MECHANISMS OF BIOCONTROL OF *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* BY *PSEUDOMONAS CORRUGATA* STRAIN 2140: GENETIC AND BIOCHEMICAL ASPECTS

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"It's easier to ask questions than to answer them."

Plato *The Republic*

"Once a man is possessed by an idea, there is no doing anything with him"

Anton Chekov (1898)

TABLE OF CONTENTS

Acknowledgments			1
Preface			2
List of Figures			3
List of Tables			6
Abbreviations			7
Summary			8
CHAPTER 1		**INTRODUCTION**	
1.1		*Gaeumannomyces graminis* var. *tritici* (*Ggt*) and Take-all	10
	1.1.1	*Ggt*: The Causative Agent of Take-all in Wheat and Barley	10
	1.1.2	Environmental Factors Conducive to Take-all	11
	1.1.3	Control of *Ggt* and Farm Management	11
	1.1.4	Control of *Ggt* with Fungicides	13
	1.1.5	Take-all Decline and Take-all Suppressive Soils	13
1.2		Biological Control of Phytopathogens with Pseudomonads	14
	1.2.1	General Overview	14
	1.2.2	Factors Affecting the Activity of Biocontrol Agents	15
1.3		Metabolites of Biocontrol Agents Antagonistic to Plant Pathogens	18
	1.3.1	Siderophores and Iron Assimilation	19
	1.3.2	Antibiotics	22
	1.3.3	Hydrogen Cyanide	25
	1.3.4	Ammonia	26
	1.3.5	Manganese Reductase	27
	1.3.6	Parasitism and cell Wall-Lytic Enzymes	28
	1.3.7	Induced Resistance	29
	1.3.8	Phytotoxicity of Metabolites of Biocontrol Agents	30
1.4		Approaches to Examining Mechanisms of Biocontrol	32
	1.4.1	Why Study Mechanisms	32

	1.4.2	The Biochemical Approach	33
	1.4.3	The Molecular/Genetic Approach	35
	1.4.4	Pleiotropy	39
1.5		Summary of Introduction	39
1.6		Background to the Project	40
1.7		Aims of the Project	41

CHAPTER 2 GENERAL METHODS

2.1		Strains	43
2.2		Storage of Cultures	43
2.3		Media and Culture Conditions	44
2.4		Determination of Bacterial Cell Numbers	45
	2.4.1	Colony Forming Units (CFU/ml)	45
	2.4.2	Absorbance (A_{550})	45
2.5		Measuring the Mean Generation Times of *P. corrugata* Strains	45
2.6		Measuring the Growth of *Ggt*	46
2.7		*In vitro* Screening of Strains for *Ggt* Antagonism	46
	2.7.1	Water-soluble Compounds	46
	2.7.2	Volatile Compounds	48
2.8		*In situ* Pot Trials	48
	2.8.1	General Overview	48
	2.8.2	Pretreatment of the Soil/Sand Mix	49
	2.8.3	Wheat Seed Preparation	49
	2.8.4	Preparation of Bacterial Inoculum	49
	2.8.5	Determination of Viability of *Ggt* Inoculum	50
	2.8.6	Construction of the Pot test	50
	2.8.7	Incubation of Pots	52
2.9		Measurement of Take-all Severity on Wheat	52
	2.9.1	Water Consumption	53
	2.9.2	Disease (Root) Rating	53
	2.9.3	Root Dry Weight	53
	2.9.4	Shoot Length and Shoot Dry Weight	54

2.10	Plant Growth Promotion by *Pc*2140 in the Absence of *Ggt*	54
2.11	Colonisation of wheat Roots by *P. corrugata* Strains	54
2.11.1	Determination of Bacterial Resistance to Chloramphenicol	55
2.12	Statistical Analysis	56

CHAPTER 3 MUTAGENESIS OF *P.CORRUGATA* 2140

3.1	Introduction	57
3.2	Materials and Methods	59
3.2.1	Strains	59
3.2.2	Suitable Systems for *Pc*2140 Mutagenesis	59
3.2.3	Mutagenesis of *P. corrugata* 2140	62
3.2.4	Screening Mutants for *In vitro* Antagonism of *Ggt*	65
3.2.5	Retention of Transposon in Mutants	65
3.2.6	Auxotrophy	66
3.2.7	Pleiotropy	66
3.2.7(i)	Growth in a Minimal Medium	67
3.2.7(ii)	Volatile Production	67
3.2.7(iii)	Colony Morphology	67
3.2.8	Bioluminescence	67
3.2.9	Testing Mutants for Single Insertions of Tn*5*::*luxAB*Tc^r	68
3.2.9(i)	Isolation and Purification of Genomic DNA	68
3.2.9(ii)	Restriction Digests of DNA	69
3.2.9(iii)	Gel Electrophoresis	69
3.2.9(iv)	Blotting DNA onto Nylon Membrane	70
3.2.9(v)	Fixing and Storage of Membrane	70
3.2.10	Preparation of Probe	71
3.2.10(i)	Extraction of Plasmid DNA	71
3.2.10(ii)	Digestion of Plasmid DNA	72
3.2.10(iii)	Gel Electrophoresis	72
3.2.10(iv)	Purification of the 3.2kb Fragment from Tn*5*::*luxAB*Tc^r	72
3.2.11	Southern Hybridisation	73
3.2.11(i)	Labelling of Probe DNA	73

	3.2.11(ii)	Prehybridisation of Membrane	74
	3.2.11(iii)	Hybridisation of Membrane	74
	3.2.11(iv)	Autoradiography	75
	3.2.11(v)	Stripping Membranes of Probe	75
	3.2.12	Pot Trials	76
3.3		Results	76
	3.3.1	Mutagenesis	76
	3.3.2	Frequency of Auxotrophy of Mutants	78
	3.3.3	*In vitro* Testing of Mutants for *Ggt* Antagonism	78
	3.3.4	Stability of Transposon in Mutants	78
	3.3.5	Southern Hybridisations of Selected Mutants for Insertion Frequency in Genome	79
	3.3.6	Testing Mutants for Pleiotropic Changes and Bioluminescence	79
	3.3.7	Pot trials: Testing Mutants for Suppression of Take-all Symptoms *In situ*	81
	3.3.7(i)	Root Disease Rating	83
	3.3.7(ii)	Water Consumption by Wheat Plants	84
	3.3.7(iii)	Shoot Lengths	85
	3.3.7(iv)	Shoot Dry Weights	85
	3.3.7(v)	Root Dry Weights	86
	3.3.8	Plant Growth Promotion by *P. corrugata* 2140	102
	3.3.9	Root Colonisation by *P. corrugata* Strains	102
3.4		Discussion	108

CHAPTER 4 COMPLEMENTATION OF MUTANT STRAIN TM1120 TO RESTORE BIOCONTROL ACTIVITY

4.1		Introduction	117
4.2		Materials and Methods	117
	4.2.1	Overview	117
	4.2.2	Strains	118
	4.2.3	Construction of the Cosmid Library	118
	4.2.4	Complementation of Strain TM1120	121

4.2.4(i)	Preparation of Cells	121
4.2.4(ii)	Triparental mating of TM1120	121
4.2.5	Screening of Transconjugant Mutants for Restored *In vitro* Inhibition of *Ggt*	122
4.2.6	Stability of Cosmids in TM1120	122
4.2.7	Probing Cosmids for Homologous Sequences	123
4.2.7(i)	General Overview	123
4.2.7(ii)	Treatment of pBluescript M13 with Calf Intestine Phosphatase	123
4.2.7(iii)	Ligation of Genomic DNA into pBluescript M13	124
4.2.7(iv)	Transformation of Cloned DNA into *E. coli* JM83	124
4.2.7(iv)(a)	Preparation of Competent *E. coli* JM83 Cells	124
4.2.7(iv)(b)	Transformation of Competent *E. coli* JM83 Cells	127
4.2.7(v)	Separation of Genomic DNA from the Tetracycline Gene	127
4.2.7(vi)	DIG-Labelling the probe for Dot-blot Hybridisation	128
4.2.7(vi)(a)	Isolation and Purification of the 2.1kb DNA Fragment	128
4.2.7(vi)(b)	Labelling the Probe with Digoxygenin	129
4.2.7(vii)	Dot-blot Hybridisation	129
4.2.7(vii)(a)	Purification of DNA	129
4.2.7(vii)(b)	Fixing DNA Samples to a Nylon Membrane	130
4.2.7(vii)(c)	Prehybridisation of the Membrane	130
4.2.7(vii)(d)	Hybridisation	130
4.2.8	Pot Trials for Complemented Mutants	131
4.3	Results	132
4.3.1	Complementation Mating	132
4.3.2	*In vitro* Antagonism of *Ggt* by Complemented Mutants	132
4.3.3	Stability of the Cosmid in *P. corrugata*	135
4.3.4	Dot-blot Hybridisation	135
4.3.4(i)	Construction of the Probe	135
4.3.4(ii)	Dot-blot Hybridisation	137
4.3.5	Pot Trials of Complemented Mutants	139
4.4	Discussion	142

CHAPTER 5 METABOLITES OF *P. CORRUGATA* 2140 AND BIOCONTROL OF *GGT.*

5.1		Introduction	148
5.2		Materials and Methods	149
	5.2.1	Strains and Culture Conditions	149
	5.2.2	Siderophores	149
	5.2.2(i)	Detection of Siderophores	149
	5.2.2(ii)	Quantification of Siderophore Production	150
	5.2.3	Volatile Production and Antagonism of *Ggt*	150
	5.2.4	Hydrogen Cyanide	150
	5.2.4(i)	Detection of Hydrogen Cyanide	150
	5.2.4(ii)	Quantification of HCN	151
	5.2.4(iii)	Sensitivity of *Ggt* to HCN	152
	5.2.5	Ammonia	152
	5.2.5(i)	Culture Conditions	152
	5.2.5(ii)	Detection of Ammonia	153
	5.2.5(iii)	Standard Curve	153
	5.2.6	Manganese Reductase	153
	5.2.7	Proteases	154
	5.2.8	Chitinase	155
	5.2.9	Cellulase	155
	5.2.10	β-1,3 Glucanase	156
	5.2.11	Water-soluble Antibiotics	157
	5.2.11(i)	Production and Initial Isolation	157
	5.2.11(ii)	Partial Purification of Antifungal Compounds	157
	5.2.11(iii)	Screening Samples for Antifungal Activity	158
	5.2.11(iv)	Thin-Layer Chromatography	158
	5.2.12	Peptides	159
5.3		Results	160
	5.3.1	Siderophore Production by *P. corrugata* 2140	160
	5.3.2	Inhibition of *Ggt* Growth by Volatile Production	162
	5.3.3	Production of Hydrogen Cyanide	162
	5.3.4	Ammonia Production	162

5.3.5 Manganese Reductase 165
5.3.6 Proteases 165
5.3.7 Cell Wall Lytic Enzymes 166
5.3.8 Antibiotics 167
5.3.9 Peptides 170
5.4 Discussion 170

CHAPTER 6 GENERAL DISCUSSION 178

APPENDICIES AND BIBLIOGRAPHY

Appendix I: Media and Other Formulae 192
Appendix II: Growth Rate of *P. corrugata* 2140 201
Appendix III: Standard Curves for Volatile Quantification 202
Appendix IV: Approval to Test GMO's in Soil 204
Appendix V: Summary of Characteristics of Mutants 205
Appendix VI: Publications and Presentations 206
Bibliography 207

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PREFACE

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

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

SIGNED. ...DATE.....30/5/96.....

LIST OF FIGURES

Figure	Title	Page
2.1	Measurement of *in vitro* radial growth of *Ggt*	47
2.2	Construction of the pot trial to assess disease suppression by *P. corrugata* strains	51
3.1	Map of plasmid pUT containing Tn*5*::*luxABTc*^{r}	63
3.2	Restriction map of Tn*5*::*luxABTc*^{r}	64
3.3	*In vitro* antagonism of *Ggt* by *Pc*2140 and TM1120	77
3.4	Southern hybridisation of *P. corrugata* total genomic DNA for single insertions of the transposon	80
3.5	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140 or mutant strain TM27 in the presence of *Ggt*	87
3.6	Comparison of water consumption of wheat plants inoculated with either *Pc*2140 or mutant strain TM27 in the presence of *Ggt*	88
3.7	Comparison of shoot lengths of wheat plants inoculated with either *Pc*2140 or mutant strain TM27 in the presence of *Ggt*	89
3.8	Comparison of shoot dry weights of wheat plants inoculated with either *Pc*2140 or mutant strain TM27 in the presence of *Ggt*	89
3.9	Comparison of root dry weights of wheat plants inoculated with either *Pc*2140 and mutant strain TM27 in the presence of *Ggt*	89
3.10	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140 or mutant strain TM129 in the presence of *Ggt*	90
3.11	Comparison of water consumption of wheat plants inoculated with either *Pc*2140 or mutant strain TM129 in the presence of *Ggt*	91
3.12	Comparison of shoot lengths of wheat plants inoculated with either *Pc*2140 or mutant strain TM129 in the presence of *Ggt*	92
3.13	Comparison of shoot dry weights of wheat plants inoculated with either *Pco*2140 or mutant strain TM129 in the presence of *Ggt*	92
3.14	Comparison of root dry weights of wheat plants inoculated with either *Pc*2140 and mutant strain TM129 in the presence of *Ggt*	92
3.15	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140 or mutant strain TM300 in the presence of *Ggt*	93
3.16	Comparison of water consumption of wheat plants inoculated with either *Pc*2140 or mutant strain TM300 in the presence of *Ggt*	94
3.17	Comparison of shoot lengths of wheat plants inoculated with either *Pc*2140 or mutant strain TM300 in the presence of *Ggt*	95

3.18	Comparison of shoot dry weights of wheat plants inoculated with either *Pc*2140 or mutant strain TM300 in the presence of *Ggt*	95
3.19	Comparison of root dry weights of wheat plants inoculated with either *Pc*2140 and mutant strain TM300 in the presence of *Ggt*	95
3.20	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140 or mutant strain TM692 in the presence of *Ggt*	96
3.21	Comparison of water consumption of wheat plants inoculated with either *Pc*2140 or mutant strain TM692 in the presence of *Ggt*	97
3.22	Comparison of shoot lengths of wheat plants inoculated with either *Pc*2140 or mutant strain TM692 in the presence of *Ggt*	98
3.23	Comparison of shoot dry weights of wheat plants inoculated with either *Pc*2140 or mutant strain TM692 in the presence of *Ggt*	98
3.24	Comparison of root dry weights of wheat plants inoculated with either *Pc*2140 and mutant strain TM692 in the presence of *Ggt*	98
3.25	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140 or mutant strain TM1120 in the presence of *Ggt*	99
3.26	Comparison of water consumption of wheat plants inoculated with either *Pc*2140 or mutant strain TM1120 in the presence of *Ggt*	100
3.27	Comparison of shoot lengths of wheat plants inoculated with either *Pc*2140 or mutant strain TM1120 in the presence of *Ggt*	101
3.28	Comparison of shoot dry weights of wheat plants inoculated with either *Pc*2140 or mutant strain TM1120 in the presence of *Ggt*	101
3.29	Comparison of root dry weights of wheat plants inoculated with either *Pc*2140 and mutant strain TM1120 in the presence of *Ggt*	101
3.30	PGPR effect on wheat plants by *Pc*2140 in absence of take-all: water consumption by wheat plants	103
3.31	PGPR effect on wheat plants by *Pc*2140 in absence of take-all: wheat shoot lengths	104
3.32	PGPR effect on wheat plants by *Pc*2140 in absence of take-all: wheat shoot dry weights	105
3.32	PGPR effect on wheat plants by *Pc*2140 in absence of take-all: wheat root dry weights	106
4.1	Map of plasmid pBluescript M13-FF (pBSM13-FF)	125
4.2	Gel electrophoresis of fragments used to construct plasmids pBSM13 and pBSM13-PD	126
4.3	Inhibition of *Ggt* by *Pc*2140, TM1120 and TM1120α8	133
4.4	Stability of cosmids in TM1120	136
4.5	Restriction enzyme digestion and electrophoresis of plasmids pBSM13-FF and pUT::Tn*5*	138

4.6	Comparison of root disease rating on roots of wheat plants inoculated with either *Pc*2140, mutant strain TM1120 and complemented strain TM1120α8 in the presence of *Ggt*	140
4.7	Comparison of water consumption of wheat plants inoculated with either *Pc*2140, mutant strain TM1120 and complemented mutant TM1120α8 in the presence of *Ggt*	141
5.1	Sensitivity of *Ggt* to cyanide (as KCN) *in vitro*	163

LIST OF TABLES

Table	Title	Page
1.1	Control of phytopathogens via release of siderophore by biocontrol agents	21
1.2	Control of phytopathogens via release of antibiotics by biocontrol agents	23
3.1	Bacterial strains utilised for the mutagenesis of *Pc*2140	60
3.2	Plasmids utilised for the mutagenesis of *Pc*2140	61
3.3	Basic characteristics of mutants of *P. corrugata* 2140 with reduced biocontrol activity	82
3.4	Wheat root colonisation by *P. corrugata 2140* and selected mutants	107
4.1	Bacterial strains utilised for the complementation of mutant strain TM1120	119
4.2	Plasmids and cosmids utilised for the complementation of mutant strain TM1120	120
4.3	Characterisation of complemented mutants with restored biocontrol activity	134
5.1	Quantification of siderophore production by *P. corrugata* strains	161
5.2	*In vitro* inhibition of *Ggt* by volatiles of *P. corrugata* strains	164
5.3	Ethyl acetate extracts of *P. corrugata* 2140 supernatants: Inhibition of *Ggt*	168
5.4	Thin layer chromatography of supernatants of *Pc*2140 and TM1120: Inspection under UV light	169
5.5	Summary of metabolite production by *P. corrugata* 2140 and mutant strain TM1120	172

ABBREVIATIONS

A_x	Absorbance @ x nm
Ap	ampicillin
CIP	calf intestinal phosphatase
Cm	chloramphenicol
cps	counts per minute
Cx	cycloheximide
DDW	double distilled water
DNA	deoxyribonucleic acid
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
gDNA	genomic DNA
Ggt	*Gaeumannomyces graminis* var. *tritici* 8
GTE	Glucose/Tris/EDTA (buffer)
Km	kanamycin
LB	Luria-Bertani (medium)
LSD	Least Significant Difference
MGT	Mean Generation Time
MMBE	modified mineral base 'E' (medium)
MR	manganese reductase
MSM	mutant selective medium
MMBE	modified mineral base E (medium)
NA	nutrient agar
NB	nutrient broth
*Pc*2140	*Pseudomonas corrugata* strain 2140
PDA	potato dextrose agar
PNS	plant nutrient supplement
PP3	proteose peptone #3 (medium)
PSM	*Pseudomonas* selective medium
SA	sucrose-asparagine (medium)
SDDW	sterile double distilled water
SDS	sodium dodecyl sulfate
SDW	sterile distilled water
SID	siderophore detection (medium)
SSC	saline sodium citrate (buffer)
STE	sucrose/tris/EDTA (buffer)
TAE	Tris/acetic acid/EDTA (buffer)
Tc	tetracycline
TE	Tris/EDTA
TM	transposon mutant
Tp	Trimethoprim
Tris	(Tris[hydroxymethyl]aminomethane
TZCA	Tetrazolium chloride agar (medium)
V	volts

SUMMARY

Pseudomonas corrugata strain 2140 (*Pc*2140), isolated from wheat field soil in Australia, antagonises the take-all fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*) *in vitro* and significantly reduces take-all symptoms on wheat in pot trials. The mechanisms by which the biocontrol agent reduces disease symptoms on wheat were investigated.

*Pc*2140 was mutated by the random insertion of the Tn*5*::*luxAB*Tc^{r} transposon. Five non-auxotrophic mutants from 2,500 isolates screened showed reduced *in vitro* fungal antagonism. When tested in a series of pot trials, these five mutants showed varying degrees of reduced disease suppression on wheat compared to the parent strain. One mutant, designated TM1120, did not antagonise *Ggt in vitro*, nor did it reduce take-all symptoms on wheat plants when compared to the parent strain in a series of independent pot tests. This mutant contained a single insertion of the transposon in its genome and was not found to exhibit any adverse pleiotropic effects.

TM1120 was complemented with a cosmid library constructed from fragments of *Pc*2140 genomic DNA partially digested with *Hind*III and ligated into pLA2917. Cosmid pMO142133, when conjugated into TM1120, restored biocontrol activity by the mutant against *Ggt* both *in vitro* and in two independent pot trials. A 2.1kb *Eco*RI/*Not*I genomic fragment from TM1120 flanking the Tn*5* insertion was used to probe the cosmid for homology. Subsequent dot-blot hybridisation confirmed that the cosmid contained DNA homologous to the genomic region containing the transposon. These results demonstrated that the loss of *in vitro* antagonism of *Ggt* by *P. corrugata* 2140 is correlated to loss of disease suppression on wheat *in situ*. Restoration of *in vitro* antagonism by cosmid complementation also restored *in situ* disease suppression.

Biochemical analysis of metabolites of *P. corrugata* 2140 revealed a number of compounds potentially antagonistic to *Ggt* and which may play a role in disease control. These included water-soluble antibiotics, siderophores, proteases, peptides and volatiles including hydrogen cyanide. Other metabolites including ammonia, manganese reductase and cell wall-degrading enzymes including chitinase and β-1,3 glucanase were not detected. Comparison of metabolite production by *Pc*2140 and TM1120 indicated that water-soluble antibiotics and peptides were not produced by TM1120. Partial purification of these compounds from supernatants of *Pc*2140 suggested that there are at least three metabolites produced by this bacterium which are antagonistic to *Ggt*.

It is concluded that the primary mechanism of biocontrol of take-all on wheat by *P. corrugata* 2140 is through the production of one or more water-soluble antibiotics and/or peptides which directly antagonise the take-all fungus.

CHAPTER 1

GENERAL INTRODUCTION

1.1 *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* AND TAKE-ALL

1.1.1 *Ggt*: The Causative Agent of Take-all in Wheat and Barley

A serious disease of wheat and barley in many agricultural regions world-wide is take-all. The causative agent is the soil-borne fungus *Gaeumannomyces graminis* (Sacc.) von Arx and Olivier var. *tritici* Walker (Cook and Rovira, 1976; Schroth and Hancock, 1982; Weller and Cook, 1983; Bockus, 1983; Weller, 1988). There are three varieties of *Gaeumannomyces graminis* described; the susceptible host(s) and virulence differs with each variety. *Gaeumannomyces graminis* var. *tritici* (*Ggt*) causes take-all in wheat and barley although a recent report (Osbourn *et al.* 1994) describes a species of oats which lacks avencin production and is susceptible to infection by *Ggt*. *Gaeumannomyces graminis* var. *avenae* targets wheat, oats and barley, and var. *graminis* causes a variety of diseases in grasses (Cook and Weller, 1987; Hollins and Scott, 1990).

Primarily a soil microorganism, *Ggt* can infest plants by hyphae found in soil debris, infected root material or through wind-blown ascospores. In winter wheat infection occurs in the seminal roots where thin-walled fungal micro-hyphae penetrate root cell walls through the production of cellulase and pectinase by the fungus (Weste, 1970; Pearson, 1974; Dori *et al.* 1990; Dori *et al.* 1992). Penetration of the cortex tissue is followed by infestation of the stele tissue which becomes discoloured and blocked with hyphae, reducing the flow of water and nutrients to the shoots. (Cook and Weller, 1987; Dori *et al.* 1992). Typical plant symptoms include deformed roots, black root lesions and

white heads devoid of viable seed (Murray *et al.* 1991; Dori *et al.* 1992). These symptoms often result in the premature death of affected plants. Yields in take-all infested crops can be reduced by up to 75%, resulting in significant economic losses for the producer (Bockus, 1983; Brennan and Murray, 1988; Rovira, 1990; Murray *et al.* 1991; Folwell *et al.* 1991).

1.1.2 Environmental Factors Conducive to Take-all

Ggt, which is generally prevalent in the top 30cm. of the soil, is favoured by moist conditions (Cook, 1981). Consequently wet climates, especially seasons of above average rainfall and soils with poor drainage are likely to be conducive to disease (Murray *et al.* 1991; Roget and Rovira, 1991; Folwell *et al.* 1991). Soil chemistry has a primary role in take-all development. The disease is most prevalent in neutral to alkaline soils (Smiley and Cook, 1973; Cook and Weller, 1987; Folwell *et al.* 1991; Ownley *et al.* 1992). As soils become more acidic the incidence of take-all lessens; below pH4.8 the disease does not occur (Murray *et al.* 1991). Levels of particular mineral compounds can affect fungal development and severity of take-all. For example increased potassium levels limits the development of *Ggt* and reduces take-all symptoms (Prade and Trolldenier, 1990) while increased iron may render soils conducive to disease (Kloepper *et al.* 1980a). Lack of particular trace elements also may contribute to wheat susceptibility to take-all. For example manganese deficiency has been recorded as a possible reason for increases in the incidence of take-all (Wilhelm *et al.* 1990).

1.1.3 Control of *Ggt* and Farm Management

The type of cultivation and sowing techniques may affect the incidence of the disease (Davies and Whitbread, 1989). In Australia and the U.S.A. it has been found that direct-drilling of wheat can lead to an increased incidence of take-all (Cook and Weller, 1987). Delayed sowing of winter wheat may reduce take-all by increasing the period of time

between crops thereby reducing *Ggt* survival between crops (Cook and Weller, 1987). Fertilisers play a role in the degree of soil nitrification (Christensen *et al.* 1989). Increased nitrification leads to increases in take-all and occurs when the soil pH is raised from 5.5 to 6.0 (Roseberg *et al.* 1986). Nitrate based fertilisers will increase soil pH whereas ammonia based fertilisers decrease pH (Smiley and Cook, 1973; Ownley *et al.* 1992).

Ggt is a poor saprophyte. Unless favourable levels of organic matter are available in the soil in the absence of host plant roots the fungus may be susceptible to attack and degradation by other soil microorganisms. Rotovating fields immediately after harvesting instead of the following autumn reduces stubble and aerates the soil. This reduces the level of plant material available which *Ggt* requires to subsist on. Increased aeration also favours competition by hardier saprophytic microflora which replace *Ggt* in the rhizosphere (Scott, 1969). Stubble is often retained after harvesting in an effort to reduce both soil erosion and moisture loss (Kollmorgen *et al.* 1987). Stubble retention increases the survival rate of *Ggt* by either supplying the fungus with plant material from the previous crop (Cook and Weller, 1987) or by the presence of volunteer wheat seedlings germinating over spring and summer (Brassett and Gilligan, 1990). The incidence of take-all has been lowered by burning stubble thereby reducing surface and soil organic matter creating a non-conducive environment for *Ggt* survival (Murray *et al.* 1991).

Rotation of wheat with unrelated crops can influence the long-term incidence of disease (MacNish, 1980; Maas and Kotze; 1990; Folwell *et al.* 1991). Rotation with soybeans, legumes, sunflowers, potatoes, alfalfa and pasture grasses such as ryegrass may lead to an increased outbreak of take-all in subsequent wheat and barley crops (Weller and Cook, 1983; Murray *et al.* 1991; Inwood and Roget, 1993). The presence of pasture grasses which act as hosts to *Ggt* over the summer allow the fungus to survive in the soil until the next wheat or barley sowing (Dewan and Sivasithamparam, 1990a). Double

cropping of fields also may be detrimental to microorganisms that flourish under wheat monoculture and which are antagonistic to *Ggt* (Maas and Kotze, 1990).

1.1.4 Control of *Ggt* with Fungicides

Applications of fungicides and other chemicals have met with varying degrees of success in suppressing take-all. Fungicides effective against *Ggt* include benzimidazole and triazole (Ballinger and Kollmorgen, 1986; Ballinger and Kollmorgen, 1988) and sterol fungicides such as fenarimol, penconazole and triadimenol (Bockus, 1983; Bateman *et al.* 1990; Smiley *et al.* 1990). Chemicals such as dicyandiamide and nitrapyrin which inhibit nitrification also may restrict fungal activity in the soil (Christensen *et al.* 1989). The use of fungicides has its drawbacks. Some can be toxic to the crops such as the effect of triadimenol on wheat seedlings (Cook and Weller, 1987). Fungicides may not be economically viable with the cost often outstripping the losses caused by take-all (Ballinger and Kollmorgen, 1986; Cook and Weller, 1987; Weller, 1988). In addition, fungicides are often not totally effective against *Ggt* and take-all outbreaks can still occur after their application (Bockus, 1983; Smiley *et al.* 1990). This may be due to the poor persistence of the compounds in the soil (Bateman *et al.* 1990).

1.1.5 Take-all Decline and Take-all Suppressive Soils

Although take-all is prevalent in wheat monoculture, after 3 to 4 years of continuous wheat monoculture the incidence of take-all may significantly decline. This phenomenon is called "take-all decline" (TAD) (Cook and Rovira, 1976; Cook and Weller, 1987; Weller, 1988; Fravel, 1988; Thomashow and Weller, 1990). It is suspected that TAD is due to either changes in the pathogen (becoming less virulent) or a shift towards a microbial community more antagonistic to *Ggt*. The host plant developing resistance to the disease has virtually been eliminated as a cause. Yield losses through wheat monoculture in the cropping seasons before TAD occurs may lead producers into

abandoning wheat monoculture and planting other crops. This practise leads to loss of natural disease suppression and increased likelihood of take-all in future wheat crops (Weller and Cook, 1983).

Experiments with microorganisms, especially plant growth promoting rhizobacteria (PGPR) isolated from take-all suppressive soils (TASS) have shown that they play a primary role in reducing take-all on wheat (Cook, 1994). These microorganisms have been found to be better at controlling take-all than organisms isolated from take-all conducive soils (TACS). Organisms isolated from TASS are also capable of converting conducive soils to suppressive soils (Schroth and Hancock, 1982; Thomashow and Weller, 1990). The identification of take-all suppressive microbial strains and the understanding of their mechanism(s) of disease suppression may lead to the development of a viable microbial biocontrol agent as an alternative to chemical control (Weller, 1988; Thomashow *et al.* 1990).

