviated;similarity=similars]\
 Vset[1...22]; TEST=cityblock
hcluster[print=amalgamations,dendrogram;method=average]similars
stop
```

INHIBITION

```
job   'invit2'
output  [w=160]1
units  [nvalues=16]
open    'invit2.dat';chan=2 ;width=199
read    [chan=2] Vset[1...7]
close   chan=2
fsimilarity[print=similarity;style=abbreviated;similarity=similars]\
 Vset[1...7]; TEST=cityblock
hcluster[print=amalgamations,dendrogram;method=average]similars
Stop
```

Procrustes comparison

Procrustes comparison was used to compare principal component plots of each of the three data sets (GC-FAME, BIOLOG or *in vitro* inhibition) with the principal component plots of the other data sets. File 'variant2.txt' contains text labels for the Pc2140 isolates.

GC-FAME-BIOLOG

```
job    'prfmbl2'
output  [w=80] 1
unit    [16]
open    'variant2.txt';2
text    variant
read    [c=2] variant
close   2
open    'blog3.dat';2
read    [c=2] biolog[1...22]
close   2
open    'fame2.dat';2
read    [c=2] fame[1...18]
close   2
matrix  [variant;2] biolscor,famescor,biolout,fameout
pcp     [prin=r,l;nr=2] biolog;score=biolscor
pcp     [prin=r,l;nr=2] fame;score=famescor
rotate  [prin=rot,sums;scal=y]xin=biolscor;yin=famescor;\
xout=biolout;yout=fameout
calc    fame1,fame2,biol1,biol2=fameout$[*;1,2],biolout$[*;1,2]
print   variant,fame1,fame2,biol1,biol2
stop
```

GC-FAME-inhibition

```
job    'prfmiv2'
output  [w=80] 1
unit    [16]
open    'variant2.txt';2
text    variant
read    [c=2] variant
close   2
open    'invit2.dat';2
read    [c=2] invitro[1...7]
close   2
open    'fame2.dat';2
read    [c=2] fame[1...18]
close   2
matrix  [variant;2] inviscor,famescor,inviout,fameout
pcp     [prin=r,l;nr=2] invitro;score=inviscor
pcp     [prin=r,l;nr=2] fame;score=famescor
rotate  [prin=rot,sums;scal=y]xin=inviscor;yin=famescor;\
xout=inviout;yout=fameout
calc    fame1,fame2,invi1,invi2=fameout$[*;1,2],inviout$[*;1,2]
print   variant,fame1,fame2,invi1,invi2
stop
```

BIOLOG-inhibition

```
job     'prbliv2'
output  [w=80] 1
unit    [16]
open    'variant2.txt';2
text    variant
read    [c=2] variant
close   2
open    'blog3.dat';2
read    [c=2] biolog[1...22]
close   2
open    'invit2.dat';2
read    [c=2] invitro[1...7]
close   2
matrix  [variant;2] biolscor,inviscor,biolout,iviout
pcp     [prin=r,l;nr=2] invitro;score=inviscor
pcp     [prin=r,l;nr=2] biolog;score=biolscor
rotate  [prin=rot,sums;scal=y]xin=biolscor;yin=inviscor;\
xout=biolout;yout=inviout
calc    biol1,biol2,invi1,invi2=biolout$[*;1,2],inviout$[*;1,2]
print   variant,biol1,biol2,invi1,invi2
stop
```

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Publications Arising From This Thesis

Barnett, S. J., Singleton, I., and Ryder, M. H. (1997). The effect of growth conditions on phenotype plasticity in *Pseudomonas corrugata* 2140, and the phenotypic characterisation of a range of new phenotype variants. In *Plant Growth Promoting Rhizobacteria, Present Status and Future Prospects,* Eds. A. Ogoshi, K. Kobayashi, Y. Homma, F. Kodama, N. Kondo, and S. Akino, pp 417-420. Hokkaido University, Sapporo, Japan.

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