1.2 BIOLOGICAL CONTROL OF PHYTOPATHOGENS WITH PSEUDOMONADS

1.2.1 General Overview

Biological control is the utilisation of organisms to control pests and pathogens. In recent decades the application of microbial biocontrol agents to control a variety of phytopathogens and restrict disease symptoms on plants has been investigated. Many different microorganisms have been found to inhibit a wide range of plant pathogens *in vitro*. These microorganisms include fungi (Wong and Siviour, 1979; Dewan and Sivasithamparam,1989; Ghisalberti *et al.* 1990; Dewan and Sivasithamparam, 1990b), yeasts (Suzzi *et al.* 1995; Walker *et al.* 1995), amoebae (Charkraborty, 1983), and many species of soil-borne bacteria including *Bacillus* (Leifert *et al.* 1995), *Agrobacterium*

(Kerr, 1980), *Aeromonas* (Inbar and Chet, 1991) and *Pseudomonas* strains as reviewed by Weller (1988).

Various strains of the bacterial genus *Pseudomonas* have been found to control a variety of plant pathogens. Examples include *Fusarium oxysporum* f. spp. suppression by *P. putida* (Scher and Baker, 1982; Simeoni *et al.* 1987), *P. fluorescens* control of *Pythium ultimum* (Gutterson *et al.* 1986; Keel *et al.* 1992), *Rhizoctonia solani* in cotton (Howell and Stiponovic, 1979; Misaghi *et al.* 1982) and *Thielaviopsis basicola* induced black rot of tobacco (Keel *et al.* 1992). Pseudomonads have also been examined for the control of post-harvest diseases such as citrus green mould caused by *Penicillium digatatum* (Smilanick and Denis-Arrue, 1992) and fungal diseases of dutch white cabbage (Stanley *et al.* 1994). Pseudomonads isolated from TASS have been found to be useful for the suppression of take-all (Cook and Rovira, 1976; Schroth and Hancock, 1982; Brisbane and Rovira, 1988; Weller, 1988).

1.2.2 Factors Affecting the Activity of Biocontrol Agents

One of the major problems of biocontrol agents is inconsistent performance in field trials (Cook, 1994). Successful *in vitro* inhibition in the laboratory and in controlled glasshouse pot trials does not always transfer to consistent suppression of disease in large-scale field trials. Furthermore, activity of biocontrol agents against specific pathogens often varies with soil and climate regimes. For example, fluorescent *Pseudomonas* strains 2-79 and 13-79 which control take-all on wheat when tested in north-west USA soils were found to have only minor biocontrol abilities against take-all when tested in fen peat and clay soils in the United Kingdom (Capper and Higgins, 1993). A potential biocontrol agent must be able to (i) colonise the host plant at sites where it is effective against a targeted pathogen, (ii) compete successfully with indigenous microflora for available nutrients, (iii) tolerate both the physical and chemical

conditions of the rhizosphere where it is introduced and (iv) remain biologically active against the targeted phytopathogen on the specific host plant.

The presence in the rhizoplane (the surface area of the plant root in contact with the soil) of a biocontrol agent actively antagonistic to a phytopathogen will often restrict the pathogen's ability to infest the plant. Microorganisms colonise the roots to take advantage of root exudates, lysates and mucilage which are utilised as sources of carbon and nitrogen (Foster, 1986). Bacteria may be attracted towards roots via chemotaxis to take advantage of the higher concentrations of these nutrients (Scher *et al.* 1985; Bashan, 1986). Colonisation of the plant root system by bacteria often occurs by attachment to primary roots of seedlings and then being carried passively through the soil by the elongating roots (Howie *et al.* 1987) or by downward movement assisted by water percolation along the root system (Chao *et al.* 1986; Parke *et al.* 1986; Davies and Whitbread, 1989a; Liddell and Parke, 1989). Some strains of *Pseudomonas* are able to produce cellulose fibrils or fimbriae which attach to roots (Dienema and Zevenhuizen, 1971; Weller, 1988).

The role of bacterial flagella in distribution on the root surface is uncertain (Weller, 1988). It has been found that Tn*5* mutants of *P. fluorescens* strains that were non-motile were poor root colonisers compared to the wild-type strain (DeWeger *et al.* 1987) yet three strains of non-flagellated *P. fluorescens* colonised root systems to the same extent as the wild-type (Howie *et al.* 1987).

Once a biocontrol agent has colonised the roots of a target plant it must successfully compete with the indigenous microflora and maintain a minimum viable population threshold to be effective (Weller, 1988; Bull *et al.* 1991). Obtaining nutrients such as trace elements which are not readily available in a potentially exotic environment is often overcome by the production of various compounds by the bacterium to facilitate nutrient acquisition. For example, compounds including iron-chelating siderophores (Loper and

Buyer, 1991) and enzymes such as manganese reductase (Trimble and Ehrlich, 1968) may be produced and secreted into the rhizosphere to convert trace elements to forms that may be assimilated by the cell.

The ability of the introduced biocontrol agent to compete may be enhanced by the production of antibiotics which reduce the populations of competing microflora. These compounds also may be the primary mechanism of biocontrol of a specific phytopathogen; antagonising the pathogen thus preventing or restricting its ability to infect a host plant. Like the general growth and viability of an introduced biocontrol agent, the production and activity of antagonistic compounds is often influenced by environmental factors such as temperature, water potential and soil pH (Schroth and Hancock, 1982; Brisbane and Rovira, 1988; Weller, 1988; Davies and Whitbread, 1989a; Tan *et al.* 1991; Ownley *et al.* 1991). Consequently, not only must the biocontrol agent adapt to a range of soil and climatic conditions, but antimicrobial compounds released by the microorganism which antagonise phytopathogens must remain biologically active in a range of environmental conditions dissimilar to the original site where the biocontrol strain was isolated.

An example where an external factor influences the activity of a compound antagonistic to a pathogen is the effect of pH on the antibiotic phenazine-1-carboxylic acid (PCA). Production of this antibiotic by a biocontrol agent has been detected in the rhizosphere of wheat (Thomashow *et al.* 1990). This metabolite has been found to be an important mechanism in the antagonism of *Ggt* by strains of *Pseudomonas* (Thomashow and Weller, 1988). In *in vitro* tests with purified PCA, it was found that this antibiotic was most active at acidic pH's down to 4.5 whereas at neutral and slightly alkaline pH's, (7.5), activity against *Ggt* was not detected, even at concentrations 500x that which inhibited the fungus at pH4.5 (Brisbane and Rovira, 1988). Subsequent examination of the biocontrol activity of a PCA-producing strain of a *P. fluorescens* strain antagonistic to *Ggt* (where PCA had been shown to be the primary mechanism of biocontrol by that

strain) confirmed that significant suppression of take-all symptoms on wheat plants only occurred in neutral to slightly acidic soils (Ownley *et al.* 1992).

In summary, the activity of a biocontrol agent, including the metabolites released into the rhizosphere, is influenced by both biotic and abiotic factors. Root colonisation and ability to compete and survive in the soil are important factors for successful biocontrol; however they usually do not directly account for the actual phytopathogen antagonism and suppression of disease. The production of compounds antagonistic to the pathogen is often the primary mechanism of biocontrol, especially by strains of *Pseudomonas*.

1.3 METABOLITES OF BIOCONTROL AGENTS ANTAGONISTIC TO PLANT PATHOGENS

Biocontrol of many plant diseases, including take-all, can be due to the production of metabolites by the biocontrol agent. These metabolites may have a variety of roles in biocontrol activity. Production of compounds which sequester nutrients from the soil such as siderophores and manganese reductase may reduce the pathogen's ability to develop and infect susceptible plants. Antibiotics, volatiles and enzymes secreted by a biocontrol agent may be directly antagonistic to the pathogen resulting in pathogen death. Other metabolites such as proteases may suppress disease by neutralising compounds released by the pathogen which are necessary for plant infection. Antagonistic compounds also may have a role in induced resistance where metabolites produced by the biocontrol agent stimulates host plant defence systems which restricts the ability of the pathogen to infect the plant. A particular biocontrol agent may produce more than one metabolite, all of which are antagonistic to a particular pathogen or different metabolites produced by a single agent can be antagonistic against different pathogens (Fravel, 1988; Keel *et al.* 1992; Maurhofer *et al.* 1994).

1.3.1 Siderophores and Iron Assimilation

Most microorganisms and plants require iron for metabolism. Iron is an important cofactor for many enzymes and complex molecules such as respiratory cytochromes, ribonucleotide reductase and the siroheme component of nitrite reductase of *Neurospora* (VanDemark and Batzing, 1986; Neilands, 1982; Smith *et al.* 1983). Iron is found naturally in two oxidative states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). In aerobic environments the less soluble Fe^{3+} form predominates, usually bound in the form of hydrated ferric oxides. The availability of free ferric iron in soil is pH dependent, increasing acidity leads to an increased concentration of free Fe^{3+}. The low solubility of Fe^{3+} ($K_{sp} = 10^{-38}$) means that the Fe^{3+} concentration usually is not greater than 10^{-17}M in many soils (Neilands, 1982; Leong, 1986; Loper and Buyer, 1991). Most bacteria require 10^{-5} to 10^{-7}M Fe^{3+} for normal growth. Plants require 10^{-4} to 10^{-9}M, depending on species and other nutritional requirements (Loper and Buyer, 1991; O'Sullivan and O'Gara, 1992). To obtain bound iron from soil most microorganisms and certain plants release special iron-chelating compounds called siderophores.

Different species of microorganisms release different siderophores. As a general rule, fungi release siderophores with low affinity hydroxamate Fe^{3+} binding groups such as ferrichrome; bacteria have siderophores with either hydroxamate or higher-affinity catechol ligands (Schroth and Hancock, 1982; Neilands, 1982; Leong,1986). Disease suppression by biocontrol agents through the production of siderophores is based on differences in siderophore-chelating activity. It has been postulated that a biocontrol agent sequesters iron through the released siderophores making iron unavailable for potential plant pathogens (Leong, 1986). This may be due to the biocontrol agent (i) releasing a higher concentration of siderophores than the pathogen, (ii) releasing siderophores with a higher stability constant or chelating activity or (iii) targeting a pathogen that does not produce any siderophores (Kloepper *et al.* 1980a). The first substantiated experiment implicating siderophores in biocontrol published involved a

fluorescent species of *Pseudomonas* that displayed *in vitro* inhibition of *Erwinia carotovora*, the causative agent of potato soft rot and seed piece decay, on iron-deficient Kings B medium (Kloepper *et al.* 1980a; O'Sullivan and O'Gara, 1992). Inhibition of antibiosis occurred when $FeCl_3$ was added to the medium - these results suggested that a siderophore acted as the biocontrol mechanism. Further experiments have been conducted on the role of siderophores in biocontrol and different biocontrol agent/target pathogen systems have been investigated. Table 1.1 summarises some of these pathogen/biocontrol relationships involving siderophore production by the biocontrol agent.

There is evidence that siderophores play a role in controlling plant diseases when soil is amended with chelated iron, often in the form of FeEDTA. *Pseudomonas putida* strain A12 was unable to suppress *Fusarium* wilt of flax and radish induced by *F. oxysporum* f. spp. *lini* and *conglutinans* when the soil was amended with FeEDTA (Scher and Baker, 1982). Fluorescent *Pseudomonas* sp. strain NZ130 is an inhibitor of *Pythium ultimum*; inhibition was antagonised when the Fe^{3+} concentration exceeded 10μM (Gill and Warren, 1987). The addition of FeEDTA to a take-all suppressive soil rendered the soil conducive to take-all (Kloepper *et al.* 1980b).

The role of siderophores in the control of *Ggt* has yet to be fully defined. *Pseudomonas* strain B10 inhibited *Ggt in situ* but when the soil was amended with Fe^{3+} the soil was converted to a conducive state. (Kloepper *et al.* 1980b). It was concluded that the soil was rendered conducive due to repression of siderophore production by the biocontrol agent. Wong and Baker (1984) concluded that iron competition was a factor in the biocontrol of *Ggt* by fluorescent *Pseudomonas* strains. However, some studies had found that siderophores produced by biocontrol agents have no significant effect on the take-all fungus. *Pseudomonas fluorescens*, strain 2-79 has been found to control *Ggt* and produces the siderophore pyoverdine. Mutants generated by transposon insertions

Biocontrol Strain	Phytopathogen	Disease	Host Plant	Reference
Alcaligenes sp. MFA1	*F. oxysporum* f. sp. *dianthi*	Wilt	Carnation	Yuen & Schroth, 1986
Pseudomonas sp.	*F. oxysporum* f. sp. *dianthi*	Wilt	Carnation	Duijff, 1994
Pseudomonas spp.	*F. oxysporum* f. sp. *lycopersici*	Wilt	Tomato	Vandenbergh *et al*, 1983
Pseudomonas sp.	*Rhizoctonia solani*, *Phytophthora megasperma* *Sclerotinia sclerotiorum* *Pythium aphanidermatum*	Various	-	Misaghi *et al*, 1982
P. fluorescens spp.	*Erwina carotovora*	Seed Piece Decay and Soft Rot	Potato	Kloepper *et al*, 1980a
P. fluorescens CHA0	*Thielaviopsis basicola*	Black Root Rot	Tobacco	Keel *et al*, 1989
P. fluorescens	*G. graminis* var. *tritici*	Take-all	Wheat	Wong & Baker, 1984
P. fluorescens	*Gaeumannomyces graminis*	Ophiobolus Patch	Agrostis turfgrass	Wong & Baker, 1984
P. putida sp.	*F. oxysporum* f. sp. *lini*	Wilt	Flax	Scher & Baker, 1982
P. putida A12	*F. oxysporum* f. sp. *cucumerinum*	Wilt	-	Simeoni *et al*, 1987
P. putida 3551	*Pythium ultimum*	Damping-off	Cotton	Loper, 1988

Table 1.1: Evidence of phytopathogen disease control as a consequence of siderophores produced by soil bacteria isolated for their biocontrol activity.

that no longer produced pyoverdine were still able to control take-all to similar levels as the parent strain (Hamdan *et al.* 1991).

1.3.2 Antibiotics

Many biocontrol agents produce water-soluble secondary metabolites that have antifungal activity (James and Gutterson, 1986). There are many such antibiotics described; Table 1.2 lists the more commonly found antibiotics produced by biocontrol agents and the phytopathogens that the antibiotics are known to inhibit.

One antibiotic that has come under investigation as an antagonist of *Ggt* in the wheat rhizosphere is the yellow-green pigmented antibiotic phenazine-1-carboxylic acid (PCA) which is produced by various strains of fluorescent pseudomonads (Brisbane *et al.* 1987; Thomashow and Weller, 1988; Brisbane and Rovira, 1988). PCA is a product of the aromatic acid biosynthesis pathway and has shikimate as the precursor and chorismate as the branch point (Calhoun *et al.* 1972; Longley *et al.* 1972). PCA is only one compound of the phenazine family, a group of compounds with known antibiotic properties (Gerber, 1973).

P. fluorescens strain 2-79, isolated from a take-all suppressive soil, is one PCA-producing strain that has been extensively tested for biocontrol activity (Weller and Cook, 1983). Production of PCA by this strain has been detected in the rhizosphere of wheat grown in growth chamber experiments (Thomashow and Weller, 1988; Thomashow *et al.* 1990). PCA production by strain 2-79 occurred in the rhizosphere regardless of whether *Ggt* was present or not. Wheat roots where the antibiotic was present showed less disease than roots where the antibiotic was absent. Transposon-induced mutants of strain 2-79 that no longer produced detectable quantities of PCA did not inhibit *Ggt* growth *in vitro* and showed reduced disease suppression on wheat seedlings (Thomashow and Weller, 1988).

Antibiotic	Producing Strain	Target Pathogen	Disease	Reference
Pyrrolnitrin	*Pseudomonas* sp. *Pseudomonas fluorescens* Pf-5 *Pseudomonas cepacia* RB425	*Rhizoctonia solani* *Rhizoctonia solani* Various	Damping-off Damping-off Various	Kempf *et al* (1994) Howell & Stipanovic (1979) Homma *et al* (1989)
Phenazine-1-carboxylic acid	*Pseudomonas fluorescens* 2-79	*Ggt*	Take-all	Thomashow & Weller (1990)
1-Hydroxyphenazine	Fluorescent *Pseudomonas* sp.	Various	Various	Levy *et al* (1989)
Pyoluteorin	*Pseudomonas fluorescens* Pf-5 *Pseudomonas fluorescens* Pf-5 *Pseudomonas fluorescens* CHA0	*Pythium ultimum* *Pythium ultimum* *Pythium ultimum*	Damping-off Damping-off Damping-off	Howell & Stipanovic (1980) Kraus & Loper (1991) Maurhofer *et al* (1994)
2,4-Diacetylphloroglucinol	*Pseudomonas fluorescens* CHA0 *Pseudomonas aureofaciens* Q2-87 Fluorescent *Pseudomonas* spp. F113	*Thielaviopsis basicola* *Ggt* Various	Black root rot Take-all Various	Keel *et al* (1990) Vincent *et al* (1991) Shannahan *et al* (1992)
Oomycin A	*Pseudomonas fluorescens* Hv37aR2	*Pythium ultimum*	Damping-off	Howie & Suslow (1991)
Anthranilic acid	*Pseudomonas fluorescens* 2-79	*Ggt*	Take-all	Thomashow & Weller (1990)
Gliovirin	*Gliovirin virens*	*Pythium ultimum*	Damping-off	Howell & Stipanovic (1983)

Table 1.2: Examples of antibiotics produced by various biocontrol agents and the phytopathogens that are antagonized by them.

P. fluorescens 2-79 also produces anthranilic acid (AA), a metabolite that also antagonises *Ggt* (Thomashow and Weller, 1990). Anthranilic acid , like PCA, is derived from chorismate and is a precursor of both PCA and tryptophan synthesis (Leisinger and Margraff, 1979; Essar *et al.* 1990). The accumulation of AA in the rhizosphere occurs in conditions of low free iron such as soils with a high pH. Under these conditions less PCA is produced, suggesting that iron availability and possibly regulation through siderophore production is a significant factor in antibiotic synthesis and biocontrol of take-all by strain 2-79 (Thomashow and Weller, 1990).

Phenazine-1-carboxylic acid is not the only antibiotic that is effective against *Ggt*. *P. fluorescens* strain CHA0 produces a range of secondary metabolites that suppress not only take-all of wheat but also black root rot of tobacco and *Pythium* and *Rhizoctonia* infection on a variety of crops (Maurhofer *et al.* 1994). As well as the siderophore pyoverdine, strain CHA0 produces the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol (DAP) as well as hydrogen cyanide. DAP production by strain CHA0 has been correlated with suppression of take-all (Keel *et al.* 1992). Genetic studies using Tn*5*-induced mutants of *P. fluorescens* CHA0 that did not produce DAP showed that production of this antibiotic was a significant factor in the biocontrol of *Ggt* on wheat as well as black root rot of tobacco caused by *Thielaviopsis basicola*. Complementation of the mutant with cosmid pME3128 containing a 11kb CHA0 genomic fragment restored both DAP production *in vitro* and disease suppression *in situ* (Keel *et al.* 1992) Production of DAP has been found in a range of *Pseudomonas* strains and has been found to have both antifungal and antibacterial properties (Keel *et al.* 1992; Shanahan *et al.* 1992; Vincent *et al.* 1992).

As well as DAP, strain CHA0 also produces pyoluteorin, another antibiotic with antifungal properties. In an attempt to improve biocontrol activity of this strain, pyoluteorin production was increased by complementation with cosmid pME3090

containing a 22kb genomic fragment from strain CHA0 (Maurhofer *et al.* 1992). The complemented strain was found to have improved biocontrol activity against *Pythium ultimum* on cucumber. No deleterious effect was observed on the host plant as a result of increased antibiotic production.

An antibiotic which is antagonistic to a range of phytopathogens is pyrrolnitrin, first described by Arima *et al.* (1964) who extracted the compound from a strain of *Pseudomonas pyrrocinia*. It has since been identified as a secondary metabolite in various fluorescent *Pseudomonas* strains under investigation for biocontrol activity. Pyrrolnitrin produced by a strain of *P. fluorescens* isolated from the rhizosphere of cotton seedlings antagonised *Rhizoctonia solani* and increased cotton seedling survival. Pyrrolnitrin also antagonised other fungal phytopathogens *in vitro* including *Thielaviopsis basicola* and a species of *Alternaria* (Howell and Stipanovic, 1979) and *Rhizoctonia solani*, *Verticillium dahliae* and *Pyricularia oryzae in vitro* (Homma *et al.* 1989). The antibiotic was found to be less active against *Pythium ultimum* and *Fusarium oxysporum* strains (Howell and Stipanovic, 1979; Homma *et al.* 1989). Pyrrolnitrin was detected in the rhizosphere of cotton roots after inoculation with a *Pseudomonas* sp. originally isolated from cotton roots (Kempf *et al.* 1994). Mutants of this strain lacking pyrrolnitrin production were generated by chemical mutagenesis and were found to be less effective in controlling damping-off (Hill *et al.* 1994). *In situ* production of pyrrolnitrin and lack of activity against *R. solani* by pyrrolnitrin-negative mutants indicated that control of damping-off of cotton was due largely to pyrrolnitrin production.

1.3.3 Hydrogen Cyanide

Many species of plants and microorganisms produce hydrogen cyanide through secondary metabolism. In plants, particular amino acids are converted to cyanogens which are subsequently hydrolysed releasing cyanide. Many fungal species produce

cyanide by utilising the cyanogens of host plants (Knowles, 1976). Various bacteria including *Pseudomonas* and *Chromobacterium* spp. produce cyanide as a consequence of glycine catabolism (Lorck, 1948; Askeland and Morrison, 1983).

Hydrogen cyanide toxicity is due to its ability to affect the activity of a range of enzymes including cytochrome oxidases (Dixon and Webb, 1964; Knowles, 1976; Astrom 1991). Cyanide also increases cell permeability, thus increasing the rate of exudate release from plant roots and thereby suppling cyanide-releasing rhizobacteria with nutrients (Knowles, 1976; Schippers *et al.* 1987).

Cyanide released by bacteria may antagonise fungal pathogens. As well as suppressing a range of phytopathogens by phloroglucinol production, *P. fluorescens* CHA0 also produces HCN and it has been shown that HCN production has a role in suppression of tobacco black rot caused by *Thielaviopsis basicola* (Keel *et al.* 1989, Voisard *et al.* 1989), *Pythium ultimum* on cucumber and cress, and *Rhizoctonia solani* on cotton (Défago *et al.* 1991, Maurhofer *et al.* 1994). However, evidence suggested that HCN did not play a significant role in the *in situ* suppression of take-all (Maurhofer *et al.* 1994). No evidence is available that HCN produced by any other biocontrol agent suppresses take-all *in situ*.

1.3.4 Ammonia

Ammonia is another volatile produced by some biocontrol agents which may have a role in the control of plant pathogens. Low levels of ammonia have been found to be toxic to fungi in soil (Pavlica *et al.* 1978). Production of ammonia by strains of *Enterobacter cloacae* were found to inhibit *in vitro* growth of *Pythium ultimum* and *Rhizoctonia solani* (Howell *et al.* 1988). The production of ammonia by these strains also was thought to be partially responsible for the control of Pythium preemergence damping-off.

1.3.5 Manganese Reductase

Increased take-all severity in plants has been correlated with deficient manganese levels in plant tissues (Graham and Rovira, 1984; Huber, 1987). Conversely, a decrease in take-all symptoms can occur when manganese is supplied to adequate levels (Wilhelm *et al.* 1990). In a series of experiments conducted by Wilhelm *et al.* (1990), wheat was grown in pots containing a calcareous sand that was low in available manganese. The wheat plants showed severe take-all infection when 4 isolates of *Ggt* were added. Take-all symptoms caused by 3 isolates were severe whereas infection by the fourth strain, a weakly virulent isolate, was less severe. In a further series of experiments, 4 wheat genotypes and 1 virulent *Ggt* strain were tested in a similar soil. Two wheat genotypes were found to be inefficient at taking up manganese and were susceptible to take-all. One genotype was manganese uptake-efficient and was showed reduced disease symptoms. The fourth genotype was intermediate for both manganese uptake efficiency and take-all severity. All four wheat genotypes were resistant to G*gt* under Mn-adequate conditions.

The oxidation state of applied manganese is an important factor in take-all severity. It has been found that manganese supplied as $MnSO_4$ reduces take-all severity but the more oxidised MnO_2 was less effective in reducing take-all symptoms (Wilhelm *et al.* 1988). In the trials described above, it was found that the 3 virulent strains of *Ggt* were efficient at oxidising manganese, making it less available to the plant. A degree of take-all control can be achieved in this situation by inoculating the wheat rhizosphere with microorganisms which are manganese reductase active such as *Bacillus* and *Pseudomonas* strains (Huber and McCay-Buis, 1993). In this situation biocontrol is achieved not through antagonism of the pathogen by metabolite production but by increasing the amount of manganese available to the plant thus making it less susceptible to *Ggt* infection. A recent review of the role of manganese in resistance of wheat to

take-all by Huber and McCay-Buis (1993) covers a range of factors believed to be the primary mechanisms of disease resistance mediated by manganese.

1.3.6 Parasitism and Cell Wall-Lytic Enzymes

Kloepper (1993) defined parasitism of fungi as "the destruction of fungal pathogens by the action of lytic enzymes that degrade fungal cell walls". A number of biocontrol agents antagonise phytopathogens by secreting lytic enzymes such as β-1,3 glucanase and chitinase which degrade the cell wall of the pathogen. An isolate of *Burkholderia cepacia* produced β-1,3 glucanase and was effective in controlling *R. solani*, *Sclerotium rolfsii* and *P. ultimum* (Fridlender *et al.* 1993). Inbar and Chet (1991) isolated a chitinase-producing *Aeromonas caviae* strain from healthy bean plants that had been previously inoculated with *Sclerotium rolfsii*. The bacterium also antagonised *R. solani* and *F. oxysporum* f. sp. *vasinfectum* in glasshouse trials as well as *S. rolfsii*. No antibiosis activity by *A. caviae* was evident. Lysis of fungal mycelium and the ability of *A. caviae* to use fungal mycelium as a sole carbon source indicated that chitinase production was involved in biocontrol. *Pseudomonas stutzeri* YPL-1 antagonised *Fusarium solani* by producing extracellular chitinase and laminarinase which degraded the cell wall of the fungus (Lim *et al.* 1991). *Trichoderma harzianum*, a fungus with biocontrol activity produced both β-1,3 glucanase and chitinase and antagonised a range of fungal pathogens (Elad *et al.* 1982).

The genus *Gaeumannomyces* belongs to the class Ascomycetes. This class of higher fungi generally have cell walls composed of chitin and glucan (Webster, 1980). Consequently biocontrol agents which antagonise *Ggt* by cell wall degradation must produce either chitinase or β-1,3 glucanase.

1.3.7 Induced Resistance

Induced resistance in plants has been defined as "the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents" (Kloepper *et al.* 1992). The addition of a PGPR/biocontrol agent (or other inducer) to the plant may induce that plant to respond in such a way, resulting in resistance to a pathogen introduced to the plant at a point spatially isolated from the region where the inducer was inoculated on the plant (systemic induced resistance). Responses by plants to this activity include production of anti-microbial compounds such as phytoalexins or synthesis of extra protective layers of lignin and callose. This induction may be due in part to a plant response to the release of metabolites by the PGPR such as HCN. Increased nutrient availability to the plant as a result of microbial activity may also be a factor in induced resistance. For example, increased manganese availability through the activity of manganese reductase-positive microorganisms has been suggested as a possible mechanism resulting in increased lignification of plant cell wall tissue and subsequent resistance to Fusarium diseases in asparagus (Elmer, 1995). Reviews by Tuzun and Kloepper (1994) and Steiner and Schönbeck (1995) discuss the mechanisms of induced resistance in more detail.

Induction of resistance may be considered as a possible mechanism of biocontrol where (i) the inducer does not antagonise the pathogen *in vitro* or (ii) is shown to protect a host plant *in situ* where it is introduced spatially isolated from the pathogen. Scheffer (1983) inoculated elm trees with fluorescent *Pseudomonas* strains which resulted in significantly reduced symptoms of Dutch elm disease. Due to the weak antagonistic activity against the pathogenic fungus *in vitro* by the inducing strains it was suggested that induced resistance was the mechanism responsible. In another experiment where induced resistance was suggested as the mechanism of biocontrol, carnation roots were inoculated with the PGPR *Pseudomonas* sp. strain WCS417. The phytopathogen *Fusarium oxysporum* f. sp. *dianthi* was stem-inoculated one week later so that pathogen

and PGPR were temporally isolated. Strain WCS417 was not isolated from stem tissue but PGPR- treated plants showed significantly reduced disease symptoms (van Peer *et al.* 1991). A different approach to investigating the role of induced resistance in biocontrol was adopted by Alström (1994). *Pseudomonas fluorescens* strain S 97 was tested for its ability to induce resistance to *P. syringae* pv. *pisi* in peas and a virulent bean-infecting strain of *P. syringae* pv. *phaseolicola* (Psp, N7, 6ar). Growth of bean plants treated with fluids collected from bacterial-treated plants were compared to control plants when challenged with the pathogen. Reduced pathogen counts in plants treated with extracts from bacterial treated plants suggest induced resistance was the mechanism involved.

1.3.8 Phytotoxicity of Metabolites of Biocontrol Agents

An important risk factor that must be considered before applying a biocontrol agent is the potential for deleterious side-effects on the host plant or other plants that may come in contact with the agent. Metabolites of biocontrol agents, whether they be the metabolites directly associated with biocontrol or unrelated compounds, may be phytotoxic to particular species of plants including important agricultural and horticultural crops. It is therefore important to consider both the antifungal and phytotoxic activities of a biocontrol agent being developed for widespread environmental release.

A number of metabolites with anti-phytopathogen properties have been shown to have deleterious effects on plants. For example increased pyoluteorin and DAP production by *P. fluorescens* strain CHA0 has been found to be toxic to cress, sweet corn and cucumber plants (Keel *et al.* 1992; Maurhofer *et al.* 1992). It was noted that cucumber was less sensitive to the increased DAP production than the other plant species indicating that different crops have different sensitivities to these active compounds. Phenazine products secreted by a strain of *Pseudomonas aurefaciens* were implicated in the phenomenon called fairy ring disease (Toohey *et al.* 1965a; Toohey *et al.* 1965b). A

low molecular weight compound produced by a non-fluorescent pseudomonad was found to have some antifungal activity against *Geotrichum candidum*. This compound also inhibited root growth of winter wheat (Bolton *et al.* 1989). Barley was also affected by the toxin but other crops such as oats and peas were unaffected which is further evidence that different plants have different sensitivities to these metabolites. Antibiotic by-products and break-down constituents can also be phytotoxic. For example, G*liocladium virens* produces the toxin viridiol which is a derivative of the antibiotic viridin. Viridiol has a deleterious effect on various crops (Howell and Stipanovic, 1983; Howell and Stipanovic, 1984; Jones *et al.* 1988).

Siderophores produced by rhizobacteria also have been associated with plant disease. Dicotyledonous plants obtain iron by releasing reductases that reduce iron(III) to iron(II) which is then taken up by the roots (Leong, 1986; Römheld, 1987). The plant pathogen *Erwinia chrysanthemi* produces the catechol siderophore chrysobactin. This siderophore has been identified as a virulence factor in plant disease (Persmark *et al.* 1989; Loper and Buyer, 1991). Pseudobactin (a common siderophore produced by *Pseudomonas* species) has been found to inhibit iron uptake in maize and peas leading to chlorosis by sequestering iron (Becker *et al.* 1985). The presence of pseudobactin secreted by fluorescent pseudomonads inhibits the uptake of iron by pea and maize roots resulting in reduced chlorophyll synthesis (Becker *et al.* 1985).

As well as targeting certain plant pathogens, hydrogen cyanide may also affect plants sensitive to this volatile compound. Two known effects of cyanide are on changes to root cell permeability and the interference with the cytochrome oxidase respiration pathway (Schippers *et al.* 1987, Alstrom, 1991). Release of cyanide by different microorganisms may be a direct cause of disease in host plants. Winter Crown Rot or Snow Mould Disease, caused by a basidiomycete fungus, is a cyanide-based disease of alfalfa (*Medicago sativa*) and other forage plants (Kosaric and Zajic, (1974). The severity of disease caused by this fungus has been found to be directly proportional to

the cyanide concentration in damaged plant tissue. Variants of the Snow Mould fungus which do not produce cyanide are non-pathogenic to otherwise susceptible plants (Ward *et al.* 1961). Examples of other plant diseases caused by cyanide-producing microorganisms include fairy ring diseases of grasses (Lebeau and Hawn, 1963) and copperspot disease (Graham, 1953). *P. fluorescens* strain A112 which produces cyanide has been found to be deleterious to wheat and pea crops (Astrom and Gerhardson, 1988). As a consequence of these findings it may be concluded that any cyanogenic biocontrol agent may have deleterious side-effects on particular crops.

Production of ammonia by a strain of *Pseudomonas tomato* has been correlated with necrosis in tomato leaves (Bashan *et al.* 1980). Necrotic symptoms in tobacco leaves when inoculated with various phytotoxic bacteria were associated with ammonia production by the bacteria (Lovrekovich et al, 1970). The presence of ammonia was found to accelerate the inactivation of glutamine synthetase by tabtoxinine-β-lactam, a phytotoxin produced by *Pseudomonas syringae* pv. *tabaci* (Langston-Unkefer *et al.* 1984). Ammonia produced as a result of mulch degradation was found to be deleterious to avocado and citrus crops (Casale *et al.* 1995).

1.4 APPROACHES TO EXAMINING MECHANISMS OF BIOCONTROL

1.4.1 Why Study Mechanisms?

To obtain the full benefit from a biocontrol agent the mechanism(s) by which the agent antagonises the pathogen and/or suppresses disease symptoms must be understood. Furthermore it is important to understand the conditions under which the mechanism(s) will function in order to exploit the full potential of the agent. Further benefits may also include the discovery of compounds such as antibiotics which may have uses beyond the control of a particular disease. Finally, an understanding of the mechanisms will assist in

predicting any potential deleterious effects of the biocontrol agent towards plant biota and non-targeted soil microflora.

In a review of the use of rhizobacteria as biocontrol agents, Kloepper (1993) states that there are two main areas of research that can be employed in understanding the mechanisms of biocontrol; the physiological or biochemical approach and the molecular approach.

1.4.2 The Biochemical Approach

Biocontrol agents that are used to antagonise a pathogen on a particular host plant are often isolated from a field site where disease symptoms are no longer present. Candidate species are then tested for their ability to inhibit the pathogen *in vitro*. Pot tests and field trials are then required for those species that displayed *in vitro* inhibition as *in vitro* inhibition does not always correlate to inhibition *in vivo* (Kloepper, 1993; Dowling and O'Gara, 1994). Some *in vitro* tests however may give a clue as to the possible mechanisms of biocontrol. These include the production of water soluble compounds creating a zone of inhibition between the growing fungal hyphae and the biocontrol agent, production of volatiles inhibiting the fungus on split plates, examination of the fungal pathogen for zones of lysis which may indicate production of lytic enzymes and the detection of metabolite production on a suitable media.

The use of these *in vitro* diagnostic tests can only be used as an initial guide for the identification of the mechanisms of biocontrol. Quite often the media and growth conditions used in these tests are not indicative of the conditions in which the biocontrol agent functions *in vivo*. The production of a particular metabolite may be artificially enhanced or suppressed by the alteration of concentrations of media constituents. For example, *in vitro* production of hydrogen cyanide can be amplified by the addition of iron or the amino acid glycine (Askeland and Morrison,1983). Antibiotic biosynthesis by

Pseudomonas fluorescens Hv37a was found to be regulated by the level of glucose present in the medium (James and Gutterson, 1986). Other examples of *in vitro* stimulation or repression of metabolites are cited in a review by Dowling and O'Gara (1994). To determine the role of any such candidate metabolite as a possible mechanism for biocontrol, *in vivo* tests must be undertaken.

The first step in determining the role of a particular metabolite in the biocontrol of a pathogen is purifying that compound. Siderophores and antibiotics are often purified from supernatants by partitioning into an organic solvent such as ethyl acetate at a particular pH then purifying the organic phase via chromatographic methods such as TLC, HPLC, column chromatography or paper electrophoresis. Purified compounds may then be tested for fungal antagonism. The purified compound may also be added to the soil in a system containing the plant to be protected and the target pathogen. This method has been used to test a range of compounds including siderophores (Kloepper *et al.* 1980a) and antibiotics such as pyrrolnitrin (Howell and Stipanovic, 1979) and 2,4-diacetylphloroglucinol (Keel *et al.* 1990).

A number of problems can arise from testing purified metabolites for *in vitro* antagonism and *in situ* suppression of disease, as metabolite production *in vitro* may not correlate to production and activity *in situ*. The compound being tested, which is produced under *in vitro* conditions, may not be produced in the rhizosphere or it is produced at much lower concentrations compared to *in vitro* yields. Consequently, the addition of high levels of the compound to soil may result in artificially enhanced biocontrol activity. Furthermore, the question of activity of metabolites in different soils must be asked. Many compounds will only be biologically active under certain soil conditions. For example pyoluteorin can be inactivated through adsorption to soil (Howell and Stipanovic, 1980) and PCA may not function in alkaline soils where it is present in ionic form (Brisbane *et al.* 1987; Ownley *et al.* 1992).

Some of these problems may be overcome by extracting the compound of interest from the rhizosphere of plants inoculated with the biocontrol agent. This can be achieved by extracting the compound from rhizosphere or total soil with an organic solvent such as acetone and running the organic phase through a HPLC column. Peaks, for example, of UV absorbing compounds, obtained by this method can then be compared to peaks produced by a pure sample of the compound in question and therefore confirm that the compound is produced in the rhizosphere. An example where production of an antibiotic with known activity against take-all was detected in the rhizosphere is PCA which is produced by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84. Production of this antibiotic in the rhizosphere of wheat plants inoculated with these bacterial strains was detected by TLC and HPLC analysis (Thomashow *et al.* 1990).

1.4.3 The Molecular/Genetic Approach

An complimentary approach to the physiological or biochemical methods described above is the genetic approach. Genes directly involved in biocontrol are mutated to disrupt gene function and reduce or abolish production of compounds of interest. The biocontrol activity of the mutated strain is then compared to the parent strain. If the mutant shows a significant reduction in biocontrol activity, this would not only confirm the role of a particular compound in biocontrol, but also quantify the importance of that compound in biocontrol. Before mutagenesis can be undertaken, some knowledge of the biocontrol strain's phenotype must be available. Prior testing of possible candidate metabolites can be achieved with simple *in vitro* tests to determine the presence of such compounds as volatiles, siderophores, enzymes and water-soluble antibiotics. By having this information available it is a much simpler process to screen mutants for loss of production of particular compounds.

The use of transposons which insert randomly into the genome has taken precedence over chemical and UV irradiation as the preferred method of mutagenesis. The reason

for this is quite simple. Transposon mutagenesis results in a single mutation for each genome (subject to confirmation by DNA hybridisation) whereas chemical and UV mutagenesis can result in multiple mutation sites in a single genome. A single transposon insertion eliminates one particular metabolite involved in biocontrol without affecting the production of other metabolites makes it easier to measure the degree of influence that metabolite has *in situ*. Furthermore, transposon mutagenesis other advantages over the more "traditional" methods. They result in mutants with a single mutation in the genome, they carry marker genes such as antibiotic resistance which aids the detection of mutants carrying the transposon both *in vitro* and *in situ* and some transposons carry reporter genes that may only function in the presence of a host promoter so that the activity of the operon under different conditions can be measured. (It should be noted that some transposons such as Tn*7* and Tn*554* insert in specific sites within the recipient chromosome and therefore are less suited for random mutagenesis (Berg *et al.* 1989)). The mechanisms by which these processes occur together with reviews of different types of transposons and their particular characteristics is covered in various publications (Mills, 1985; Berg *et al.* 1989).

Once a mutant generated by transposon mutagenesis has been isolated which shows reduced biocontrol activity it may be complemented with genes from the parent strain to restore wild-type phenotype activity. This process is similar to the original mutagenesis itself whereby a cosmid containing genes from the parent strain is inserted into the mutant. These corresponding gene sequences replace the activity of genes disrupted by the mutation, thereby restoring biocontrol activity.

Although transposon mutagenesis is often sufficient to produce mutants defective in biocontrol there are situations where further genetic manipulation is required to obtain suitable mutants for study. Such cases occur where no mutants containing the defective gene are detected during screening or where mutations result in pleiotropic phenotypic changes. Gene replacement offers an alternative method to obtain a desired mutation.

This method involves similar mechanisms as transposon mutagenesis but instead of a foreign transposon being inserted, the gene is replaced in the recipient genome with a copy of itself which has been altered, often by base-pair deletions or through gene insertions.

An example where gene replacement was utilised to produce a mutant with compromised biocontrol activity is HCN production by *P. fluorescens* CHA0 and its role in controlling tobacco black root rot. Mutants generated by standard transposon procedures that were defective in HCN production were invariably shown to be pleiotropic mutations (Voisard *et al.* 1989). A mutant defective in HCN production only, designated CHA5, was produced by cloning a 5kb fragment containing *hcn* genes into pME3041 and then inactivating the *hcn* gene by insertion of the transcription/translation stop element Ω-Hg (Felley *et al.* 1987). Strain CHA5 was then generated by insertion of the new plasmid pME3043 and subsequently tested for lack of HCN production and biocontrol *in situ*. Complementation with pME3013 containing wild-type *hcn* genes restored HCN production and biocontrol activity in this strain.

An alternative to disrupting metabolite production in strains to test their role in biocontrol is to increase their production by introducing multiple copies of a gene operon into the strain. Not only does this give an indication of the role of a particular metabolite in biocontrol, it can also result in a strain with improved biocontrol activity. Increasing metabolite production by this method is usually done after a particular gene sequence has been identified and has been cloned into a suitable vector that is stable in the recipient strain.

An example is the insertion of a 22kb fragment from *P. fluorescens* CHA0 responsible for antibiotic production to create cosmid pME3090 (Maurhofer *et al.* 1992). Subsequent sub-cloning of this wild-type DNA produced a 2.3kb fragment that, when

cloned into the parent strain via the vector pVK100, resulted in a 1.5 fold increase in DAP and a 6 fold increase in pyoluteorin production (Schnider *et al.* 1994).

The technique of introducing cloned genes can be extended to the introduction into strains that either do not have these genes or are not biocontrol agents. Genes for DAP production from *P. fluorescens* strain F113 were cloned into the vector pCU203 and subsequently transformed into non-biocontrol/non-DAP producing *Pseudomonas* strain M114 (Fenton *et al.* 1992). The transformed derivative displayed DAP production and biocontrol activity against *Pythium ultimum*.

As mentioned previously, transposons containing reporter genes can be used to mutate genes or they can be used to investigate the activity of genes in different conditions. Reporter genes are gene sequences which require a promoter sequence in the host genome to function. Therefore a reporter gene sequence can only be read when the host promoter is activated. Correlations between reporter gene activity and normal gene activity/biocontrol can then be determined.

There are examples where reporter genes have been used to determine the role of particular metabolites in biocontrol of plant diseases under specific conditions. *Pseudomonas aureofaciens* PGS12 produces the antibiotic phenazine-1-carboxylic acid. Insertion of the ice nucleation reporter system under the control of the phenazine biosynthesis locus was undertaken to determine phenazine production under a variety of media and crop seed conditions (Georgakopoulos *et al.* 1994). It was found that expression of the phenazine genes was dependent on nutrient availability and therefore seed exudates may play a role in *in situ* gene expression. Fusion of the *lacZ* gene with the Tn*3*HoHo-1 transposon element enabled the measurement of production of the antibiotic compound Oomycin-A by *P. fluorescens* Hv37aR2 and subsequent control of *Pythium* (Howie and Suslow, 1991). Measurement of gene activity with this reporter

system was through β-galactosidase activity. The activity of this enzyme was barely detectable in non-producing strain WH103.

1.4.4 Pleiotropy

Although the use of transposons has eliminated many of the undesired side-effects of chemical and UV mutagenesis the problem of secondary effects or pleiotropy can still occur. A pleiotropic mutation occurs when a single gene (often a regulatory gene) disruption affects the production of more than one compound involved in biocontrol or has a broader effect on the strains behaviour *in situ* as previously discussed. As a consequence mutants generated by transposon insertion must be tested for the production of compounds involved in biocontrol and their general ability to survive in the soil. Assessment of mutants with reduced biocontrol activity for pleiotropic mutations usually includes testing for metabolite production, growth in minimal media, and ability to colonise roots of host plants *in situ*.

1.5 SUMMARY OF INTRODUCTION

A serious disease of wheat and barley is take-all, caused by the fungus *Ggt*. Control of this disease by current methods often meets with mixed results. Biocontrol of take-all (as well as many other fungal diseases of plants) offers an alternative to current practices to suppress disease symptoms. Biocontrol agents for take-all control are often isolated from the rhizosphere or soil of a healthy but potentially susceptible plants. Strains of *Pseudomonas* are usually selected for further testing of biocontrol activity because of their excellent root colonisation capabilities and their ability to compete successfully in the soil against indigenous microflora and relative ease of genetic manipulation for in depth study.

Because the ability of a particular biocontrol strain to control take-all varies in different soil/environmental regimes an understanding of the mechanisms of biocontrol is necessary. This variation in suppression activity often results in inconsistent results in field trials, an important problem for the commercial development of a biocontrol agent. Knowledge of what mechanisms the agent utilises to antagonise *Ggt* and/or suppress disease symptoms and under what conditions these mechanisms function gives a better understanding of the potential of the biocontrol agent. In the case of bacterial biocontrol agents, particularly those of the genus *Pseudomonas*, the primary mechanisms of biocontrol are through the release of metabolites with antifungal activity. Quite often these compounds are water-soluble secondary metabolites with antibiotic properties.

To determine what these mechanisms of take-all control actually are, a combination of microbiological, biochemical and molecular techniques are used to identify the mechanisms. A series of simple microbiological and biochemical tests can often identify candidate metabolites. The use of molecular techniques including transposon mutagenesis and gene complementation may confirm the role of any particular metabolite in biocontrol and assist in determining the conditions required for its synthesis and biological activity in the rhizosphere.

1.6 BACKGROUND TO THE PROJECT

Pseudomonas corrugata 2140 (*Pc*2140) was originally isolated from a wheat field at Wagga Wagga, New South Wales. The strain was isolated as part of a project to identify potential biocontrol agents for *Ggt* and *Rhizoctonia*. Strain 2140 was identified as *P. corrugata* by gas chromatography of fatty acid methyl esters (GC-FAME) and organic carbon source utilisation (Biolog GN Version 3.00) (Ryder and Rovira, 1993).

In the laboratory strain 2140 was identified as antagonistic to *Ggt* on a potato dextrose agar medium (PDA). When strain 2140 was grown on a PDA plate near a spreading *Ggt*

hyphal mat a zone of fungal inhibition around the bacterial colony was observed. The zone of inhibition was found to be maximised at temperatures around 15°C. This initial observation suggested that compounds released by 2140 were diffusing through the medium and inhibiting fungal growth.

In a pot trial conducted at 15°C strain 2140 was tested for its ability to suppress take-all lesions on the roots of wheat plants. The results of this trial suggested that strain 2140 significantly reduced lesions on the wheat roots ($P = 0.01$); incidence of lesions was reduced by approximately 50% (Ryder and Rovira, 1993).

Field trials were subsequently carried out with strain 2140 in the mid-North of South Australia during the late 1980's - early 90's. In four of six trials conducted during this period *P. corrugata* 2140 reduced take-all symptoms on wheat, in one trial the disease suppression was significant ($P < 0.05$) (Rovira *et al.* 1992). Variations in responses observed in these trials were attributed to the effects of water potential and soil temperature.

Although *P. corrugata* 2140 showed potential as a biocontrol agent for take-all the mechanism by which *Pc*2140 antagonised the fungus and suppressed take-all symptoms was not known. However, presence of zones of inhibition of *in vitro* fungal growth surrounding a *Pc*2140 colony on a PDA medium suggested that metabolites, in particular water-soluble compounds with antifungal activity, produced by the bacterium were responsible for take-all control.

1.7 AIMS OF THE PROJECT

The overall aim of this project was to determine what the primary mechanism(s) by which *P. corrugata* strain 2140 suppresses take-all symptoms on wheat caused by *Ggt*.

The mechanism(s) would be studied through both genetic and biochemical analysis. The project was divided into three sections.

The first stage of the project was to mutate *P. corrugata* 2140 by transposon mutagenesis to produce a collection of mutants with reduced *in vitro* antagonism towards *Ggt*. Mutants with reduced antagonism would then be tested in a series of pot trials to determine whether loss of antagonism was related to suppression of take-all symptoms on wheat roots.

Second, a cosmid library of *P. corrugata* 2140 genomic DNA was screened to identify gene sequences involved in biocontrol. Mutants that showed reduced take-all suppression in the pot trials described above would be complemented with *wild-type* DNA packaged in a cosmid library. Complemented mutants would then been screened for *in vitro* antagonism of *Ggt* and suppression of take-all on wheat as before.

The third stage of the project would be to determine what compounds are produced by strain 2140 which may have an effect upon *Ggt*. These compounds include water-soluble antibiotics, siderophores, manganese reductase, volatiles such as hydrogen cyanide and ammonia, proteases, and lytic enzymes including chitinase and glucanase. The identification of these compounds was undertaken utilising microbiological and biochemical techniques. Next, comparisons would be made on metabolite production of the biocontrol strains and suitable mutants to determine which of these groups of compounds have a role in take-all suppression.

Completion of theses aims will give us a better understanding of the biocontrol potential of strain 2140 and facilitate development of a commercially viable biocontrol agent for the control of take-all.

CHAPTER 2

GENERAL METHODS

2.1 STRAINS

Pseudomonas corrugata strain 2140 (*Pc*2140) was isolated from the rhizosphere of wheat seedlings grown in soil collected from a wheat field in Wagga Wagga, New South Wales. The isolate was identified as a strain of *P. corrugata* by gas chromatography of fatty acid methyl ester profile analysis (GC-FAME) and organic carbon source utilisation (Biolog GN Version 3.00) (Ryder and Rovira, 1993).

Fungal isolate *Gaeumannomyces graminis* var. *tritici* 8 (*Ggt*) was isolated from infected wheat roots by H. McDonald collected near Avon, South Australia in 1979.

All bacterial strains used in the mutagenesis and complementation work are described in the relevant chapters.

2.2 STORAGE OF CULTURES

Strains were grown to stationary phase in nutrient broth (NB) medium with appropriate antibiotics. Cultures were then centrifuged to pellet the cells (12,000g for 2 minutes). The supernatant was discarded and the pelletted cells were resuspended in 0.9% NaCl, vortexed and pelletted as before. After removal of the supernatant, cells were resuspended in a 15% w/v glycerol/0.45% w/v NaCl solution and stored at -70°C. Routine sub-culturing from these stocks was undertaken by scraping the frozen surface of the culture and placing the scrapings on an appropriate medium and incubating.

Ggt was stored at -20°C as viable hyphae on killed rye grass seeds (propagules) by the method of Simon *et al.* (1987). Cultures were regenerated by placing propagules on PDA and incubating at 25°C. The rye grass seeds were killed and sterilised prior to inoculating with *Ggt* by autoclaving at.121°C for 15 minutes once daily on 3 consecutive days.

2.3 MEDIA AND CULTURE CONDITIONS

P. corrugata strains were routinely grown on modified tetrazolium agar medium (TZCA) (after Kelman, 1954). *E. coli* and non-*P. corrugata* pseudomonad strains were grown on nutrient agar (NA) and in nutrient broth (NB) (Difco, Detroit) and Luria-Bertani medium (LB) (Sambrook *et al.* 1989). Unless otherwise stated all *Pseudomonas* cultures were grown at 25°C and *E. coli* strains were grown at 37°C. All media are listed in Appendix I.

When antibiotics were added to media the following concentrations were used unless otherwise stated; ampicillin (Ap), 50μg.ml^{-1}; tetracycline (Tc), 10μg.ml^{-1}; trimethoprim (Tp), 100μg.ml^{-1}; kanamycin (Km), 25μg.ml^{-1}; chloramphenicol (Cm), 40μg.ml^{-1}and cycloheximide (Cx), 75μg.ml^{-1}. All antibiotics were prepared as stock solutions in a suitable solvent (Sambrook *et al*, 1989) and stored at -20°C.

Ggt was routinely grown on half strength potato dextrose agar (PDA) (Difco, Detroit) at 25°C. A culture plate of *Ggt* was prepared from stocks by aseptically transferring a single propagule to the centre of a PDA plate and incubating. *Ggt* was routinely sub-cultured from one plate to another by removing a 4mm diameter agar plug from the leading edge of a growing hyphal mat and placing it hyphal-side down in the centre of a fresh plate. All plates were incubated 'lid-side-up'.

2.4 DETERMINATION OF BACTERIAL CELL NUMBERS

2.4.1 Colony-Forming-Units (CFU/ml)

Determination of viable cells in culture or on wheat roots (Section 2.11) was undertaken by dilution spread-plating cell suspensions on appropriate media and counting colonies. A suspension of cells was serially diluted tenfold in 0.9% w/v sterile NaCl and 100µl of each dilution was spread-plated onto solid media containing appropriate antibiotics. After incubation the colonies on the plates were counted (only for plates containing 30-300 colonies) and the cell densities of the original solution were determined.

2.4.2 Absorbance (A_{550})

Cell density was determined spectrophotometrically. A cell suspension was placed in a plastic cuvette (3 mls) and read in a spectrophotometer (Beckman DU 640) at 550nm. A blank was prepared with the appropriate solution the cells were suspended in. A graph relating A_{550} to CFU/ml was subsequently prepared (Appendix II).

2.5 MEASURING THE MEAN GENERATION TIMES OF *P. corrugata* STRAINS

The mean generation time (MGT) of *P. corrugata* strains was measured by growing strains in a mineral base E medium (MMBE) (modified after Owens and Keddie, 1969) (Appendix I). Strains were grown overnight on TZCA medium with appropriate antibiotics at 25°C. A single colony was removed and resuspended in 0.9% w/v sterile NaCl by vortexing and then pelleting cells by centrifugation (12,000g, 2 minutes). The supernatant was discarded and the cells were resuspended in sterile distilled water (SDW). The A_{550} was adjusted to 0.5 (approx) by further addition of SDW. 10µl of cell suspension was then added to 100mls MMBE in 250ml conical flasks. The cultures

were incubated at 25°C on a shaker-incubator (200 RPM). After 9 hours growth samples were taken for A_{550} readings and CFU counts. Samples and measurements were taken during log phase growth. Incubation of the cultures was continued until stationary phase was reached. Samples were taken every three hours for analysis.

2.6 MEASURING THE GROWTH OF *Ggt*

In vitro growth of *Ggt* was determined by measuring the radius of the spreading hyphal mat. Four measurements were taken at right angles to each other from the edge of the original agar plug (Section 2.3) to the leading edge of the hyphae. The average of these four measurements was then calculated. Figure 2.1 illustrates the method.

2.7 *IN VITRO* SCREENING OF STRAINS FOR Ggt ANTAGONISM

2.7.1 Water-Soluble Compounds

A 4mm diameter plug of agar containing the leading edge of *Ggt* hyphae growing on PDA was removed and placed in the centre of a fresh PDA plate and incubated for 3 - 4 days at 25°C. *P. corrugata* strains were grown overnight on TZCA with appropriate antibiotics. A single *P. corrugata* colony was suspended in 1ml SDW, vortexed and the cells pelleted in a microcentrifuge. (12,000g, 2 minutes). The supernatant was removed and the cells were resuspended in an equal volume of SDW. A 10µl aliquot was spotted approximately 1cm from the leading edge of the *Ggt* hyphae that had been incubated for 3 - 4 days. The bacterial spot was allowed to dry and the plates were incubated as before. Plates were inspected daily for zones of inhibition of *Ggt* growth surrounding the bacterial colonies.

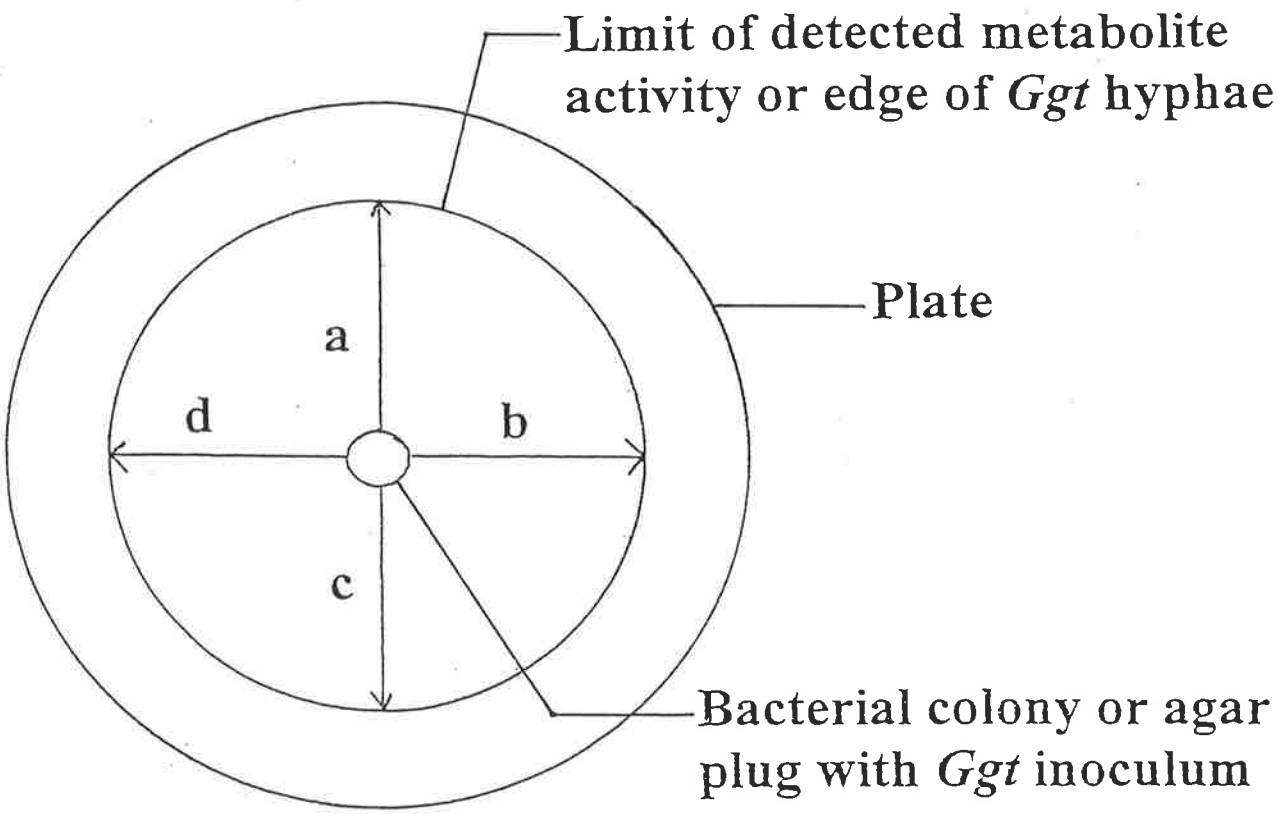


Figure 2.1: Measurement of growth of *Ggt* hyphae on PDA medium. Four measurements (a,b,c,d) were taken perpendicular to each other. The four measurements were averaged to give a final measurement of radial growth. For determination of the effects of volatiles on *Ggt in vitro* one of the measurements was directly towards the bacterial inoculum on split plates and the other three measurements were at 90° intervals. Measurement of *in vitro* metabolite production by *P. corrugata* including siderophores, proteases and manganese reductase was also performed by this method; measurements taken from the outer edge of the colony to the edge of metabolite activity on the plate. Note: where measurements were taken more than once, e.g. daily over a period of time, measurements were taken on the same regions of the plate to ensure consistency of results.

2.7.2 Volatile Compounds

Antagonism of *Ggt* growth by volatile compounds produced by *P. corrugata* strains was tested on split petri plates. *P. corrugata* strains were grown on TZCA and resuspended in sterile saline solution as described in Section 2.5. The bacterial strains were inoculated onto half of the split plate containing 2% Proteose peptone #3 agar medium (PP3) (Difco) by spotting 20µl of the cell suspension onto the medium and spreading the culture fluid over the entire surface of the medium. After drying the plates (approximately 30 minutes) a *Ggt* plug (Section 2.3) was added to the centre of the second half of the split plate containing PDA:Km^{25}. The plates were sealed with plastic wrap to prevent leakage of volatiles and then incubated at 25°C. Plates were inspected daily for fungal growth and the radial growth of the hyphae from the central plug was measured (Figure 2.1).

2.8 *IN SITU* POT TRIALS

2.8.1 General Overview

Pot trials were conducted on *P. corrugata* strains to determine whether biocontrol activity observed on petri dishes could be transferred to a soil/wheat root environment. A pot assay developed for this purpose (Ryder and Rovira, 1993) was used to see if loss of suppression of take-all symptoms on wheat inoculated with strains of *P. corrugata* could be related to loss of *in vitro* antagonism against *Ggt*. The assay consisted growing wheat initially inoculated with *P. corrugata* strains for 4 weeks in a clay soil/sand mix in the presence of a pathogenic strain of *Ggt*. Wheat plants were harvested after 4 weeks and examined for take-all symptoms. Growth promotion by *P. corrugata* 2140 and root colonisation rates of selected strains were also examined. These pot tests were used to measure loss of biocontrol activity of transposon-induced mutants of strain 2140

(Chapter 3) and restoration of activity in selected mutants through complementation with a cosmid library containing *Pc*2140 genomic DNA (Chapter 4).

2.8.2 Pretreatment of the Soil/Sand Mix

Soil used for testing biocontrol activity of *P. corrugata* strains was a Kapunda clay soil mixed with washed river sand. The Kapunda clay soil is classified as a sodic red-brown earth and was collected from the top 10cms of a legume pasture with a low natural take-all inoculum level (Stace *et al.* 1968; Ryder and Rovira, 1993). The soil was prepared by air-drying for 2-3 days then sieving, retaining soil particles < 3 mm. Coarse river sand from Waikerie, South Australia was treated in the same manner. After sieving, the sand and soil were stored in plastic-lined steel tins.

2.8.3 Wheat Seed Preparation

Wheat (*Triticum aestivum* L. var. "Spear") was prepared by pregerminating seeds 2 days before planting. Seeds were germinated at 25°C on Whatman filter paper dampened with distilled water. For long-term storage, seeds were placed in air-tight containers with added Dichlorvos (applied as per manufacturer's instructions). Seeds were stored at room temperature.

2.8.4 Preparation of Bacterial Inoculum

P. corrugata strains were prepared by streaking on TZCA medium 2 days before seed sowing. Plates were incubated at 25°C for 24 hours. A single colony was transferred to NB (wild-type strain) or NB:Tc^{10} (mutants) and incubated on a shaker (250 RPM) at 25°C for a further 24 hours. Cells from the culture broth were pelleted by centrifugation (Sorvall GS3, 5,000 RPM, 30 minutes) and resuspended in one-tenth strength plant nutrient supplement (PNS) (Hoagland and Aaron, 1938) (refer Appendix 1). This

bacterial suspension was then diluted to $A_{550} = 1.0 \pm 0.05$ with one-tenth strength plant nutrient solution (PNS/10); this correlated to a CFU count of approximately 10^9 per ml (Ryder and Rovira, 1993).

2.8.5 Determination of Viability of *Ggt* Inoculum

Ggt was added to the soil as inoculum growing on dead rye-grass seed (propagules) (Simon *et al*, 1987) at a rate of 120 viable propagules per kilogram of soil/sand mix. Propagules were pretested for purity and viability by placing *Ggt*-inoculated rye-grass seeds on ½ strength PDA and incubating plates at 25°C until mycelial growth was observed. Viability was determined as a percentage of seeds where hyphal growth was observed after 3-4 days incubation compared to the total number of propagules placed on the plates. This percentage figure was used to determine the total number of propagules from the stock supply that would be added to the soil mix.

2.8.6 Construction of the Pot Test

Soil and sand were premixed by placing 1.4kg of sand and 0.6kg Kapunda field soil in a plastic bag and shaking vigorously until the two components were thoroughly mixed. Prior to adding the soil mix to pots, 68mls PNS/10 solution were added to each kilogram of soil/sand mix and remixed as described above. The wetted mix was then added to 300 ml. non-draining plastic pots as follows: layer 1 comprising 250g soil mix, either uninoculated or inoculated with 120 viable *Ggt* propagules per kilogram soil mix. Propagules were added to the wetted soil mix and the bag reshaken gently as to mix propagules throughout the mix but to avoid damaging inoculum on the seed. The second layer comprised 50g uninoculated wetted soil mix placed on top of the first layer without any mixing of the layers.

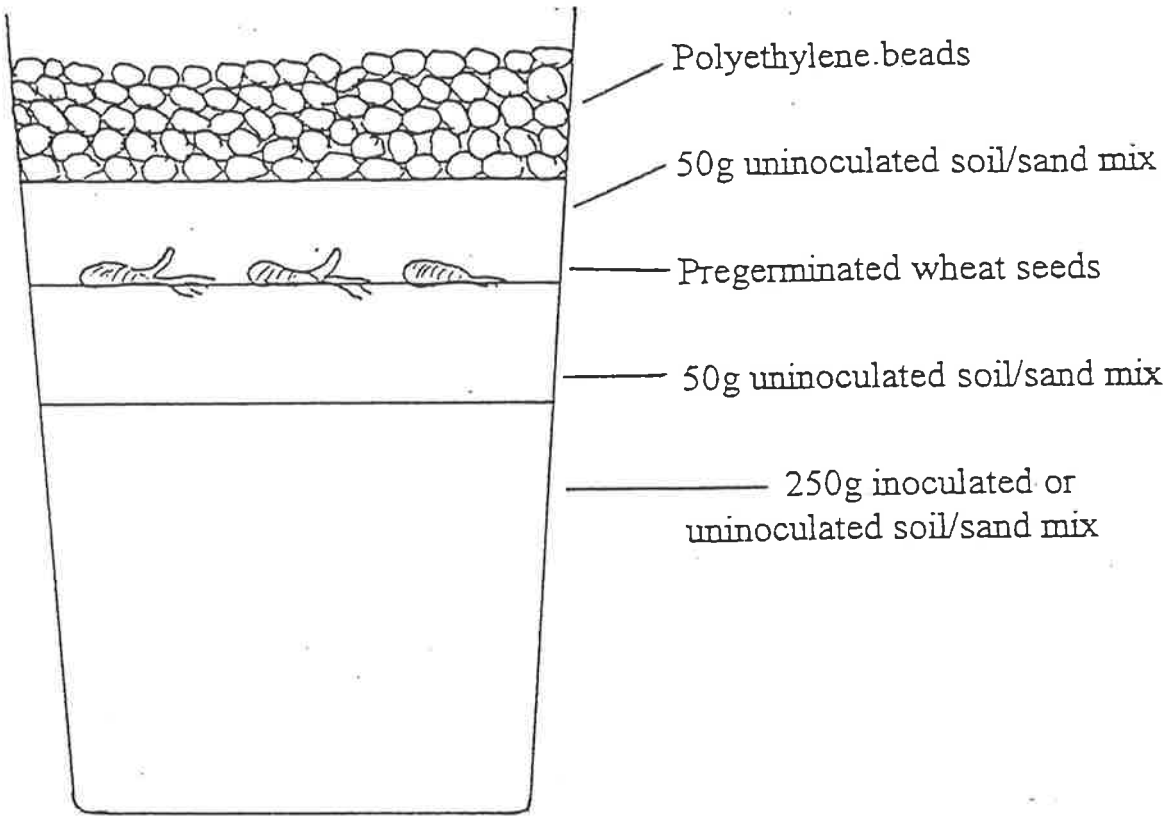


Figure 2.2: Pot test for assaying of biocontrol of take-all by *P. corrugata* strains *in situ*. Sand and Kapunda soil were premixed in 2kg batches as dry material then water (as 1/10th strength PNS solution) was added and the mix reshaken before adding to pots. Batches were distributed equally amongst treatments to minimize effects of any variations in the soil/sand preparations. Inoculation of the bottom layer with *Ggt* propagules was achieved by adding viable propagules to the soil-sand mix and shaking briefly before adding PNS. Bacterial drenches consisted of *P. corrugata* cells suspended in 1/10th strength PNS solution. Controls received 8 mls of the 1/10th strength PNS solution only as a drench.

Seven pregerminated wheat seeds per pot were added to the top of layer 2. Before adding the seeds to the pots they were dipped in a 10^{-2} diluted bacterial suspension (see above). On top of the seeds another 50g non-inoculated wetted soil mix was added. The bacterial suspension was then added as a drench to the top of layer 3. Eight mls. of non-diluted suspension was added per pot. Bacteria-free treatments (controls) received 8mls PNS/10 only. The top layer of the pot (layer 4) comprised polyethylene beads, added to prevent water loss from the pots by evaporation from the soil surface. Beads were added so the final gross weight of the pot was 400g. Figure 2.2 illustrates the different layers in the pot. Ten replicates of each treatment were prepared per trial. Each strain was tested at least twice in independent trials.

2.8.7 Incubation of Pots

Pots were placed in a controlled-environment growth cabinet. The cabinet was set at 15°C day and night with a 12 hour day/night cycle. The daytime light intensity was set at 250μmoles.m^{-2}.s^{-1}. Plants were routinely watered with ¼ strength PNS (PNS/4), total pot weight was not allowed to fall by more than 15g (approximately 50% of the total water content of the pot) through water transpiration. Water was added to restore the gross weight of the pot to 400g. Seven days after seedling emergence, plant number per pot was reduced to five by culling the emerging seedlings. Surplus seedlings were removed by gently loosening the soil around selected plants and severing the roots just below the seed to prevent re-emergence of the wheat seedling. Pots were incubated for a total of four weeks before harvesting and examination of take-all symptoms.

2.9 MEASUREMENT OF TAKE-ALL SEVERITY ON WHEAT

Wheat plants were harvested by removing soil and plant matter from the plastic pot and soaking in water for approximately five minutes. After this time the soil was gently removed from the roots and then the roots were washed in fresh water to remove

remaining soil particles. Shoots were separated from roots by cutting approximately 1 cm above the crown at the base of the stem. Five factors were examined to determine the extent of disease in the plant and the degree of biocontrol activity by *P. corrugata* strains.

2.9.1 Water Consumption

Water uptake and transpiration was measured by weight change in the pot. During the final two weeks of incubation in the growth cabinet the pots were weighed each time before watering. The weight loss was recorded and each pot was then adjusted to 400g with quarter-strength PNS. During the first two weeks the weights were not taken as the fungus would have yet to become established within the plant tissue. Total water uptake for the final two weeks was measured for each pot/treatment.

2.9.2 Disease (Root) Rating

After separating the shoots from the roots the seminal roots of each plant were examined for lesions. The length of each primary root (to a maximum of 12 cms. below the crown) and the total lengths of the lesions on the 12 cms. of each root were measured. Lesion length per plant was calculated as a percentage and the average percentage length of roots affected by lesions per plant for each pot was derived.

2.9.3 Root Dry Weight

After the disease ratings were obtained the roots were placed in paper bags and dried in an oven at 60°C for 3 days. The dry weights of the roots for each pot and treatment were then obtained.

2.9.4 Shoot Length and Shoot Dry Weight

Immediately after separating shoots from roots at harvesting the length of each shoot was taken by measuring the longest leaf on each plant. The average shoot length for each pot was then calculated. Shoots were placed in paper bags and then dried in an oven for 2 days at 60°C and the dry weight of shoots for each pot/treatment determined.

2.10 PLANT GROWTH PROMOTION BY STRAIN 2140 IN THE ABSENCE OF *Ggt*.

The effect on the growth of wheat by *P. corrugata* 2140 in the absence of take-all was examined. Water consumption, root dry weight, shoot length and dry weight of wheat plants treated with strain 2140 were measured in four independent trials. Ten replicates of each treatment were prepared per trial. The pot trials were prepared and incubated as described in Section 2.8. Determination of growth promotion parameters of wheat plants was carried out as described in Section 2.9.

2.11 COLONIZATION OF WHEAT ROOTS BY *P. corrugata* STRAINS

Mutants were compared to the wild-type strain for their ability to colonise wheat roots in pot tests. Pots were prepared and wheat roots inoculated with bacterial strains as per previous pot tests (refer Section 2.8). No *Ggt* inoculum was added to the pots. Five replicates for each strain and control (no added bacteria) were prepared.

After 4 weeks the plants were removed from the pots and loose soil carefully dislodged from the roots. Water was withheld from the pots for 48 hours immediately prior to harvesting to facilitate soil removal. Remaining soil clinging to the roots was carefully

brushed away with a soft-haired brush. The weight of each plant root system plus rhizosphere soil was taken and the roots placed in 10 mls SDW and vortexed for 30 seconds to dislodge rhizosphere soil from the roots. The roots were then placed in a sonicator (Branson B-220) for 10 seconds to dislodge microbial cells from the root surface (Ryder and Borrett 1991). The liquid suspension was transferred to a clean sterile vial for viable cell counts (see below). The roots were removed, blotted to surface dryness and reweighed. Separate fresh root and rhizosphere weights were calculated for each sample.

Suspensions containing cells isolated from the surface of roots were serially diluted and 100µl spread-plated on modified *Pseudomonas* selective medium (PSM) (after Gasson, 1980) supplemented with Cm^{50} (see below), Ap^{50} (Simon and Ridge, 1974), and Cx^{75}. The medium for the isolation of mutants also contained Tc^{10}. Plates were incubated at 25°C until colonies appeared. These were then patched onto modified TZCA medium to confirm typical *Pc*2140 colony morphology. Colony forming units per gram fresh root weight were then calculated.

2.11.1 Determination of Bacterial Resistance to Chloramphenicol

A level of natural resistance of *P. corrugata* 2140 to chloramphenicol was exploited as a means to reisolate *P. corrugata* strains from wheat roots. The concentration of chloramphenicol used in the medium was determined by streaking a solution of *P. corrugata* 2140 cells in SDW onto PSM media containing 0, 10, 20, 50, 100, 150 and 200 $\mu g.ml^{-1}$ chloramphenicol and incubating for 48 hours at 25°C. *P. corrugata* was grown on TZCA medium for 24 hours prior to suspending in SDW. The maximum concentration was determined where colonies of *P. corrugata* were greater than 1mm in diameter after 2 days incubation.

2.12 STATISTICAL ANALYSIS

Statistical analysis of data was undertaken with the assistance of Statistix version 3.5 (Analytical Software). All data obtained from experiments with multiple replication of treatments were subjected to General Analysis of Variance with comparisons of Least Significant Difference between Means at $P = 0.05$. Data was analysed for residuals and subjected to normal distribution analysis before ANOVA and LSD analysis. Where necessary data was transformed by square root, log, or natural log to fit a normal distribution. Percentage data from the root disease ratings (Section 2.9.2) was subjected to arcsin transformation before analysis (Gomez and Gomez, 1984).

CHAPTER 3

MUTAGENESIS OF *P. CORRUGATA* 2140

3.1 INTRODUCTION

Due to the ability of *P. corrugata* 2140 to produce a zone of fungal inhibition around the bacterial colony (Section 1.6) it was hypothesised that biocontrol was due to the production of at least one metabolite that inhibited fungal growth. As these earlier experiments were conducted in unsealed petri dishes and the distinct semi-circular nature of the zone (Ryder and Rovira, 1993) it was further hypothesised that the metabolite (or metabolites) were diffusible water-soluble compounds and not volatiles. Volatiles, by their very nature, would tend to diffuse throughout the whole plate thereby reducing *Ggt* growth in general and not restrict inhibition to the vicinity of the bacterial colony. Furthermore, volatiles would tend to diffuse from the unsealed plate, reducing their efficiency over time (Ahl *et al.* 1986). In previous research on biocontrol of take-all by pseudomonad strains as reviewed in Chapter 1 it was often found that water-soluble antibiotics were the primary mechanism of take-all control by these bacteria.

The first stage of the project was to generate mutants of *P. corrugata* 2140 that were unable to antagonise *Ggt in vitro* or suppress take-all symptoms on wheat in pot trials. Suitable mutants with these two characteristics would suggest that disease suppression was related to fungal inhibition. Furthermore, the continued production of various metabolites by mutants with little or no biocontrol activity, ie, those metabolites unaffected by the mutation which reduces biocontrol activity, would then be eliminated as likely mechanisms, thus reducing the number of mutants and mechanisms to be examined. For example, if *Pc*2140 produces a number of compounds potentially

antagonistic to *Ggt* and a mutant with little or no activity produces any of them, then those compounds can be eliminated as potential mechanisms of biocontrol.

As there is a current preference for transposon mutagenesis over chemical and UV treatments (Chapter 1) it was decided to attempt to mutate *P. corrugata* 2140 with a range of transposons with different antibiotic markers (Table 3.2). A range of transposons on different vectors would be trialed as it has been found in the past that not all systems are suitable for the random mutagenesis of a specific bacterium (Mills, 1985). Previous genetic manipulation of *Pc*2140 has only involved insertion of marker genes such as the site-specific Tn*7*::*lacZY* cassette (Barry, 1988) to track the strain in field trials (Ryder, 1994); the strain has not been characterised genetically by the random insertion of transposons such as Tn*5* and Tn*10*. It would be expected however, that at least one of the systems trialed would produce a library of mutants with stable, single insertions of the transposon.

Once a collection of suitable mutants was generated, the mutants would initially be screened for loss of *in vitro* antagonism towards *Ggt*. These mutants would be screened by the method described in Section 2.7.1. Mutants with reduced *in vitro* antagonism would then be tested in a series of pot trials (Section 2.8) to determine whether loss of *in vitro* antagonism would result in loss of take-all suppression on wheat.

Mutants that were found to have reduced antagonism of *Ggt in vitro* and reduced ability to suppress take-all symptoms on wheat in pot trials would then be examined for pleiotropic changes. Pleiotropy refers to secondary effects of the mutation as detailed in Section 1.4.4 and may have an effect on the biocontrol activity of the mutant independent of the actual mutation. Mutants would be compared with the parent strain for growth rate in a minimal salts/glucose medium, production of volatiles possibly antagonistic to *Ggt in vitro* and ability to colonise the wheat root system. Changes in colony morphology on TZCA medium would also be examined.

Following mutagenesis and the isolation of mutants with reduced ability to suppress take-all symptoms on wheat, selected mutants would be complemented with a *Pc*2140 genomic library to restore biocontrol activity (Chapter 4). Finally, these mutants would be compared with the parent strain for the production of various metabolites including water-soluble compounds and volatiles that may have a role in the antagonism of *Ggt* and suppression of disease (Chapter 5).

3.2 MATERIALS AND METHODS

3.2.1 Strains

All *E. coli* strains used in the mutagenesis of *P. corrugata* 2140 are listed in Table 3.1. The transposons and the suicide vectors carrying them are listed in Table 3.2. All mutants of *P. corrugata* selected for further examination after screening for *in vitro* antagonism of *Ggt* are also listed in Table 3.1. The mutants generated by transposon insertion were given the designation 'TM' (transposon mutant) and a number, eg. TM1120.

3.2.2 Suitable Systems for *Pc*2140 Mutagenesis

All *E. coli* strains listed in Table 3.1 carrying a particular vector/transposon system were trialed as suitable donors for *Pc*2140 mutagenesis. All matings were carried out in accordance with the appropriate literature (Table 3.1). Of all the systems trialed, only mutagenesis with the Tn*5*::*luxAB*Tc^r contained in *E. coli* strain S17-1λ*pir* resulted in stable mutations in strain 2140. Consequently, mutagenesis and screening of mutants with this system only will be described in the text. Problems arising from mutagenesis with the other transposon/vector systems will be addressed in the discussion.

Strain	Phenotype/Characteristics	Source/Reference
Pseudomonas corrugata 2140	Tp^r, Cm^r, Ap^r, Tc^s, Km^s	Ryder and Rovira (1993)
P. corrugata TM27	*Pc*2140::Tn*5* Tc^r, reduced take-all suppression	This study
P. corrugata TM129	*Pc*2140::Tn*5* Tc^r, reduced take-all suppression	This study
P. corrugata TM300	*Pc*2140::Tn*5* Tc^r, reduced take-all suppression	This study
P. corrugata TM692	*Pc*2140::Tn*5* Tc^r, reduced take-all suppression	This study
P. corrugata TM1120	*Pc*2140::Tn*5* Tc^r, reduced take-all suppression	This study
Escherichia coli S17-1 λpir	contains pUT::Tn*5*::*luxABTc*^{r}	deLorenzo *et al* (1990)
E. coli K381	contains pJB4JI::Tn*5*	Hirsch & Beringer (1984)
E. coli SM10	contains pSUP1011::Tn*5*	Simon *et al* (1983)
E. coli J53	contains pJB4JI::Tn*10*	Way *et al* (1984)
E. coli K1259	contains pRU670::Tn*1731*	Ubben &Schmitt (1986)
E. coli K1261	contains pME305::Tn*1732*	Altenbuchner *et al* (1983)

Table 3.1: Strains used in the mutagenesis of *P. corrugata* 2140. Mutants generated from the mutagensis work that were selected for further evaluation are also described. These mutants were given a designation of 'TM' (transposon mutants) and a number corresponding to their original isolation and *in vitro* screening for loss of *Ggt* antagonism (refer text for details).

Plasmid	Characteristics	Reference
pUT	Tn*5*::*luxAB*Tc^r, *amp*, *ori*	deLorenzo *et al* (1990)
pJB4JI::Tn*5*	Tn*5*::Km^r,	Hirsch & Beringer (1984)
pSUP1011::Tn*5*	Tn*5*::Km^r,	Simon *et al* (1983)
pJB4JI::Tn*10*	Tn*10*::Tc^r,	Way *et al* (1984)
pRU670::Tn*1731*	Tn*1731*::Tc^r, *kam*,	Ubben &Schmitt (1986)
pME305::Tn*1732*	Tn*1732*::Km^r, *tet*,	Altenbuchner *et al* (1983)

Table 3.2: Plasmids containing transposons used in the mutagenesis of *P. corrugata* 2140. Antibiotic markers on the mobile element are signified as X^r. Antibiotic marker on the suicide vector are given normal genotype designations (in italics).

3.2.3 Mutagenesis of *P. corrugata* 2140

Transposon mutagenesis of strain 2140 was undertaken with plasmid pUT containing transposon Tn*5*::*luxAB*Tc^r (Herrero *et al.* 1990; de Lorenzo *et al.* 1990) (Figure 3.1). The donor strain was *E. coli* S17-1λ*pir*. Mutants were generated by conjugation between *E. coli* S17λ*pir* and *P. corrugata* 2140. *E. coli* strain S17-1λ*pir* containing Tn*5*::*luxABTc*^{r} was obtained from Prof. Ken Killham, University of Aberdeen.

P. corrugata 2140 was grown overnight on TZCA medium at 25°C. A single colony was transferred to NB and incubated with gentle shaking at 25°C. The culture was grown to early log phase (A_{550} = 0.06). *E. coli* S17-1λ*pir* was grown overnight on NA:Tc^{10}. A single colony was transferred to NB:Tc^{10} and incubated with gentle shaking at 37°C for 24 hours. Cells from both cultures were pelleted (Sorvall SS34, 10,000 RPM, 15 minutes) and resuspended in an equal volume of SDW. Cells were repelletted and resuspended in SDW as before: the *E. coli* cells were resuspended in an equal volume of SDW, the *P. corrugata* cells in a $^1/_{10}$th volume of same.

After washing and resuspending in SDW donor and recipient cells were mounted on sterile 0.22um millipore paper filter discs as follows: 1ml of *E. coli* S17λ*pir* containing Tn*5*::*luxAB*Tc^r suspension was mounted on the filter disc. Two mls of donor cells mixed with 1 ml of *P. corrugata* suspension was then placed on top. After drying, the filters were placed "cell-side up" on NA plates and incubated overnight at 30°C. Bacteria from the filters were then suspended in 10mls. of sterile 0.03M NaCl by vortexing for 15 seconds. A tenfold dilution series was performed on the cell suspension and samples were spread-plated on TZCA:Tc^{10}:Tp^{100}. After drying, the plates incubated at 25°C until growth was detected. Controls were prepared by streaking both *E. coli* S17-1λ*pir* containing the vector/transposon and *Pc*2140 onto TZCA:Tc^{10}:Tp^{100} and incubating at 25°C to ensure that only *Pc*2140 cells that received the transposon would grow on the selective medium.

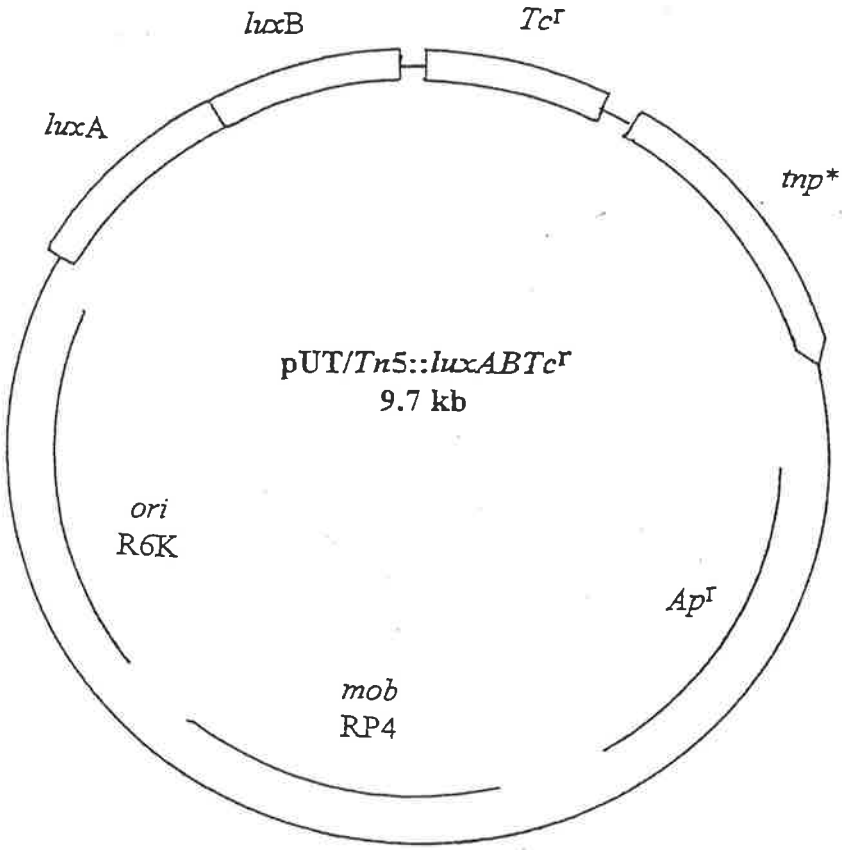


Figure 3.1: Schematic diagram of pUT with the Tn*5*::*luxABTc*^{r} insert based on data provided in the literature (see text). The construction of the suicide vector pUT/Km is described in Herrero *et al* (1990). The modified transposase gene *tnp**, also described in the above reference, is located adjacent to but outside the transposon at the I end. The kanamycin gene is deleted when the *lux* transposon cassette is inserted (*E. coli* S17-1*pir* carrying the pUT/Tn*5* vector does not grow in NB:Km^{25}).

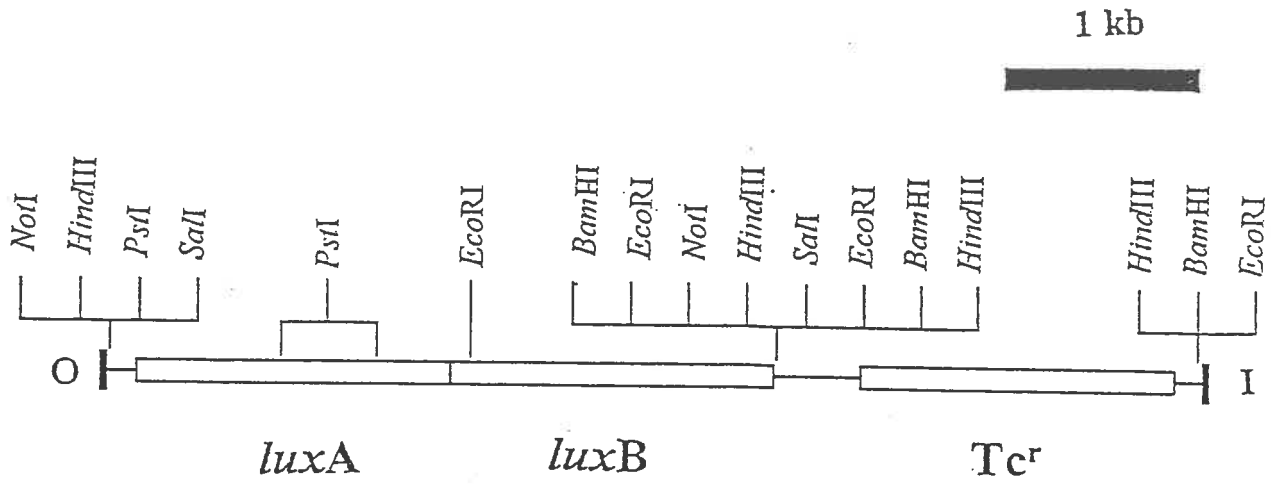


Figure 3.2: Restriction map of Tn5::*luxAB*Tc^r showing relevant restriction sites (deLorenzo *et al*, 1990). The probe for transposon insertions in mutants of strain 2140 was derived from a 3.2kb *Sal*I fragment comprising the *lux*A/*lux*B genes. Construction of a probe for the cosmid library came from a 4.5kb *Not*I fragment from TM1120 genomic DNA (gDNA) comprising the *tet* gene plus TM1120 gDNA flanking the I end of the transposon. This fragment was then ligated into pBluescript M13 together with the *lux* genes. The resultant plasmid (pBSM13-FF) was subsequently cut with *Eco*RI to separate the *tet* and *lux* genes from the gDNA and transposon (refer Chapter 4). The gDNA, comprising a 2.1kb *Eco*RI/*Not*I fragment was then used to probe the cosmid library.

Frequency of conjugation was determined by plating out equal volumes of the diluted samples of the cell suspension on both TZCA:Tc^{10}:Tp^{100} and TZCA:Tc^{10}:Tp^{100} and comparing *Pc*2140 cell numbers on each of the two media. Plates were incubated at 25°C and colony forming units counted.

3.2.4 Screening Mutants for *In vitro* Antagonism of *Ggt*

P. corrugata 2140 transposon mutants that grew on the selection medium were transferred to PDA plates preinoculated with *Ggt* to identify mutants with reduced ability to inhibit *in vitro* growth of *Ggt* (refer Section 2.7.1). As each mutant was tested it was given a strain designation number, eg, TM1120, as described in Section 3.2.1. Mutants found to have reduced activity were restreaked for single colonies on TZCA:Tc^{10} medium and single colonies from these plates were retested for reduced *in vitro* inhibition. Strains showing reduced antagonism in two independent trials were selected for further screening.

3.2.5 Retention of Transposon in Mutants

Mutants were initially streaked on TZCA:Tc^{10} and incubated overnight at 25°C. A single colony was then transferred to 10mls LB:Tc^{10} broth and grown overnight at 25°C in a rotary incubator (200 RPM). The next day 100µl of culture was added to 100mls MMBE broth. The cultures were grown on a rotary incubator for 24 hours (200 RPM) at 25°C. The culture was then serially diluted tenfold and each dilution was then spread-plated on LB agar plates with and without 10µg.ml^{-1} tetracycline (100µl diluted culture sample per plate). Each dilution for each culture was spread-plated on two plates (100µl per plate) for each of the two LB media. The plates were dried and incubated at 25°C for 48 hours before counting colony-forming-units (refer Section 2.4.1). The number of colonies on each type of medium was then compared for each strain. Transposon

retention was calculated as a percentage of CFU's growing on LB agar supplemented with kanamycin compared to CFU's on LB agar medium only.

Controls were prepared by streaking *Pc*2140 on both LB and LB:Tc^{10} media to ensure only mutants carrying the transposon would grow on the LB:Tc^{10} plates.

After samples were removed from the culture, 100µl of the culture was added to 100mls fresh MMBE broth and the culture reincubated a further 24 hours when fresh samples were taken for diluting and testing as described. This process was repeated daily for 7 days.

3.2.6 Auxotrophy

Mutants of *P. corrugata* 2140 generated by transposon mutagenesis were tested for frequency of auxotrophy. The first 1000 mutants isolated from the conjugal mating were streaked onto modified PMS agar medium (Gasson, 1980) supplemented with 10ug.ml^{-1} tetracycline. Controls were prepared with NA:Tc^{10} to ensure viability of each mutant strain. All plates were incubated at 25°C and inspected daily for growth. Mutants that grew on nutrient agar but not on the minimal medium after 48 hours were scored as auxotrophs.

3.2.7 Pleiotropy

All strains of *P. corrugata* derived from the wild-type strain that displayed reduced biocontrol activity against *Ggt* both *in vitro* and in pot trials (see Section 3.2.12) were compared with the parent strain for pleiotropic effects.

3.2.7(i) Growth in a Minimal Medium

The ability of selected mutants to grow at a similar rate to the parent strain was tested in a minimal medium broth. 100 µl of cell suspension was added to 100 mls MMBE medium. The cultures (5 replicates per strain) were incubated on a shaker at 25°C. After 18 hours incubation A_{550} readings were taken (refer Section 2.4.2) and converted to total cell numbers (Appendix II). Samples were also taken for CFU counts by dilution plating.

3.2.7(ii) Volatile Production

Mutants were tested for production of inhibitory volatiles including HCN. Split plates were prepared by the method described in Section 2.7.2. Ten replicates for each strain and control (no bacteria) were prepared. The radial growth of *Ggt* was measured after four days incubation at 25°C (refer Section 2.6)

3.2.7(iii) Colony Morphology

Colony morphology of *P. corrugata* strains was determined on modified TZCA:Tc^{10} medium (after Kelman, 1954) (Appendix I). Strains were streaked onto the solid medium and incubated for 48 and 96 hours at 25°C. Single colonies on the plate were compared with the colony morphology of the parent strain.

3.2.8 Bioluminescence

Bioluminescence of mutants carrying Tn*5*::*luxAB*Tc^r was visually detected by the addition of n-decyl aldehyde to growing cultures. *P. corrugata* strains were grown on LB agar medium with appropriate antibiotics for 24 hours at 25°C. A 10% v/v solution of n-decyl aldehyde (Sigma) in 100% ethanol was prepared and 1µl was added to the

inside of the lid of the petri dish containing the culture. The plates were sealed and incubated at room temperature in the dark until visible bioluminescence was detected (usually after several minutes).

3.2.9 Testing Mutants for Single Insertions of Tn*5*::*luxAB*Tc^r: Overview

Selected mutants were checked for the number of Tn*5* insertions within the genome by probing with a 3.2kb DNA fragment derived from a *Sal*I digestion of the transposon labelled with [α-^{32}P]-dCTP. Genomic DNA from selected mutants and *Pc*2140 was completely digested with *Pst*I, run on a 0.7% w/v agarose gel, and blotted onto a nylon membrane. Plasmid pUT DNA digested with *Sal*I was included in the gel as a positive control for the probe. The probe was prepared by digesting pUT containing Tn*5*::*luxABTc*^{r} (Figure 3.1) with *Sal*I and purifying a 3.2kb fragment containing the *lux* genes from the transposon (Figure 3.2). The *Sal*I fragment was labelled with [α-^{32}P]-dCTP and hybridised with the genomic DNA mounted on the nylon membrane. Bands containing homologous DNA signifying transposon insertions were then detected by autoradiography.

3.2.9(i) Isolation and Purification of Genomic DNA

P. corrugata strains were grown overnight and a single colony transferred to NB and incubated for 24 hours on a shaker at 25^{o}C. 1.5ml of the grown culture was transferred to a microfuge tube and centrifuged to pellet cells (12,000g, 2 mins.). The pellet was resuspended in 0.8ml Tris/EDTA buffer, pH 8.0, (TE8) (Sambrook *et al.* 1989), vortexed and pelleted as before. After removal of the supernatant the cells were resuspended in 565µl TE8 with vortexing. Isolation and purification of genomic DNA from *P. corrugata* strains was undertaken by the method of Ausbel *et al.* (1994).

3.2.9(ii) Restriction Digests of DNA

Genomic DNA from *P. corrugata* strains was digested with *Pst*I restriction enzyme (Boehringer Mannheim). The restriction digest mixture was as follows: 10 µl DNA solution, 2.5 µl 10x buffer H, 2.0 µl appropriate restriction enzyme (10 units per µl stock solution) and 2.5 µl 40mM spermidine. The volume was made up to 24 µl with DDW. The contents were gently mixed and pulse centrifuged in a benchtop microcentrifuge. The mixture was placed in a 37°C water bath and incubated for 2 hours. 1 µl of 10 $mg.ml^{-1}$ DNAase free pancreatic RNAase solution (Boehringer Mannheim) was then added, the contents mixed and spun as before and then incubated overnight at 37°C.

3.2.9(iii) Gel Electrophoresis

An aliquot of digested genomic DNA was mixed with a 0.2x volume of 6x loading buffer comprising 0.25% bromophenol blue and 0.25% xylene cyanol FF in 30% aqueous glycerol (Sambrook *et al.* 1989). After gently mixing and pulse centrifugation the samples were loaded into the bottom of wells in the agarose gel. The DNA was run on 0.7% w/v agarose (Pharmacia) dissolved in TAE buffer (Sambrook *et al.* 1989). An aliquot of *Sal*I-digested pUT DNA (see below) was included as a positive control. Ethidium bromide was added to the melted agarose solution immediately prior to pouring into a tray at a rate of 1.5µg per 10 mls agarose solution.

All gels were run at 5.0 $V.cm^{-1}$. TAE was used as the running buffer. Gels were run until the bromophenol blue dye had traversed approximately 80% the length of the gel.

3.2.9(iv) Blotting DNA onto Nylon Membranes

Digested DNA to be probed was run on a gel as previously described. The gel was then washed in 100mls 0.25M HCl for 10 minutes and in 100mls denaturation solution (0.4M NaOH, 0.6M NaCl) for 30 minutes. The gel was then transferred to a neutralising solution (1.5M NaCl, 0.5M Tris, pH 7.5) and washed twice for 15 minutes (100mls per wash).

DNA was blotted from the gel to a nylon membrane by Southern blotting (Southern, 1975) as follows. A plastic tray was half filled with 20x SSC solution (3M NaCl, 0.3M tri-sodium citrate). A flat sponge presoaked in the SSC solution was laid in the tray, then 4 pieces of Whatman filter paper #1 presoaked in SSC was laid on top. A plastic frame was then laid over the top. This frame was used so that only SSC solution absorbed by the sponge and filter paper would reach the gel and membrane. Excess SSC solution in the tray was removed so that the level of the SSC solution was below the plastic frame. The gel was then placed on top of the filter papers and all air bubbles removed. A Hybond N+ nylon membrane (Amersham) was then placed on top of the gel and all air bubbles were removed. Two sheets of presoaked filter paper as described above was then placed on top the membrane and two flat, presoaked sponges placed on top of the filter papers. Three cms. of absorbent tissue was then placed on the sponges and a light weight placed on the tissue. Transfer of DNA from the gel to the membrane was completed overnight. Confirmation that the DNA had transferred to the membrane was undertaken by inspecting the gel under UV light.

3.2.9(v) Fixing and Storage of Membrane

DNA was fixed to the membrane by heating and subsequent alkali treatment as described by the manufacturer (Amersham). The membrane was placed between two sheets of Whatman filter paper #1 and placed in a 60-80^{o}C oven where it was heated for 2 hours.

The corners of the membrane were held down with weights to prevent possible curling. After heating and cooling to room temperature the membrane was placed DNA-side up on 2 pieces of Whatman #1 filter paper predampened with 0.4M NaOH and incubated at room temperature for 20 minutes. The membrane was then rinsed for 30 seconds in 5x SSC solution.

After removal of excess moisture following the SSC wash, the damp nylon membrane was stored by placing between two dry pieces of Whatman #1 filter paper, wrapping in commercial alfoil and sealing in an airtight plastic bag. The membrane was stored at 4°C until required.

3.2.10 Preparation of Probe

3.2.10(i) Extraction of Plasmid DNA

Strains were grown overnight on NA:Tc^{10} at 37°C. A single colony was transferred to 10 mls NB:Tc^{10} and incubated on a shaker for a further 24 hours at 37°C. 1.5 mls of the bacterial suspension was transferred to a microfuge tube and spun at 12,000g in a bench-top microcentrifuge for 4 minutes to pellet the cells. The supernatant was discarded and replaced with 1 ml STE buffer (Appendix I) (Sambrook *et al.* 1989). Cells were resuspended by vortexing and then pelleted as above and the supernatant was discarded. Plasmid pUT containing Tn*5*::*luxAB*Tc^r was then extracted from *E. coli* strain S17-1λ*pir* by the alkaline-lysis method of Sambrook *et al.* (1989). After removal of the chloroform/isoamyl alcohol phase, the plasmid DNA was recovered and washed by the method of Dillon *et al.* (1985).

3.2.10(ii) Digestion of Plasmid DNA

Plasmid DNA was totally digested with *Sal*I (Boehringer Mannheim) by the method described in Section 3.2.9(ii) An aliquot of *Sal*I-digested pUT DNA was reserved for probing along side *P. corrugata* genomic DNA.

3.2.10(iii) Gel Electrophoresis

The 3.2kb *Sal*I fragment from Tn5::*luxAB*Tc^r was separated from all other DNA fragments by gel electrophoresis by the method described in Section 3.11.3.

3.2.10(iv) Purification of the 3.2kb Probe DNA from Tn5::*luxAB*Tc^r

The 3.2 kb *Sal*I fragment from plasmid pUT containing the *lux* genes from the transposon (Figure 3.1) was used as a probe. This fragment was detected under UV light and excised from the gel. The fragment was extracted from the gel and purified by the "Bresa-Clean™ Nucleic Acid Purification" method (Bresatec, Adelaide, Australia). Briefly, the excised agarose gel containing the fragment was weighed and 3 volumes of Bresa-Salt™ (1 gm gel ≡ 1ml) was added and the mixture incubated for 5 minutes to melt the agarose. To the melted mixture 5µl of Bresa-Bind™ silica matrix was added and incubated for 5 minutes at room temperature. The matrix was kept in suspension with occasional gentle flick mixing during this incubation. The bound DNA was pelleted by brief centrifugation and the supernatant was removed. The pellet was washed with Bresa-Wash™, spun and the supernatant removed and the pellet air-dried. The DNA fragment was recovered by adding 20µl TE buffer, spinning and recovering the supernatant. The fragment was stored at 4^{o}C for subsequent labelling and probing.

3.2.11 Southern Hybridisation

The probe was labelled with [α-^{32}P]-dCTP and hybridised with genomic DNA by the method of Feinberg and Vogelstein (1983). Primers and other reagents, unless otherwise stated, were obtained from Boehringer Mannheim.

3.2.11(i) Labelling of Probe DNA

Approximately 60ng of probe DNA was placed into a 1.5ml eppendorf tube. Random labelling primers (3µl) were added, gently mixed and pulse-spun to settle contents. The solution was placed in boiling water for 6 minutes to denature the DNA. After boiling the tube was immediately placed on ice to prevent reannealing. Oligolabelling buffer (12.5µl of 2x solution) was added, the solution mixed and pulse-spun as before. Both the random primers and buffer were prepared as per the protocol of Feinberg and Vogelstein (1983). To this solution 3µl [α-^{32}P]-dCTP (Amersham) and 1.5 units Klenow enzyme (Boehringer Mannheim) was added, mixed carefully and incubated at 37°C for 30 minutes in a water bath.

After incubation the probe solution was passed through a Sephadex G-100 column to separate labelled probe from unlabelled DNA. The column was prepared by stuffing glass wool at the tip end of a pasteur pipette, rinsing with SDDW and then filling with medium grade Sephadex G-100 beads suspended in TE8 buffer. After filling the column the matrix was flushed with 2 volumes TE8. The column was not allowed to dry out at any stage. The labelled probe was then passed through the column by flushing with 1ml TE8 buffer. The labelled probe was detected with a Geiger counter when the counts per minute (cpm) of the effluent exceeded 200. The collection continued as the cps exceed

500 (peaking >1000) and ceased when the cps of the effluent dropped below 250 approx. The collected probe was then placed on ice ready for hybridisation.

3.2.11(ii) Prehybridisation of Membrane

A prehybridisation solution was prepared comprising 2mls sterile double distilled water (SDDW), 4mls 10% w/v dextran sulphate, 3 mls 5x HSB (3M NaCl, 100mM PIPES and 25mM Na_2EDTA, pH 6.8) and 2mls Denhardt's III solution (2% gelatin, 2% ficoll, 2% polyvinyl pyrollidone (PVP), 10% SDS and 5% tetrasodium pyrophosphate). The prehybridisation solution was mixed and warmed to 65°C for a minimum of 15 minutes. A 0.5ml aliquot of salmon sperm DNA solution (50mg in 10mls DDW and then autoclaved) was boiled for 5 minutes, immediately chilled on ice and then added to the prewarmed prehybridisation solution. The solution was then incubated a further 15 minutes at 65°C.

A piece of nylon mesh slightly larger than the membrane was soaked in 2x SSC until saturated. The membrane prepared in Section 3.10.4 was then placed DNA-side-up onto the nylon mesh. The nylon mesh and membrane were then rolled into a tube and placed in a glass cylinder. Approximately 40 mls 2x SSC was added to the cylinder and the mesh and membrane were allowed to unwind so that the mesh was flush with the inner wall of the cylinder and no air bubbles present. The cylinder was drained of all excess fluid and replaced with the warm prehybridisation solution. The membrane was then incubated overnight with gentle agitation at 65°C.

3.2.11(iii) Hybridisation of Membrane

200µl of $5mg.ml^{-1}$ salmon sperm DNA was added to the labelled probe prepared in Section 3.2.11(i) and the probe was boiled for 5 minutes. The tube was then immediately placed on ice and then added to the overnight incubated prehybridised

membrane system described in Section 3.2.11(ii). The probe was incubated with the membrane as before at 65°C overnight.

After overnight incubation the membrane was sequentially washed with gentle shaking in 2x SSC/0.1% SDS; 1x SSC/0.1% SDS and 0.5xSSC/0.1% SDS respectively at 65°C for 20 minutes for each wash. After each stringency wash the membrane was checked for exogenous radioactivity with a Geiger counter. After background radiation was not detected the membrane was prepared for autoradiography.

3.2.11(iv) Autoradiography

The membrane was then exposed to X-ray film (Kodak X-Omat™ Diagnostic Film). A piece of film was placed in a cassette and the membrane was placed DNA-side down onto the film. The cassette was kept at -70°C for 3 days before developing.

The film was developed by soaking in developing solution for up to 4 minutes, rinsing briefly in water and then fixing for a further 4 minutes. Both developing and fixing solutions were prepared as per the manufacturer's (Ilford, Australia) directions. The film was then washed thoroughly in water and then air-dried.

3.2.11(v) Stripping Membranes of Probe

Labelled probes were removed from membranes before reprobing or storage. The membrane was incubated at 45°C with gentle agitation for 30 minutes on 0.4M NaOH solution. The membrane was then transferred to a stripping solution comprising 0.1x SSC, 0.1% SDS and 0.2M Tris-Cl, pH 7.5. The membrane was incubated for 15 minutes at 45°C with gentle agitation. After stripping, the membrane was blot-dried and stored in a sealed plastic bag at 4°C.

3.2.12 Pot Trials

The five mutants listed in Table 3.1 that were found to have reduced *in vitro* antagonism towards *Ggt* were tested in a series of pot trials to determine whether loss of *in vitro* antagonism could be translated to loss of disease suppression on wheat. Pot trials were prepared as described in detail in Section 2.8. Each strain was tested in at least two independent trials. All trials were carried out in accordance with the guidelines specified by the Genetic Manipulation Advisory Committee of Australia (Appendix IV).

3.3 RESULTS

3.3.1 Mutagenesis

Successful mutagenesis of strain 2140 was achieved by the random insertion of the Tn*5*::*luxAB*Tc^r cassette containing the promoterless *luxAB* genes with *E. coli* S17-1λ*pir* as the donor strain (de Lorenzo *et al.* 1990). Conjugation and subsequent incorporation of the transposon in the recipient strain occurred at a frequency of approximately 10^{-6}. Both the donor strain and non-mutated *Pc*2140 cells failed to grow on the selective medium at 25°C.

Attempts to generate mutants of *P. corrugata* 2140 with the other transposon/vector systems (refer Tables 3.1 and 3.2) failed to produce mutants. In most cases no colonies carrying the appropriate resistance marker of the transposon were isolated on suitable media. Successful conjugation and insertion of the transposon occurred when *Pc*2140 was mated with *E. coli* SM10 containing Tn*5*::*Km*^{r} on vector pSUP1011 (Simon *et al.* 1983). Subsequent experimentation with some of these mutants indicated a degree of instability of the transposon; mutant strains either lost kanamycin resistance or the phenotype generated by the insertion was unstable.

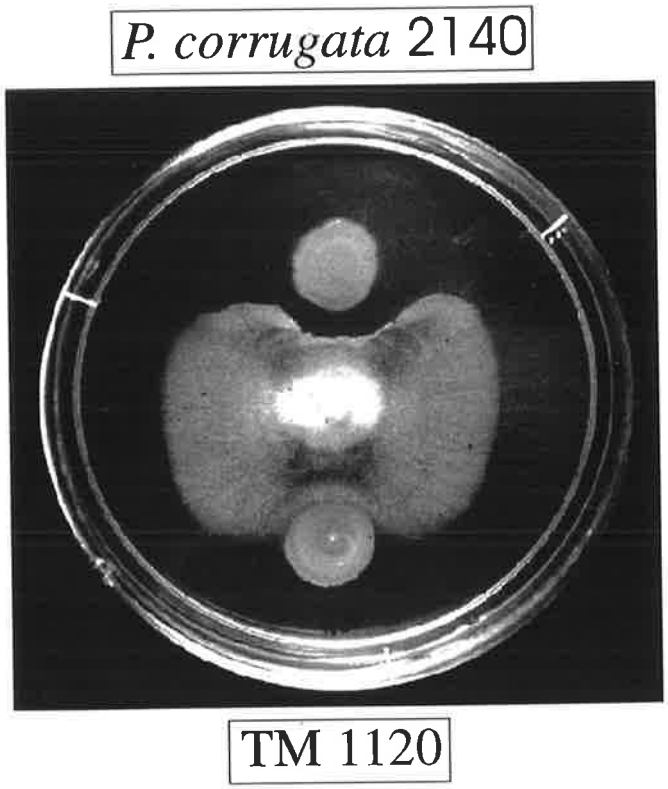


Figure 3.3: *In vitro* antagaonism of *Ggt* by *P. corrugata* 2140 and mutant strain TM1120. An agar plug containing growing *Ggt* hyphae was placed in the centre of a PDA plate and incubated for 3 days at 25°C. Bacteria were added by spotting 20µl of bacterial supension approximately 1 cm from the growing edge. The plates were returned to the 25°C incubator and inspected daily for zones of incubation. Distinct zones of fungal inhibition produced by *Pc*2140 were usually apparent after 2 - 3 days incubation.

3.3.2 Frequency of Auxotrophy of Mutants

Of the 1,000 mutants selected for auxotrophy screening, 7 failed to grow on the minimal salts/glucose medium. All 1,000 mutants tested grew on NA:Tc^{10}. The rate of auxotrophy was 0.7%. One of the auxotrophs (TM986) appeared to have reduced activity against *Ggt* on PDA medium. Because of its auxotrophy this strain was not considered for further testing for disease suppression. The remaining six auxotrophs isolated were all found to antagonise *Ggt in vitro* to a similar level as the parent strain. The seven auxotrophic mutants were not characterised for metabolic deficiencies.

3.3.3 *In vitro* Testing of Mutants for *Ggt* Antagonism

Of the 2,500 transposon-induced mutants isolated, five non-auxotrophic mutants displayed reduced levels of *in vitro* antagonism of *Ggt*. Three strains, designated TM27, TM129 and TM300 showed slightly reduced fungal antagonism whereas two strains, TM692 and TM1120, did not exhibit any antagonism towards the fungus (Table 3.3 and Figure 3.3). All five mutants were subsequently tested for *in situ* suppression of take-all symptoms on wheat in pot trials.

3.3.4 Stability of Transposon in Mutants

Strain TM1120 was the only mutant tested for transposon retention. CFU counts on both agar media were similar for the 7 days indicating that the transposon insertion was stable.

Routine streaking of *Pc*2140 as a negative control indicated that this strain failed to grow on the medium containing tetracycline.

3.3.5 Southern Hybridisation of Selected Mutants for Insertion Frequency in Genome

Digestion of DNA from the *P. corrugata* mutants listed in Table 3.1 with *Pst*I produced a series of cuts within the transposon including one fragment containing most of the element and *Pc*2140 chromosomal DNA downstream from the transposon (Figure 3.2). Hybridisation of the 3.2kb *Sal*I fragment to *P. corrugata* DNA is shown in Figure 3.4. Each band per lane signifies a separate transposon insert in the chromosome. Only one Tn*5* insertion was detected for all of the transconjugants tested. No hybridisation of the probe to genomic DNA from the parent strain was detected.

Mutant strains TM129, TM692 and TM1120 displayed a similar sized fragment containing the Tn*5* DNA and flanking genomic DNA (gDNA). This suggested that these three strains may have been clones of each other. However, variations in colony morphology, visible bioluminescence and *Ggt* antagonism (Table 3.3) and take-all suppression (Section 3.3.7) suggested that although all three strains produced near identically sized hybridising fragments, phenotypic variations indicated that they were separate mutations, each with a unique insertion event into the chromosome.

3.3.6 Testing Mutants for Pleiotropic Changes and Bioluminescence

The five mutants were tested for colony morphology on TZCA medium and bioluminescence. Results are summarised in Table 3.3. Mutants with a similar colony appearance to the parent strain showed reduced antagonism towards *Ggt in vitro* while TM692 and TM1120, which became mucoid on TZCA medium more rapidly than the other mutants and the parent strain, did not display any antagonism. It should be noted that the other *P. corrugata* strains including *Pc*2140 became mucoid to a similar degree to both TM692 and TM1120 after a further 2-3 days incubation.

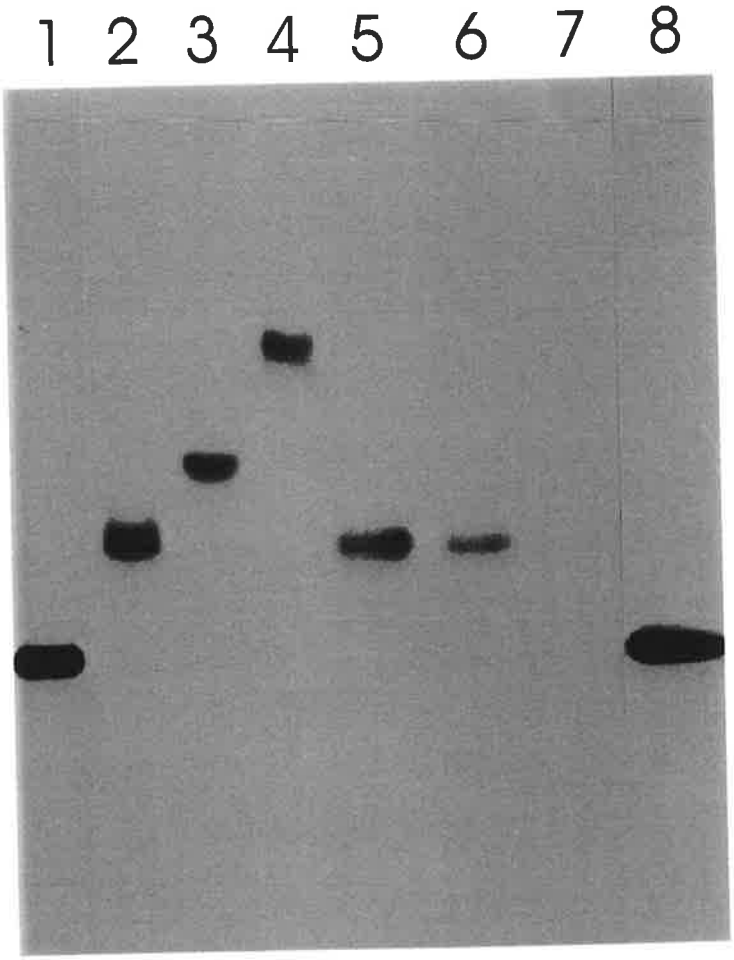


Figure 3.4: Probing genomic DNA of mutants of *P. corrugata* 2140 for single insertions of Tn*5*::*luxAB*Tc^r. Genomic DNA from *P. corrugata* strains was digested with *Pst*I and probed with a labelled 3.2kb *Sal*I fragment derived from the transposon. Each insertion in the genome of the mutants would produce a unique sized fragment when cut with *Pst*I. Lane 1, control (3.2kb probe DNA); 2, TM27; 3, TM129; 4, TM300; 5, TM692; 6, TM1120; 7, *Pc*2140. Results from the Southern hybridisation indicate that each mutant has a single copy of the transposon in its genome. The probe failed to hybridise with *Pc*2140 genomic DNA.

Mutants TM1120 and TM300 were compared with the parent strain for growth in a chemically defined minimal salts/glucose medium. No significant variation in the growth (measured as mean generation time (MGT) during log phase growth) was found between the three strains (P = 0.05). *P. corrugata* 2140 was found to have a MGT of 0.614 generations per hour, strain TM1120 had a MGT of 0.601 and strain TM300 had a MGT of 0.632.

Results obtained indicated that no mutant was compromised in the production of volatiles as there was no significant difference in *Ggt* growth when exposed to the volatiles of each *P. corrugata* strain. Full details of this trial as well as detection and quantification of volatiles are presented in Chapter 5.

Of the five mutants selected for further screening, TM27, TM300 and TM692 showed visible bioluminescence in the dark when exposed to the vapours of n-decyl aldehyde. The bioluminescence was usually detected within 2 minutes of adding the aldehyde to the petri dish. Strains TM129, TM1120 and the parent strain failed to exhibit visible bioluminescence when tested under the same conditions.

The variation in detectable bioluminescence, colony morphology, biocontrol activity (as discussed in Section 3.3.5) and the difference in banding patterns for the Southern hybridisation indicate that the insertion of the transposon in the five mutants were unique events and no mutant was a clone of any other.

3.3.7 Pot Trials: Testing Mutants for Suppression of Take-all Symptoms

The five mutants were tested for reduced biocontrol activity in a total of 6 independent trials. Each mutant was tested at least twice. Because of resource limitations it was impractical to test all the mutants in one pot trial. Therefore each trial comprised the

Strain	*In vitro* antagonism of *Ggt* on PDA medium	Appearance on TZCA medium [y]	Visible Bioluminescence
*Pc*2140	strong [x]	rough	-
TM27	reduced	rough	+
TM129	reduced	rough	-
TM300	reduced	rough	+
TM692	not evident	mucoid	+
TM1120	not evident	mucoid	-

Table 3.3: Basic characteristics of mutants of *P. corrugata* 2140 with reduced biocontrol activity.

x A clear zone of approximately 5mm occurs between strain 2140 and *Ggt* hyphae due to inhibition of fungal growth. This zone was reduced to 1-2mm with strains TM27, TM129 and TM300. When TM692 and TM1120 were tested for *in vitro* antagonism, the fungus was able to grow to the edge of the bacterial colony (see Figure 3.3).

y Colony morphology was examined after 2 days incubation at 25°C. Strains that appeared rough after this time became mucoid after a further 2-3 days incubation at the same temperature.

following bacterial-inoculum treatments: a control (no added bacteria), *Pc*2140 and 3 mutants. Each treatment consisted of 10 pots each of added *Ggt* and no added *Ggt*, making a total of 100 pots per trial. Details of mutants tested in each trial are presented in Appendix V. Data from each trial was analysed as an 'original experiment' by ANOVA and LSD between means (Chapter 2) and the results separated for each mutant for ease of presentation.

Results and specific comments of the pot trials for suppression of take-all symptoms on wheat by mutants of *P. corrugata* 2140 are contained in Figures 3.5 to 3.29. The results for all treatments where *Ggt* inoculum was added and the control treatment with no added *Ggt* inoculum were analysed for variations in the five parameters examined.

3.3.7(i) Root Disease Rating

When *Ggt* inoculum was added to soil sown with pregerminated wheat seeds approximately 20-30% of seminal root length was affected by black vascular take-all lesions after 4 weeks growth. Addition of the biocontrol agent, *P. corrugata* 2140, reduced the total lesion length by 60% ± 10%. This represents a significant reduction in take-all lesions. No lesions were observed on control plants which were not inoculated with either *Ggt* or *Pc*2140.

In all trials, wheat roots of plants inoculated with the mutant strains had a significantly greater amount of *Ggt*-induced lesion damage on the seminal roots compared to the parent strain. Of the five mutant strains tested TM1120 showed a similar incidence of root lesions as the disease-only control treatment; in two tests TM1120-treated plants were not significantly different from the controls and in the third test a small but significant biocontrol effect was observed ($P < 0.05$). In one of the tests (test #1) lesion lengths were slightly greater on the TM1120-inoculated plants compared to the *Ggt* controls.

Of the four other mutants tested, the extent of lesions on roots on plants inoculated with TM129 were not significantly different from the *Ggt*-treated control plants although the actual lesion length was less in all 3 trials. Both TM27 and TM300 displayed significantly reduced biocontrol activity; one trial showing no significant difference between the mutant-inoculated plants and the *Ggt* control plants and the other trial showing biocontrol activity approximately halfway between *Pc*2140 and the control treatments. In the second trial for each of these two mutants, lesions on wheat roots were significantly different from both the *Pc*2140-inoculated plants and the control plants ($P < 0.05$).

Mutant TM692 displayed the most variation in biocontrol activity of the five mutants. In the first trial there was a small but significant reduction in biocontrol activity ($P < 0.05$) compared to the parent strain but lesion damage was significantly less than the control plants. In the second trial there was significant difference between the *Pc*2140 and TM692-inoculated treatments but no significant difference between the TM692-inoculated plants and the control plants. In the third trial there were three statistically significant groups; plants inoculated with TM692 displaying total lesion lengths approximately halfway between *Pc*2140 and control treatments.

3.3.7(ii) Water Consumption by Wheat Plants

In nearly all trials conducted, plants inoculated with *P. corrugata* 2140 and *Ggt* utilised more water during the last 14 days of each trial than control plants where *Ggt* only was added. In one trial control plants used slightly more water than the *Pc*2140-inoculated plants but usage was not significantly greater (see Figure 3.6, test 2 as an example). In all other trials the uptake of water by *Pc*2140-inoculated plants was significantly greater than uptake by *Ggt* control plants ($P < 0.05$).

Water usage by plants inoculated with mutant strains was generally midway between *Pc*2140-inoculated plants and the controls. In no cases was water usage significantly greater than *Pc*2140-treated plants or significantly less than the controls for all treatments containing *Ggt*. Overall the uptake of water by plants treated with mutant strains was not significantly different from either *Pc*2140-treated plants or the controls. The measurement of water consumption as an indicator of take-all suppression or disease will be addressed in the discussion.

3.3.7(iii) Shoot Lengths

Shoot lengths of plants inoculated with *Pc*2140 were generally greater than control plants. Only in one trial (see Figure 3.12, test 3 as an example) was the average shoot length of control plants greater than *Pc*2140-treated plants although there was no significant statistical difference between the two treatments. For the rest of the trials the difference in shoot length between *Pc*2140-inoculated plants and control plants was either not significant or marginally significant at $P = 0.05$.

Shoot lengths of plants inoculated with the mutant strains showed a similar trend as water consumption, that is, no discernible differences were observed between the treatments with mutants or with *Pc*2140 and control treatments. In most trials shoot lengths of mutant-inoculated plants were between the two other treatments but not significantly different from either treatment (see shoot lengths of TM1120-treated plants, Figure 3.27 as an example). Where shoot lengths of control plants were greater than plants treated with *Pc*2140, shoot lengths of mutant-inoculated plants were not significantly different from either other treatment.

3.3.7(iv) Shoot Dry Weights

In all trials the shoot dry weights of control plants were less than plants treated with *P. corrugata* 2140. Significant differences ($P < 0.05$) were only observed for approximately half the trials conducted, in other trials there was no statistical difference between the two treatments.

Shoot dry weights of plants treated with the mutant strains were not statistically different from either the *Pc*2140-inoculated plants or the controls. In one trial shoot dry weights of plants treated withTM300 and TM692 were significantly greater than the other two treatments (Figure 3.18, test 2 and Figure 3.23, test 3). This was not observed in the other tests for these strains although the results of the second test for TM692 showed a similar trend without being significantly different. Shoot dry weights of wheat plants inoculated with the other three mutants were generally not significantly different from *Pc*2140 and control treatments.

3.3.7(v) Root Dry Weights

Root dry weights of control plants were generally less than plants inoculated with *P. corrugata* 2140. In one trial the control plants displayed root dry weights significantly greater than *Pc*2140-inoculated plants (see Figure 3.14, test 1 as an example). In all other trials however, root dry weights of control plants were lower, approximately half of the remaining trials showing significant differences ($P = 0.05$).

The overall results for root dry weights for plants inoculated with the mutant strains showed similar results as both shoot lengths and shoot dry weights, that is, no discernible pattern was observed. Root dry weights were often similar to either or both of *Pc*2140-treated plants and the controls. No trials with any mutant strain displayed any trend in

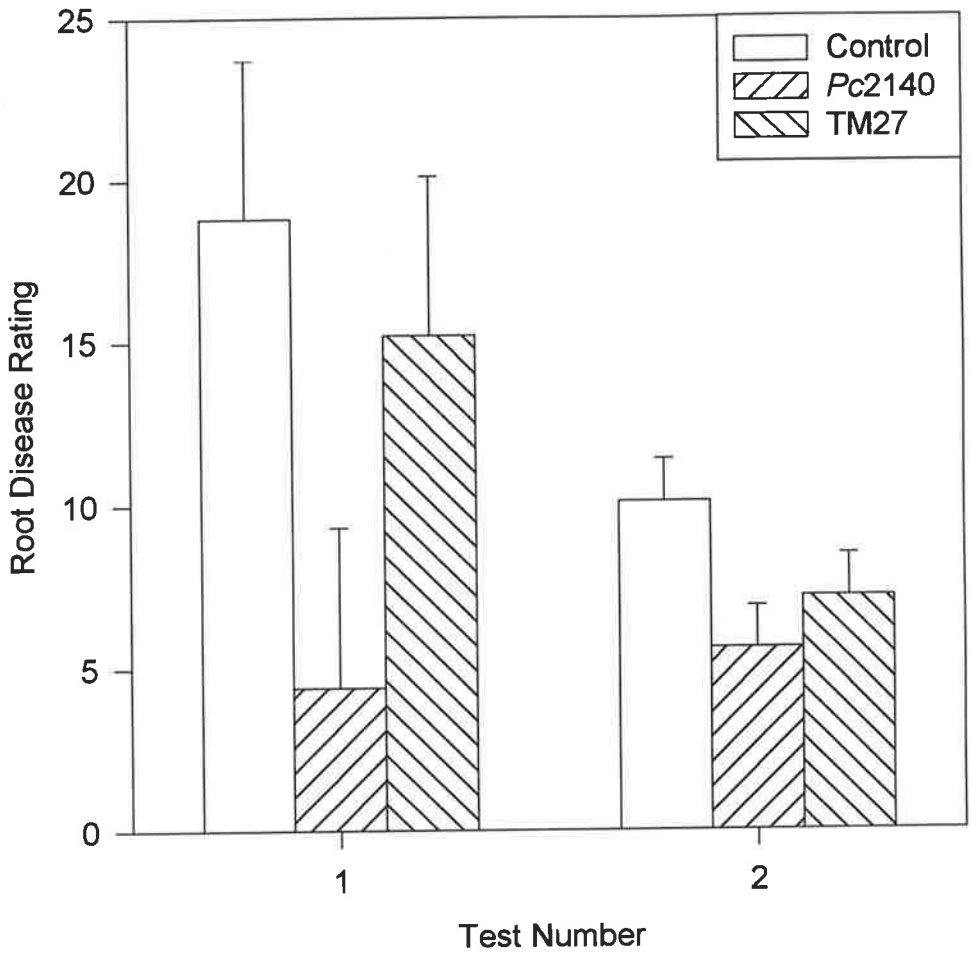


Figure 3.5: Comparison of root disease rating between *P. corrugata* 2140 and mutant strain TM27 for treatments with added *Ggt* inoculum. Severity of root disease was measured as the percentage seminal root length affected by black vascular take-all lesions (refer text). In both trials lesion length on seminal roots of wheat plants was significantly greater on roots inoculated with TM27 compared to *Pc*2140 ($P < 0.05$). In the first trial the lesion length on TM27-inoculated roots was less than but not significantly different to the control treatment at $P = 0.05$. In the second trial root lesions were signicantly less on TM27-inoculated roots compared to the control plants. Bars represent LSD between means ($P = 0.05$).

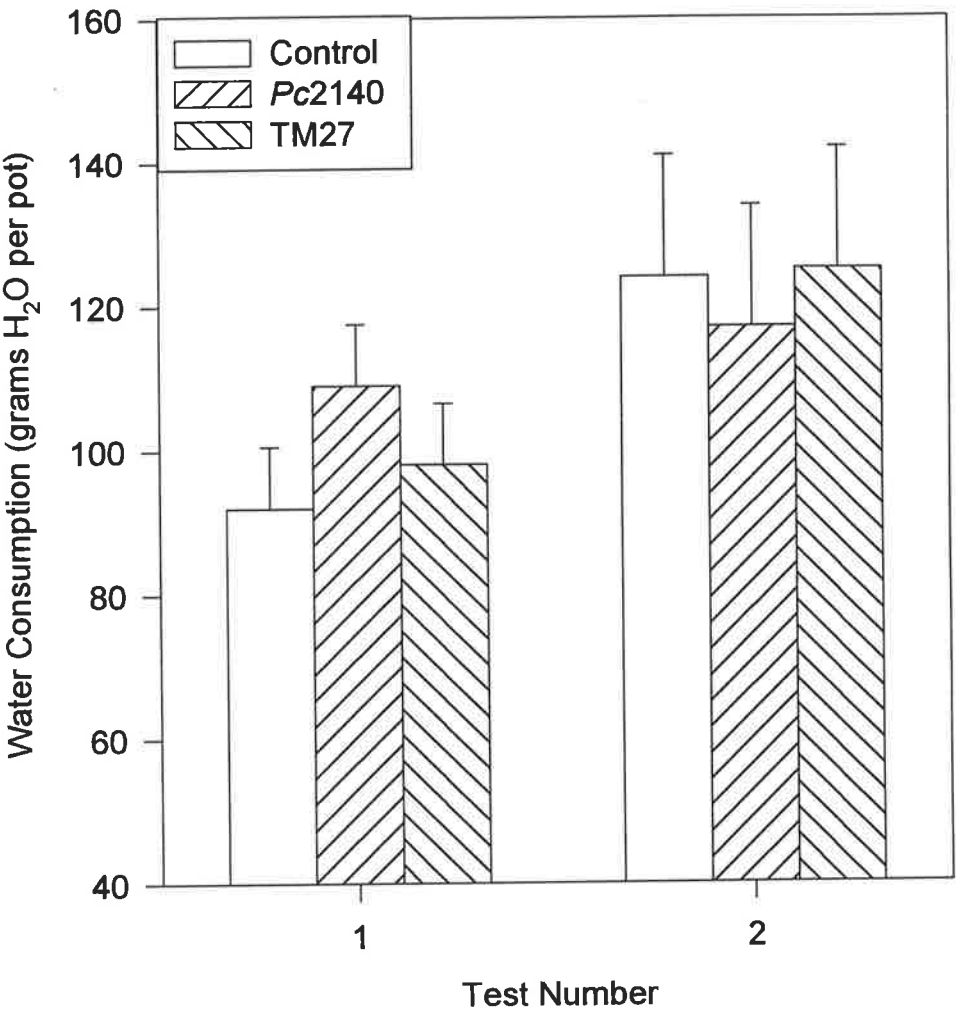
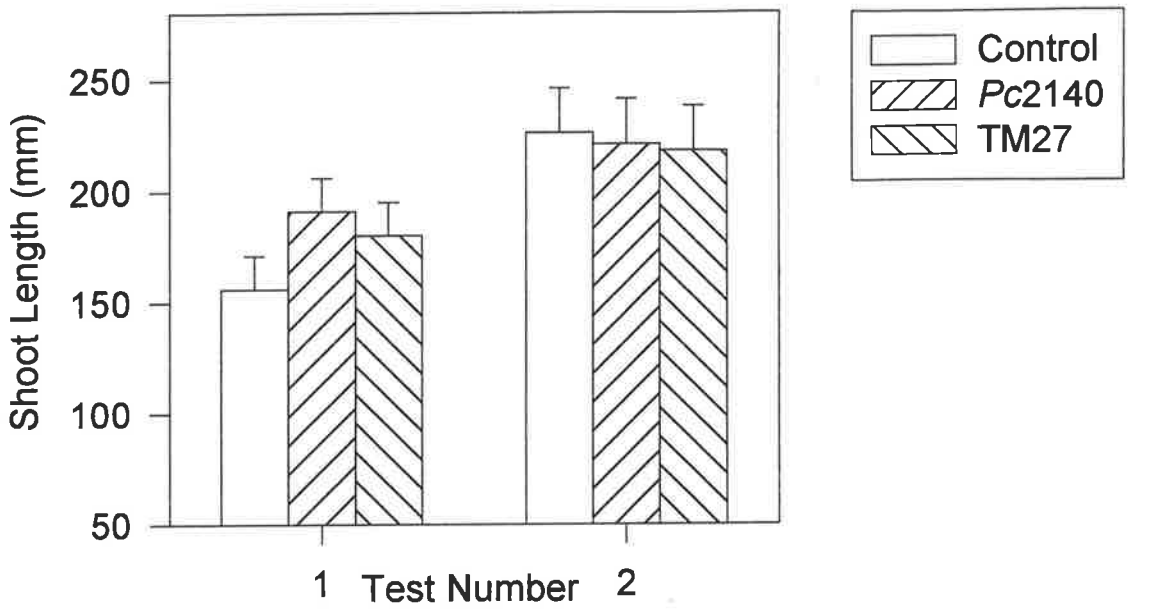


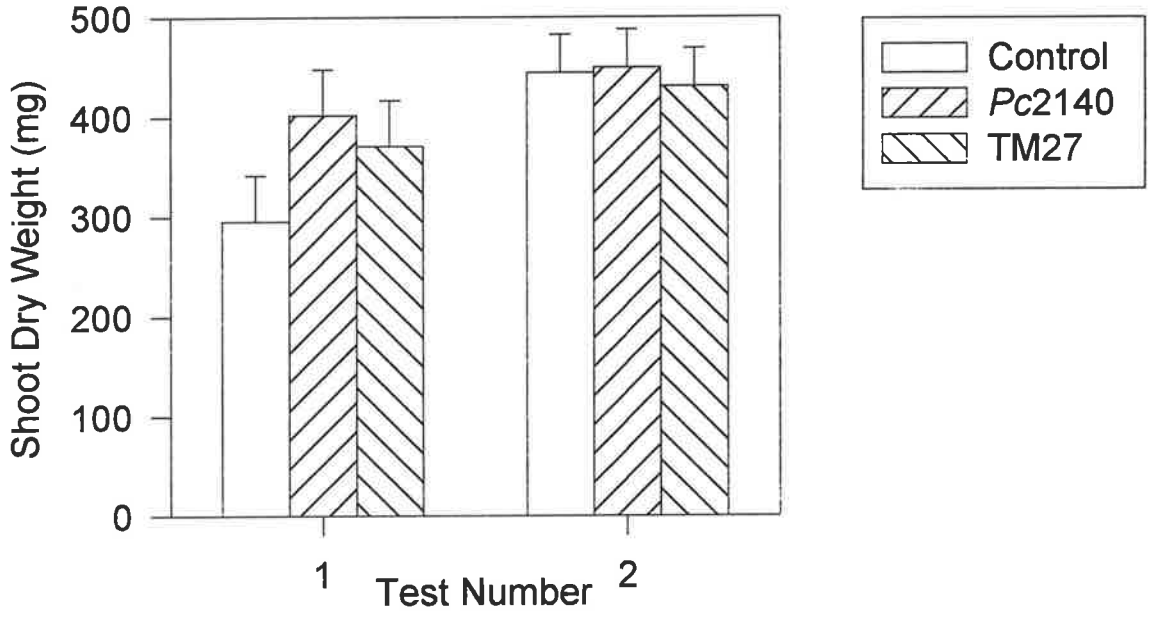
Figure 3.6: Comparison of water consumption by wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM27. Water consumption was measured over the last two weeks of the four week trials (refer text for details). In trial 1 plants inoculated with *Pc*2140 consumed significantly more water than plants inoculated with TM27 and control plants ($P < 0.05$). Plants inoculated with TM27 consumed more water than the control plants but consumption was not significantly different at $P = 0.05$. In the second trial there was no significant difference in water consumption between all three treatments. Bars represent LSD between means ($P = 0.05$).

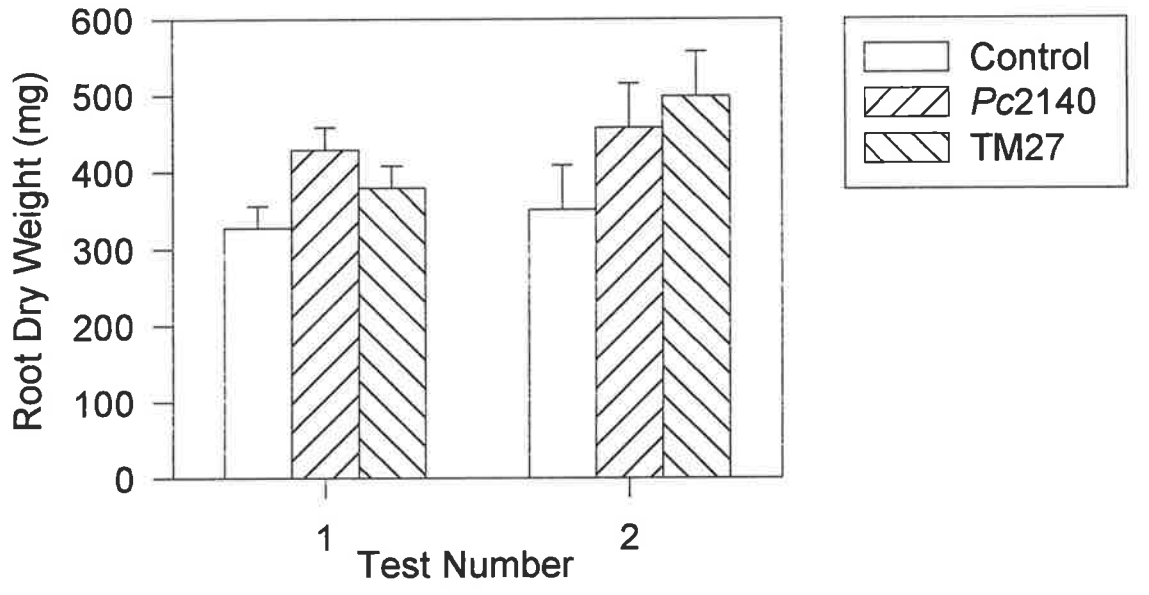
Figure 3.7: Comparison of shoot lengths of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM27 for treatments with added *Ggt* inoculum. In the first trial shoot lengths on both *Pc*2140 and TM27-inoculated plants were significantly greater than control plants ($P < 0.05$). There was no significant difference between plants inoculated with either bacteria. In the second trial there was no significant difference in shoot lengths between all three treatments. Bars represent LSD between means ($P = 0.05$).

Figure 3.8: Comparison of shoot dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM27 for treatments with added *Ggt* inoculum. In the first trial shoot dry weights for both *Pc*2140 and TM27-inoculated plants were significantly greater than control plants ($P < 0.05$). There was no significant difference between plants inoculated with either bacteria. In the second trial there was no significant difference in shoot dry weights between all three treatments. Bars represent LSD between means ($P = 0.05$).

Figure 3.9: Comparison of root dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM27. In the first trial there was a significant difference between all three treatments; root weights of plants inoculated with *Pc*2140 were significantly greater than TM27-inoculated plants ($P < 0.05$). Both bacteria-inoculated treatments were significantly greater than the control plants. In the second trial there was no significant difference between both bacteria-inoculated treatments. These two treatments were significantly greater than the control treatment. In the second trial root dry weights of TM27-inoculated plants was greater than *Pc*2140-inoculated plants. Bars represent LSD between means ($P = 0.05$).


Shoot Length (mm)
250
200
150
100
50
1
Test Number
2
Control
Pc2140
TM27


Shoot Dry Weight (mg)
500
400
300
200
100
0
1
Test Number
2
Control
Pc2140
TM27


Root Dry Weight (mg)
600
500
400
300
200
100
0
1
Test Number
2
Control
Pc2140
TM27

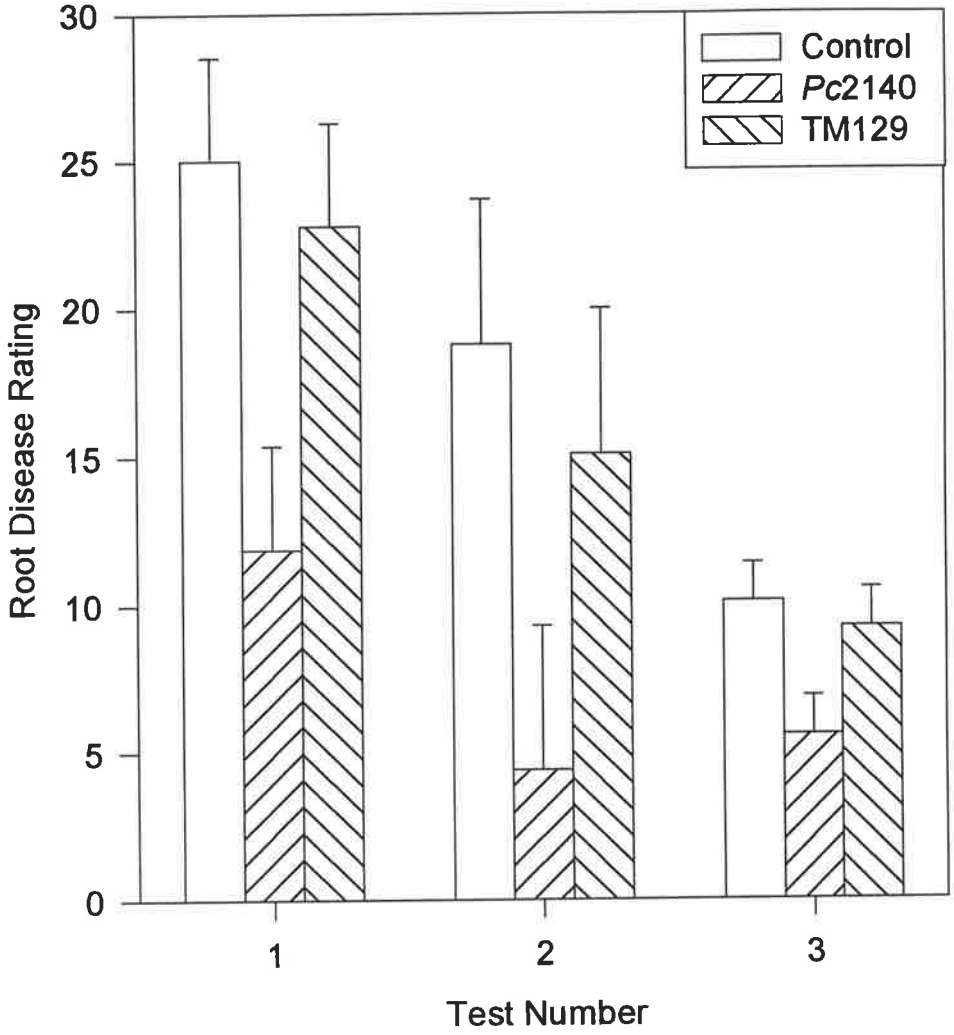


Figure 3.10: Comparison of root disease rating between *P. corrugata* 2140 and mutant strain TM129 with added *Ggt* inoculum. Severity of root disease was measured as the percentage seminal root length affected by black vascular lesions (refer text). In all three trials roots inoculated with *Pc*2140 had significantly less lesion damage than control plants and plants inoculated with TM129 ($P < 0.05$). There was no significant difference between control plants and plants inoculated with the mutant strain. In all cases however, the lesion length of TM129-inoculated plants was less than the controls. Error bars represent LSD between means ($P = 0.05$).

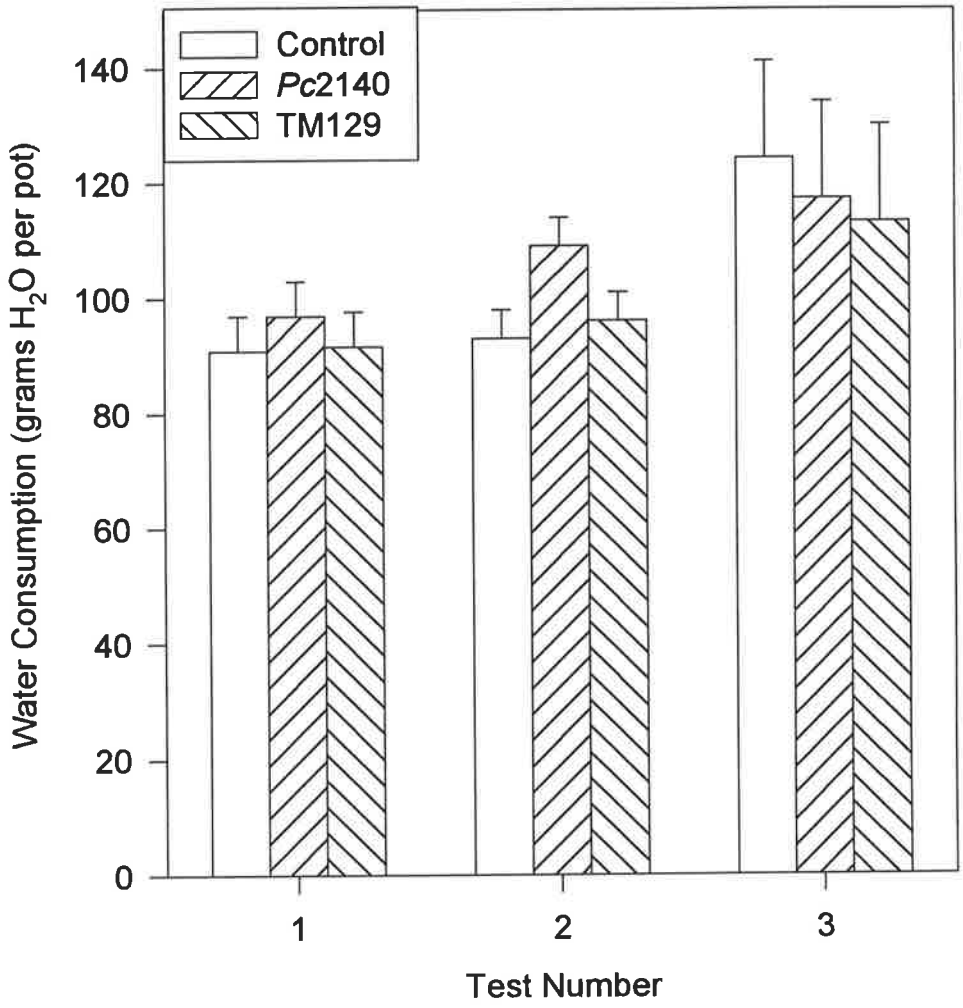
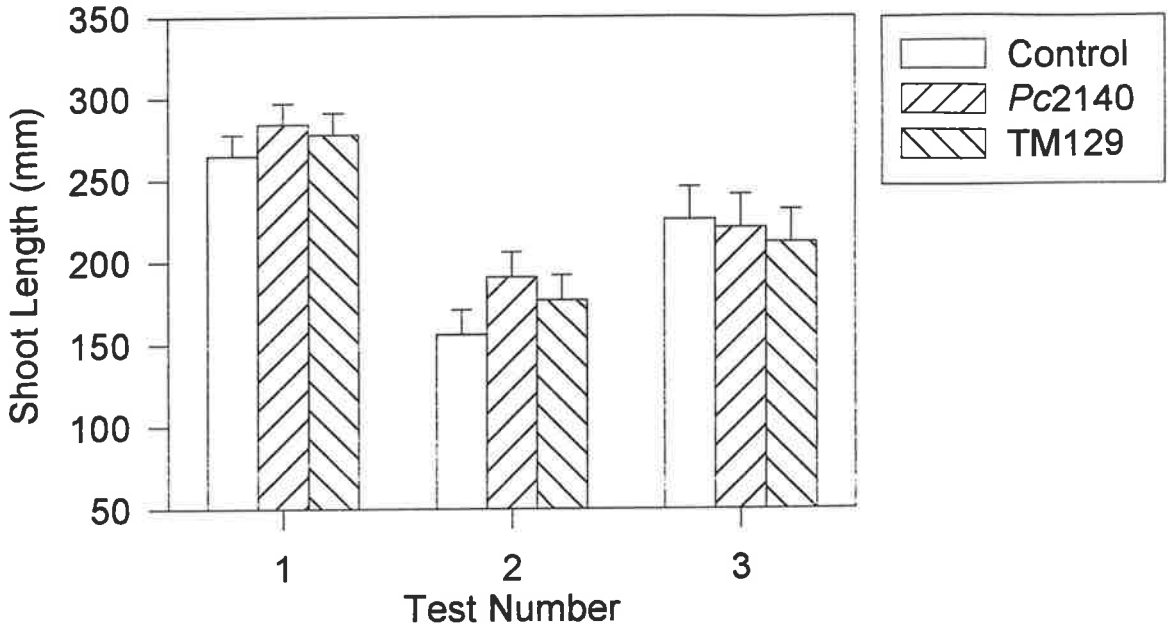


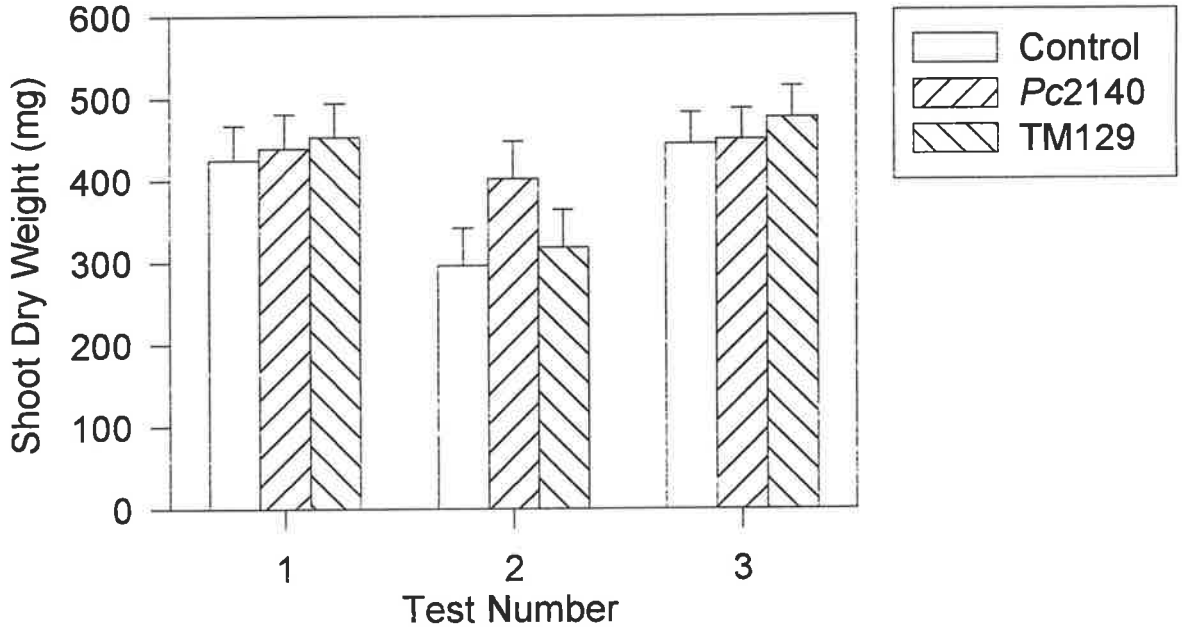
Figure 3.11: Comparison of water consumption by wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM129 for treatments with added *Ggt* inoculum. Water consumption was measured over the last two weeks of the four week trials (refer text for details). There was no significant difference in water consumption by wheat plants for all three treatments in trials 1 and 3. In the second trial water consumption by *Pc*2140-inoculated plants was significantly greater than the other two treatments. There was no significant difference in water consumption between TM129-inoculated plants and control plants in the second trial. Bars represent LSD between means (P = 0.05).

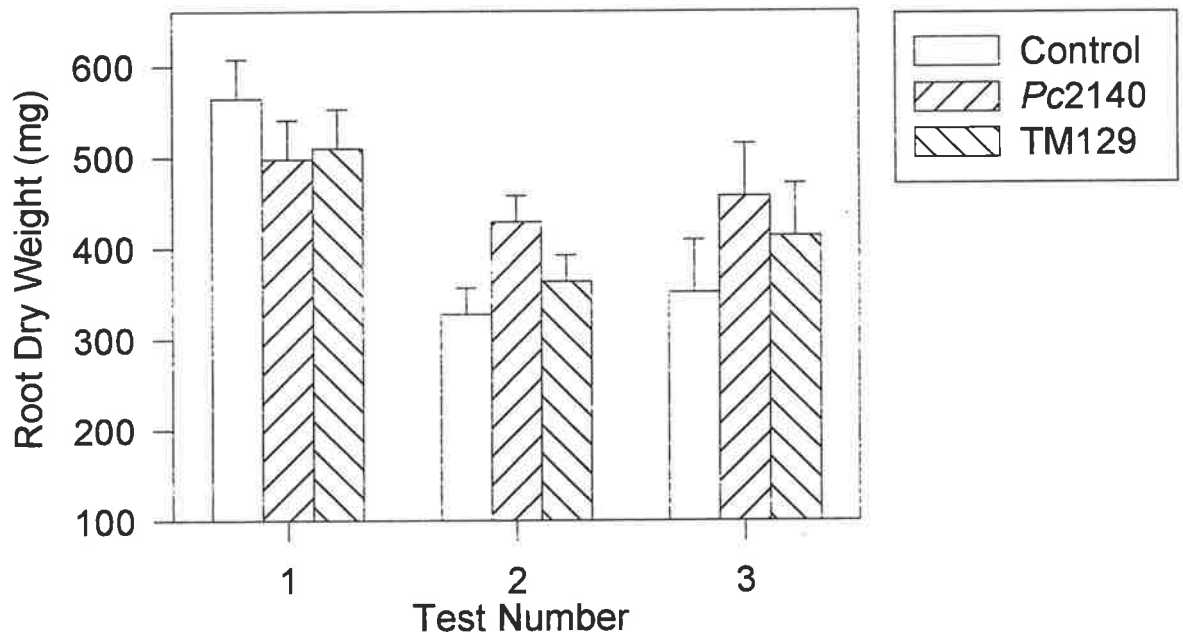
Figure 3.12: Comparison of shoot lengths of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM129 for treatments with added *Ggt* inoculum. In all three trials there was no significant difference in shoot lengths of plants inoculated with either *Pc*2140 or mutant strain TM129. Shoot lengths of control plants were significantly less than bacteria-inoculated plants in the first two trials ($P < 0.05$). In the third trial there was no significant difference in shoot lengths for all three treatments. Bars represent LSD between means ($P = 0.05$).

Figure 3.13: Comparison of shoot dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM129 for treatments with added *Ggt* inoculum. In trials 1 and 3 there were no significant differences in shoot dry weights between all three treatments. In the second trial shoot dry weights of plants inoculated with *Pc*2140 were signicantly greater than the other two treatments ($P < 0.05$). There was no significant difference between control plants and the TM129-inoculated plants in the second trial.Bars represent LSD between means ($P = 0.05$).

Figure 3.14: Comparison of root dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM129 for treatments with added *Ggt* inoculum. In the first trial there was significant difference between the control treatment and the bacteria-inoculated plants; the root dry weights of control plants was significantly greater than the other two treatments. In trials 2 and 3 the root dry weights of bacteria-inoculated plants were significantly greater than control plants ($P < 0.05$). Only in trial 2 were weights for *Pc*2140-inoculated plants significantly greater than plants inoculated with TM129.Bars represent LSD between means ($P = 0.05$).


Shoot Length (mm)
350
300
250
200
150
100
50
1
2
3
Test Number
Control
Pc2140
TM129


Shoot Dry Weight (mg)
600
500
400
300
200
100
0
1
2
3
Test Number
Control
Pc2140
TM129


Root Dry Weight (mg)
600
500
400
300
200
100
1
2
3
Test Number
Control
Pc2140
TM129

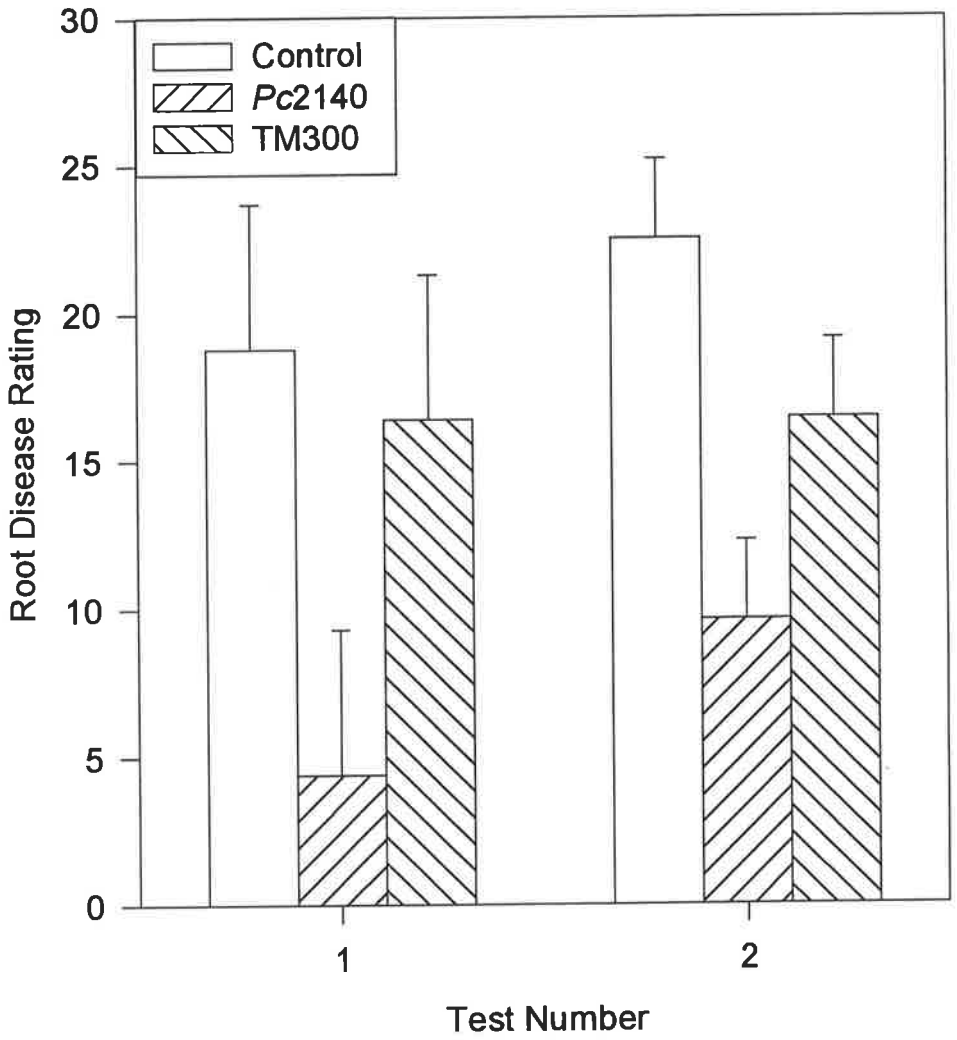


Figure 3.15: Comparison of root disease rating between *P. corrugata* 2140 and mutant strain TM300 with added *Ggt* inoculum for treatments with added *Ggt* inoculum. Severity of root disease was measured as the percentage seminal root length affected by black vascular lesions (refer text). In both trials roots inoculated with *Pc*2140 had significantly less lesion damage than control plants and plants inoculated with TM300 ($P < 0.05$). Lesion damage on roots inoculated with TM300 were less than control plants in both trials but only significantly less at $P = 0.05$ for the second trial. Bars represent LSD between means ($P = 0.05$).

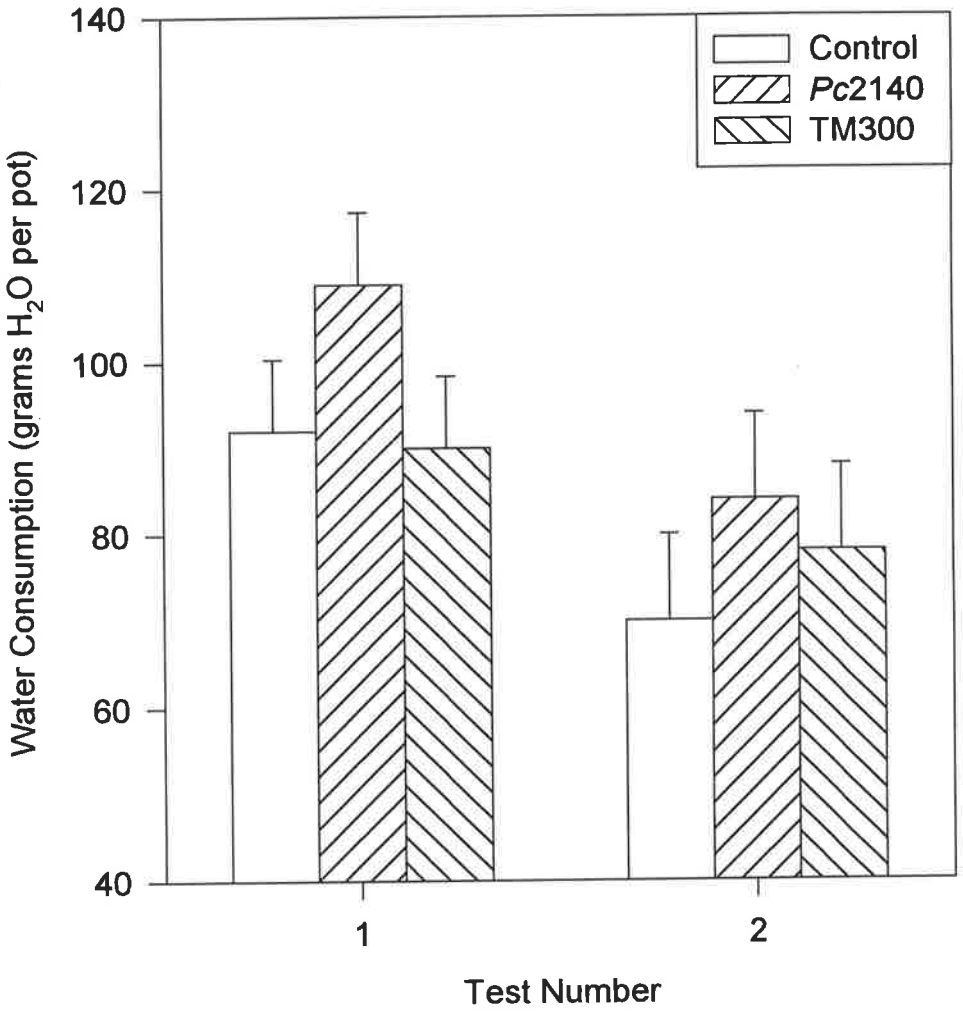
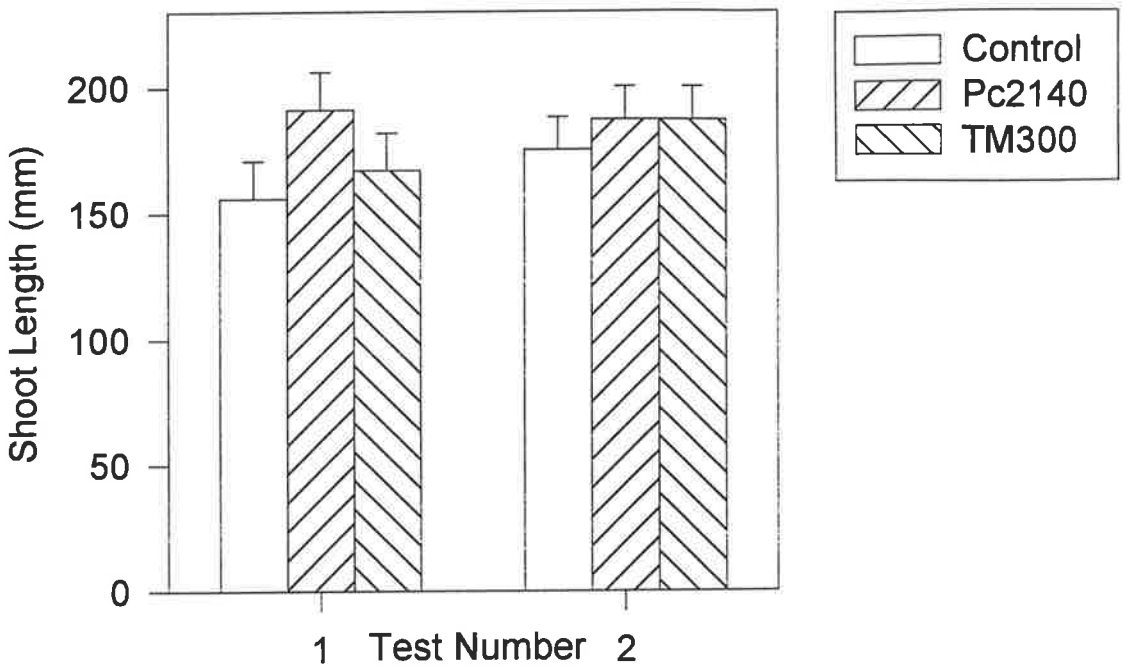


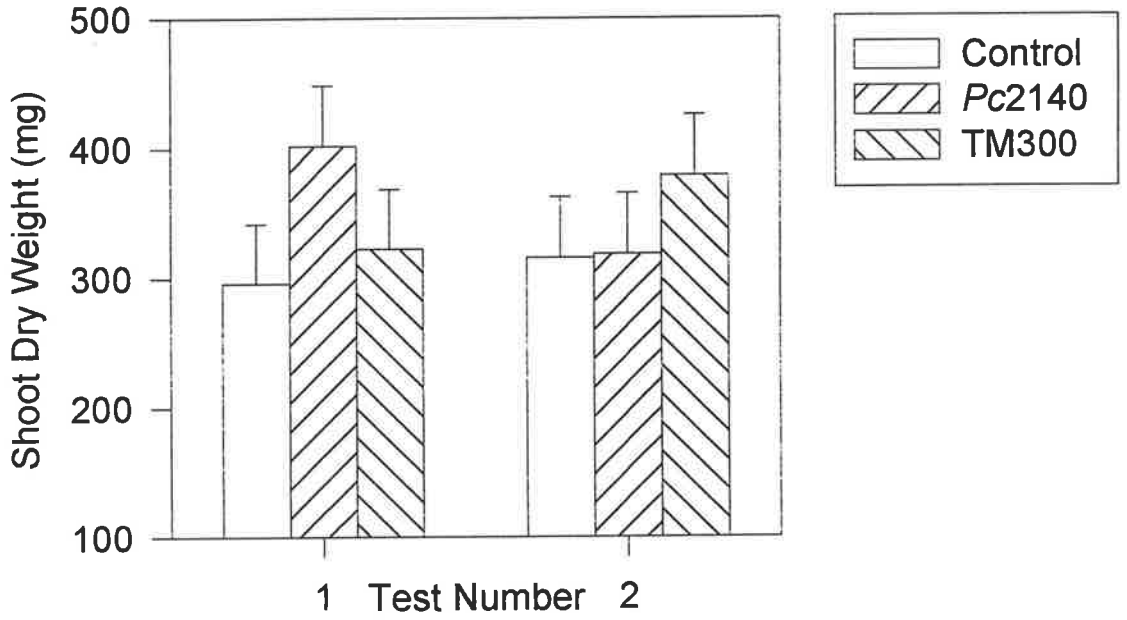
Figure 3.16: Comparison of water consumption by wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM300 for treatments with added *Ggt* inoculum. Water consumption was measured over the last two weeks of the four week trials (refer text for details). In the first trial water consumption by plants inoculated with *Pc*2140 was significantly greater than the other two treatments ($P < 0.05$). There was no significant difference in water consumption by wheat plants for both control plants and TM300-inoculated plants. In the second trial water consumption by plants inoculated with *Pc*2140 was significantly greater than control plants only. Plants inoculated with TM300 consumed water at a rate between the other two treatments but was not significantly different to either of these two treatments.Bars represent LSD between means ($P = 0.05$).

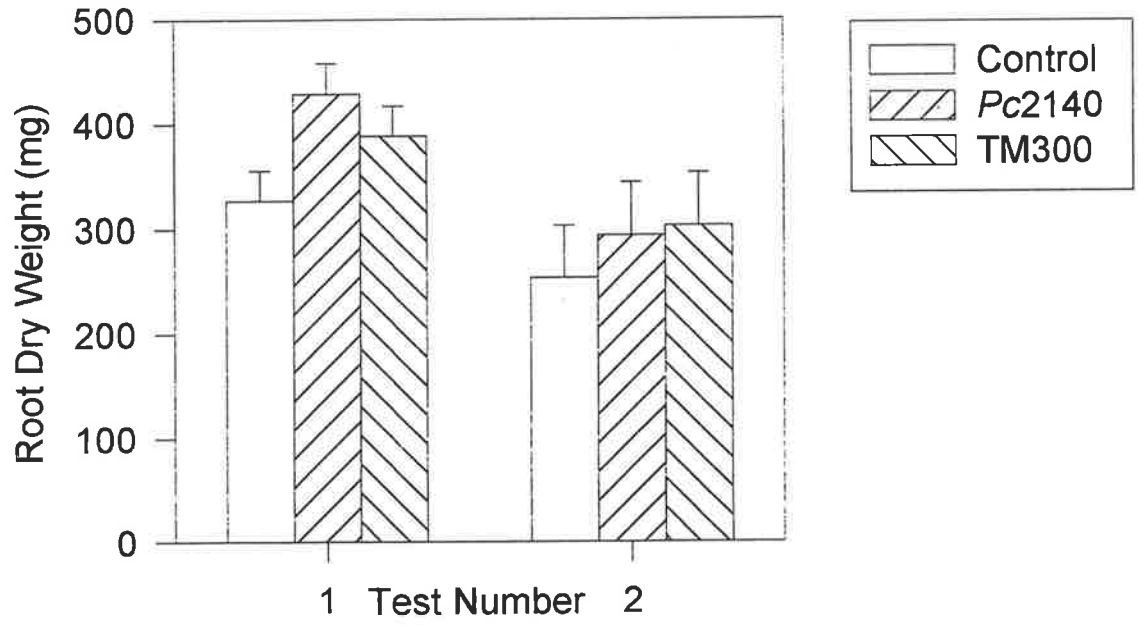
Figure 3.17: Comparison of shoot lengths of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM300 for treatments with added *Ggt* inoculum. In the first trial, the shoot length on *Pc*2140 was significantly greater than the other two treatments ($P < 0.05$). The shoot length of TM300-inoculated plants was greater than control plants but not signicantly different at $P = 0.05$. In the second trial there was no significant difference in shoot lengths between all three treatments.Bars represent LSD between means ($P = 0.05$).

Figure 3.18: Comparison of shoot dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM300 for treatments with added *Ggt* inoculum. In the first trial shoot dry weights on *Pc*2140 were significantly greater than the other two treatments ($P < 0.05$). Shoot dry weights of TM300-inoculated plants were greater than control plants but not signicantly different at $P = 0.05$. In the second trial there was no significant difference between controls and *Pc*2140-inoculated plants. Plants inoculated with TM300 showed significantly greater shoot dry weight than the other two treatments for this trial ($P < 0.05$).Bars represent LSD between means ($P = 0.05$).

Figure 3.19: Comparison of root dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM300 for treatments with added *Ggt* inoculum. In the first trial there was a significant difference between all three treatments; root weights of plants inoculated with *Pc*2140 were significantly greater than TM300-inoculated plants ($P < 0.05$). Both bacteria-inoculated treatments were significantly greater than the control plants. In the second trial there was no significant difference between all three treatments for root dry weights. Error bars represent LSD between means ($P = 0.05$).


Shoot Length (mm)
200
150
100
50
0
1 Test Number 2
Control
Pc2140
TM300


Shoot Dry Weight (mg)
500
400
300
200
100
1 Test Number 2
Control
Pc2140
TM300


Root Dry Weight (mg)
500
400
300
200
100
0
1 Test Number 2
Control
Pc2140
TM300

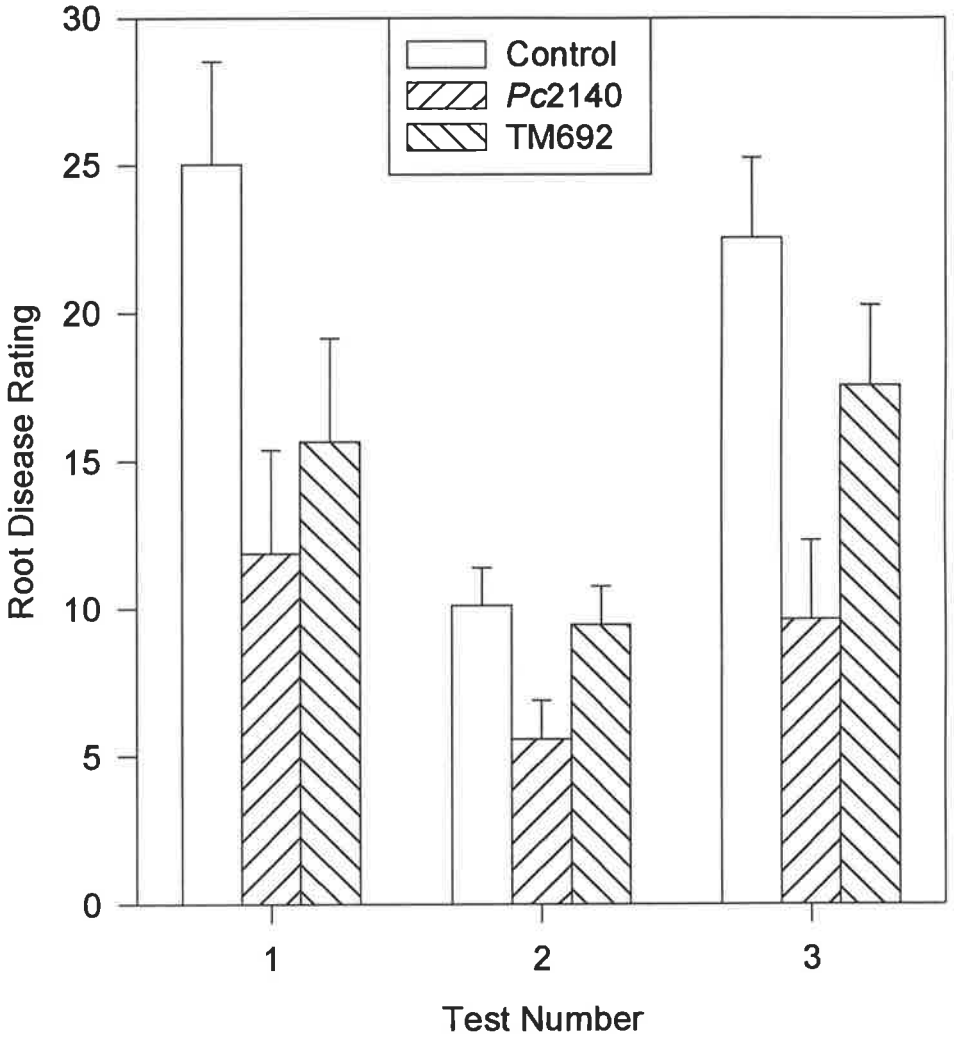


Figure 3.20: Comparison of root disease rating between *P. corrugata* 2140 and mutant strain TM692 with added *Ggt* inoculum. Severity of root disease was measured as the percentage seminal root length affected by black vascular lesions (refer text). In all three trials lesion damage on roots inoculated with *Pc*2140 was significantly less than control plants and plants inoculated with TM692 ($P < 0.05$). In two trials (1 and 3) lesion damage was signifcantly less on TM692-inoculated plants than on control plants. In trial number 2 there was no significant difference between these two treatments although lesion damage was slightly less on the roots inoculated with the mutant strain. Error bars represent LSD between means ($P = 0.05$).

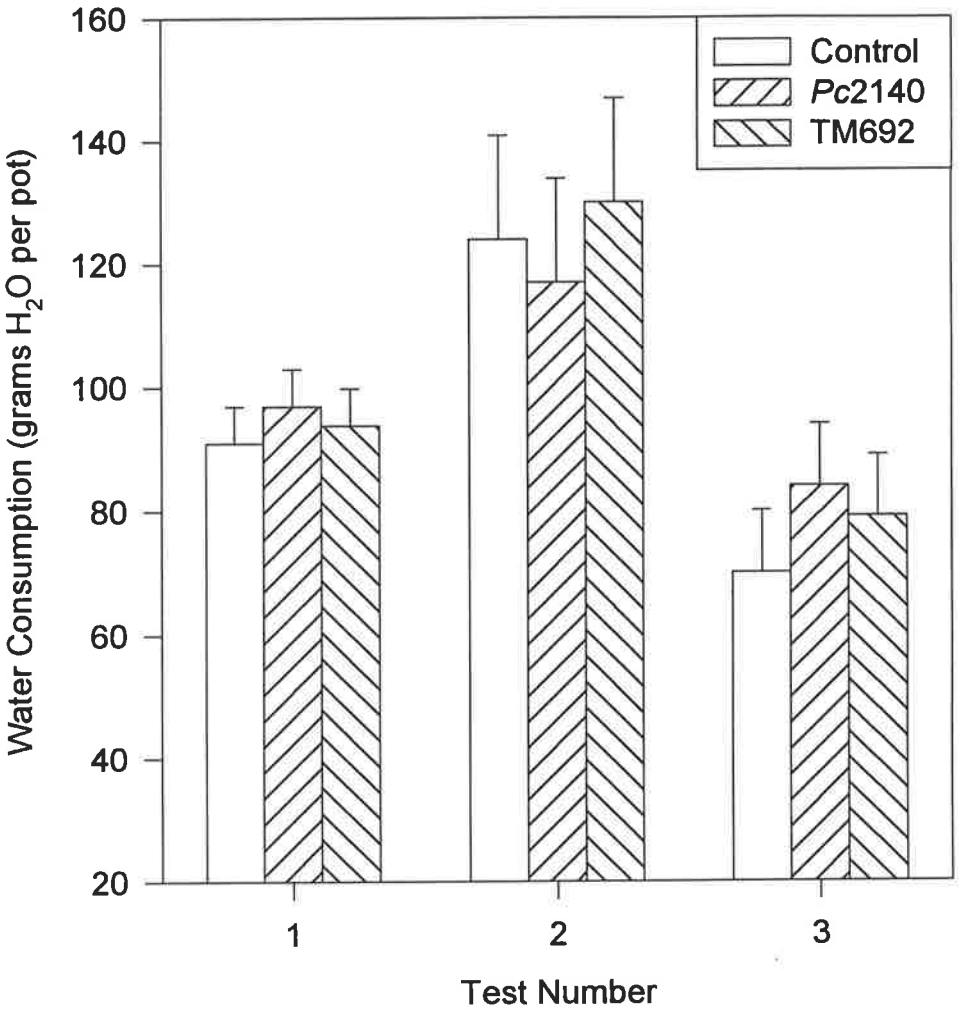
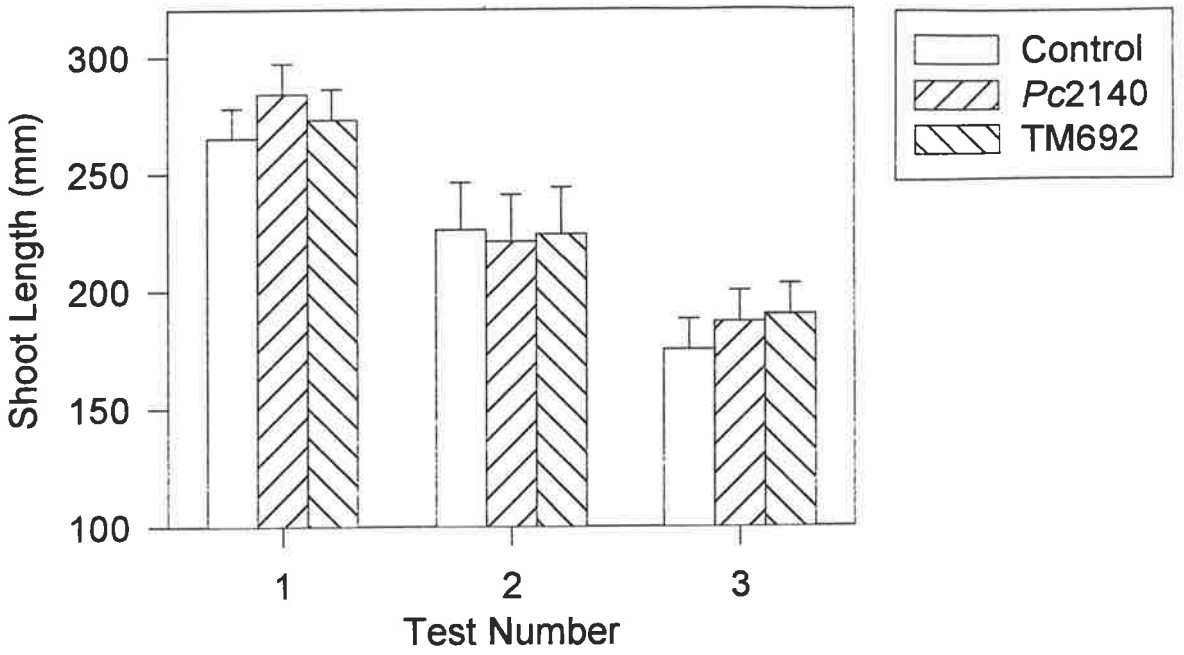


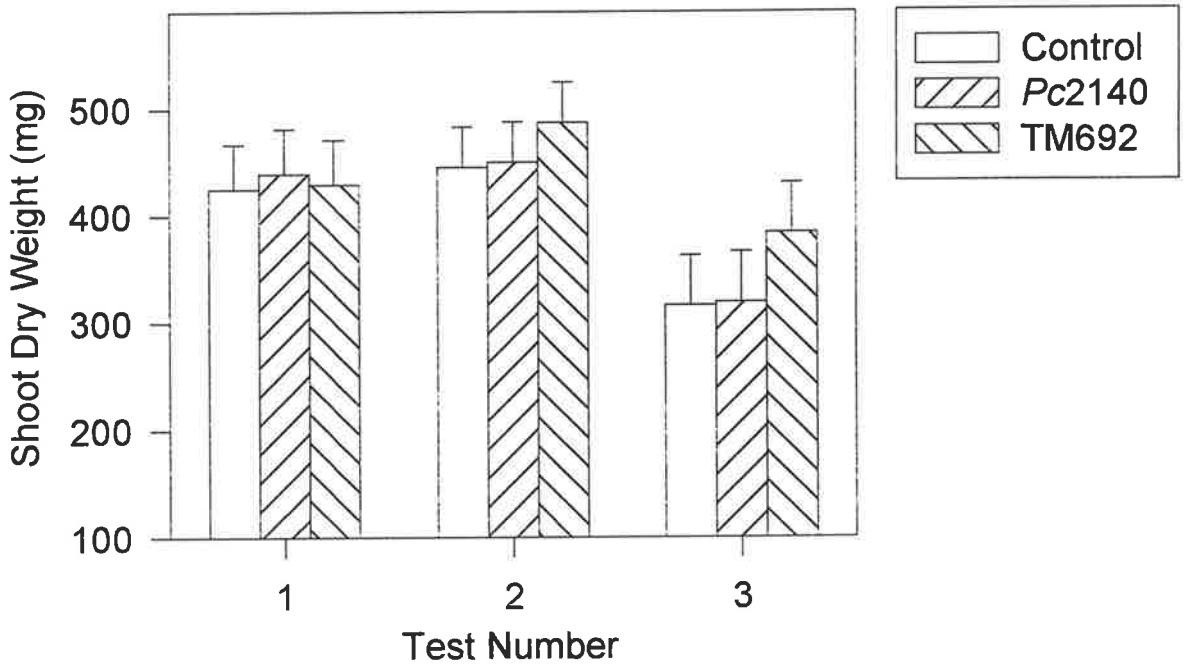
Figure 3.21: Comparison of water consumption by wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM692 for treatments with added *Ggt* inoculum. Water consumption was measured over the last two weeks of the four week trials (refer text for details). In the first two trials there was no significant difference in water consumption between each treatment. In the third trial, plants inoculated with *Pc*2140 consumed significantly more water than control plants ($P < 0.05$). Plants inoculated with TM692 consumed less water than the *Pc*2140-inoculated plants but more water than the control plants although the consumption rate was not significantly different at $P = 0.05$ than either of the other two treatments. Error bars represent LSD between means (P=0.05).

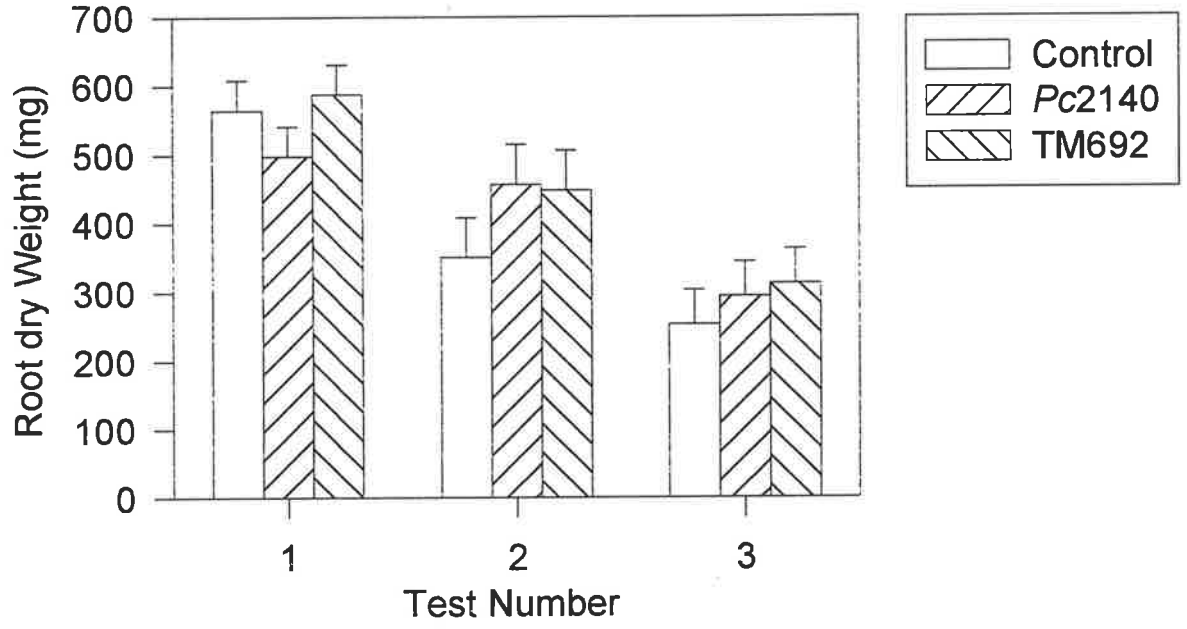
Figure 3.22: Comparison of shoot lengths of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM692 for treatments with added *Ggt* inoculum. In the first trial shoot lengths of plants inoculated with *Pc*2140 were significantly greater than control plants ($P < 0.05$). Shoot lengths of plants inoculated with TM692 were less the *Pc*2140-inoculated plants and greater than the control plants but the lengths were not significantly different at $P = 0.05$ than either of the other two treatments. In the second trial there was no significant difference in shoot length for all three treatments. In the third trial shoot lengths of TM692-inoculated plants were significantly greater than the control plants but not significantly different to *Pc*2140-inoculated plants. There was no significant difference in shoot lengths of *Pc*2140-inoculated plants and control plants. Error bars represent LSD between means ($P = 0.05$).

Figure 3.23: Comparison of shoot dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM692 for treatments with added *Ggt* inoculum. In the first trial there was no significant difference in shoot dry weights between all three treatments. In the second trial shoot dry weights of TM692-inoculated plants were significantly greater than the control plants but not significantly different to *Pc*2140-inoculated plants. In the third trial shoot dry weights of plants inoculated with TM692 were significantly greater than the other two treatments. There was no significant difference in shoot lengths of *Pc*2140-inoculated plants and control plants in trials 2 and 3. Error bars represent LSD between means ($P = 0.05$).

Figure 3.24: Comparison of root dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM692 for treatments with added *Ggt* inoculum. In the first trial there was a significant difference between *Pc*2140-inoculated plants and the other two treatments; root dry weights of *Pc*2140-inoculated plants were significantly less ($P < 0.05$) than the other two treatments. In the second trial there was no significant difference between both bacteria-inoculated treatments but both were significantly greater than control plants. In the third trial root weights of TM692-inoculated plants were significantly greater than the control plants but not significantly different to *Pc*2140-inoculated plants. There was no significant difference in root weights of *Pc*2140-inoculated plants and control plants. Error bars represent LSD between means ($P = 0.05$).


Shoot Length (mm)
300
250
200
150
100
1
2
3
Test Number
Control
Pc2140
TM692


Shoot Dry Weight (mg)
500
400
300
200
100
1
2
3
Test Number
Control
Pc2140
TM692


Root dry Weight (mg)
700
600
500
400
300
200
100
0
1
2
3
Test Number
Control
Pc2140
TM692

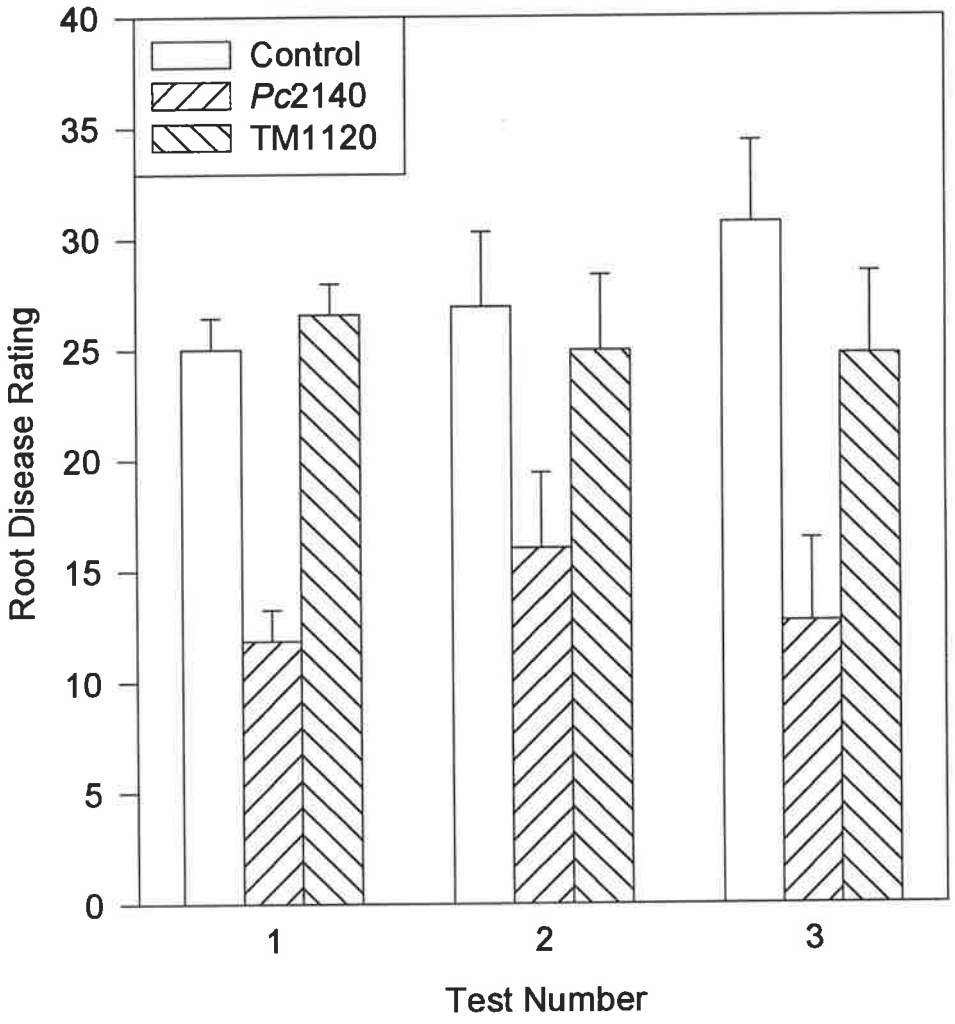


Figure 3.25: Comparison of root disease rating between *P. corrugata* 2140 and mutant strain TM1120 for treatments with added *Ggt* inoculum. In all three trials there was a significant difference in lesion damage between *Pc*2140 and the other two treatments ($P < 0.05$); lesion damage on roots being significantly less on plants inoculated with *Pc*2140. In the first two trials the lesion length on TM1120-inoculated roots was not significantly different to the control treatment at $P = 0.05$. In the third trial root lesions were signicantly less on TM1120-inoculated roots compared to the control plants. Error bars represent LSD between means ($P = 0.05$).

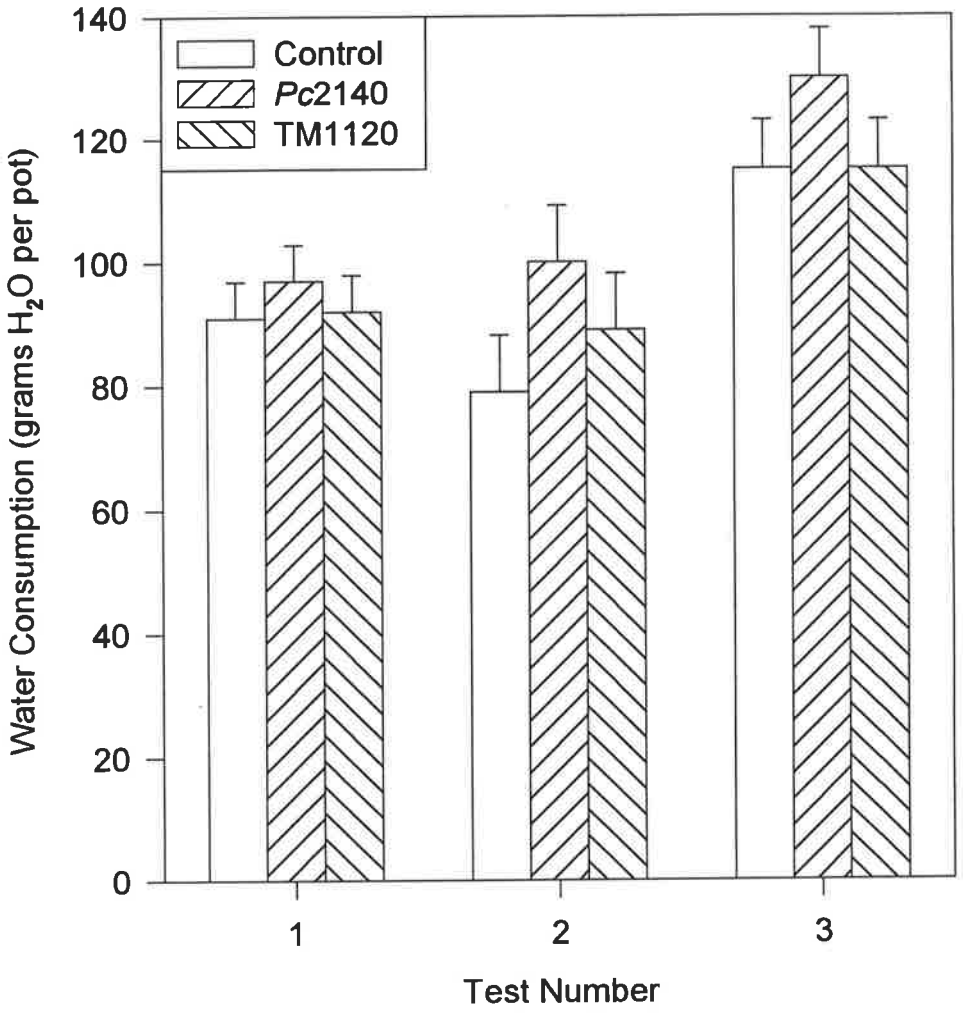
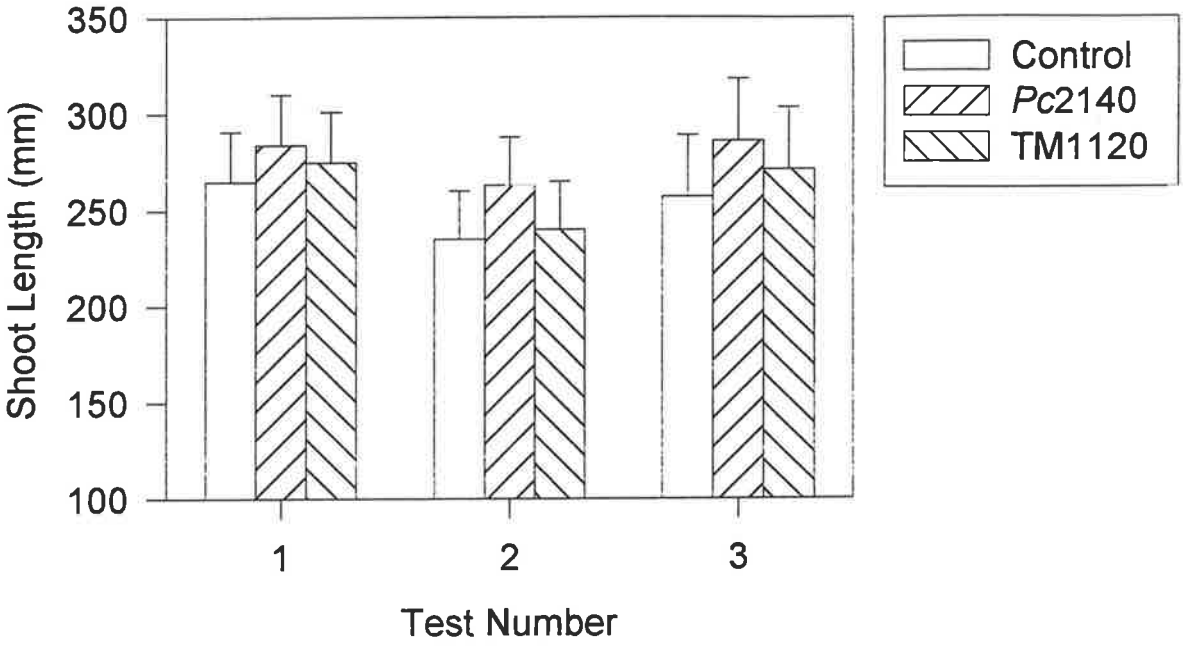


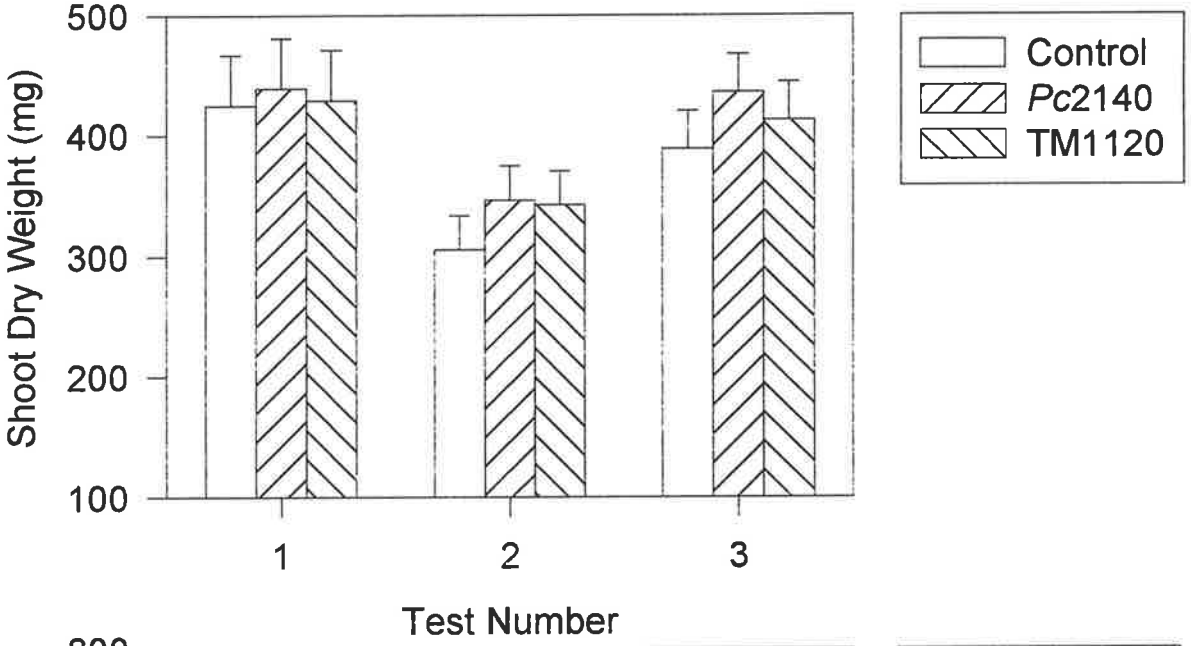
Figure 3.26: Comparison of water consumption by wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM1120 with added *Ggt* inoculum. Water consumption was measured over the last two weeks of the four week trials (refer text for details). Severity of root disease was measured as the percentage seminal root length affected by black vascular lesions (refer text). In the first trial there was no significant difference in water consumption between all three treatments. In the second and third trials water consumption by *Pc*2140-inoculated plants was significantly greater than both TM1120-inoculated palnts and control plants ($P < 0.05$). Only in trial two was water consumption by TM1120-inoculated plants greater than control plants; in the third trial there was no significant difference between these two treatments. Error bars represent LSD between means ($P = 0.05$).

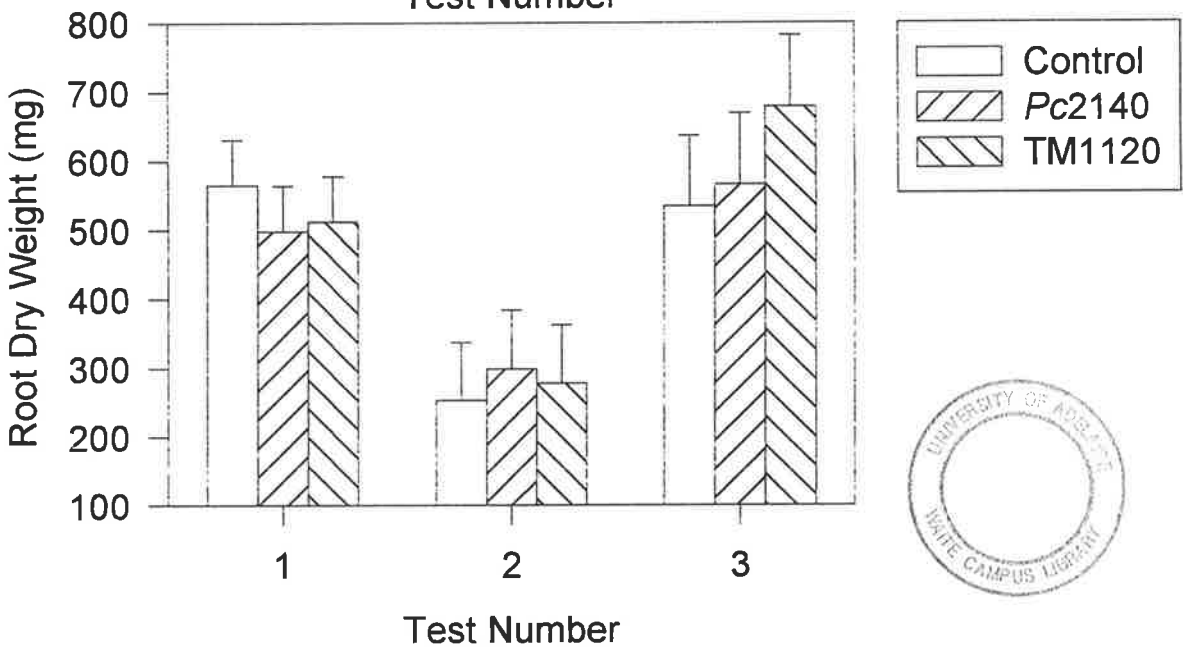
Figure 3.27: Comparison of shoot lengths of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM1120 for treatments with added *Ggt* inoculum. In trials one and three there was no significant difference in shoot lengths between all three treatments. In the second trial there was a significant difference in shoot lengths between the *Pc*2140 treatment and the control ($P < 0.05$). There was no significant difference between TM1120-inoculated plants and the other two treatments in the second trial. Error bars represent LSD between means ($P = 0.05$).

Figure 3.28: Comparison of shoot dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM1120 for treatments with added *Ggt* inoculum. In the first trial there was no significant difference in shoot dry weights between all three treatments. In the second trial shoot dry weights of bacteria-inoculated plants were significantly greater than the control plants ($P < 0.05$). In the third trial shoot dry weights of plants inoculated with *Pc*2140 were significantly greater than control plants only. There was no significant difference in shoot lengths of TM1120-inoculated plants and either *Pc*2140-inoculated or control plants. Error bars represent LSD between means ($P = 0.05$).

Figure 3.29: Comparison of root dry weights of wheat plants inoculated with either *P. corrugata* 2140 or mutant strain TM1120 for treatments with added *Ggt* inoculum. In the first and second trials there were no significant differences in shoot dry weights between all three treatments. In the third trial root weights of TM692-inoculated plants were significantly greater than both *Pc*2140-inoculated and control plants ($P < 0.05$). There was no significant difference in root weights of *Pc*2140-inoculated and control plants in the third trial. Error bars represent LSD between means ($P = 0.05$).







relation to root dry weight results, no two tests for any mutant gave exactly the same pattern of results.

3.3.8 Plant Growth Promotion by *P. corrugata* 2140

The plant growth-promoting ability of *P. corrugata* 2140 on wheat in the absence of disease was measured by water consumption, shoot length, shoot dry weight and root dry weight in four separate trials. Plants inoculated with *Pc*2140 were compared to healthy, disease free plants with no added bacterial inoculum. Water consumption showed little difference between *Pc*2140-treated plants and control plants. In three trials *Pc*2140-inoculated plants utilised more water than the control plants but the uptake was not statistically significant in any trial (Figure 3.30). In the first trial control plants utilised significantly more water than the bacterial-treated plants ($P < 0.05$). Shoot lengths of *Pc*2140-treated plants were greater than the control plants in all four trials (Figure 3.31) but only significantly greater at $P = 0.05$ in one trial only (test #1). Shoot and root dry weights (Figure 3.32 and 3.33 respectively) showed an identical trend to water consumption, that is, no significant difference in three trials and a slight but significant increase in dry weights of control plants in test #1.

3.3.9 Root Colonisation by *P. corrugata* Strains

The ability of three of the mutants of *P. corrugata* 2140, TM300, TM692 and TM1120, to colonise the roots of wheat plants compared to the parent strain was examined. Results are summarised in Table 3.4. Results from this trial indicated that the three mutant strains were not compromised in their ability to colonise and survive on the wheat roots during the 4 week pot trial.

Growth of *P. corrugata* strains on a selective agar medium containing chloramphenicol was not different to growth in the absence of the antibiotic when the concentration of

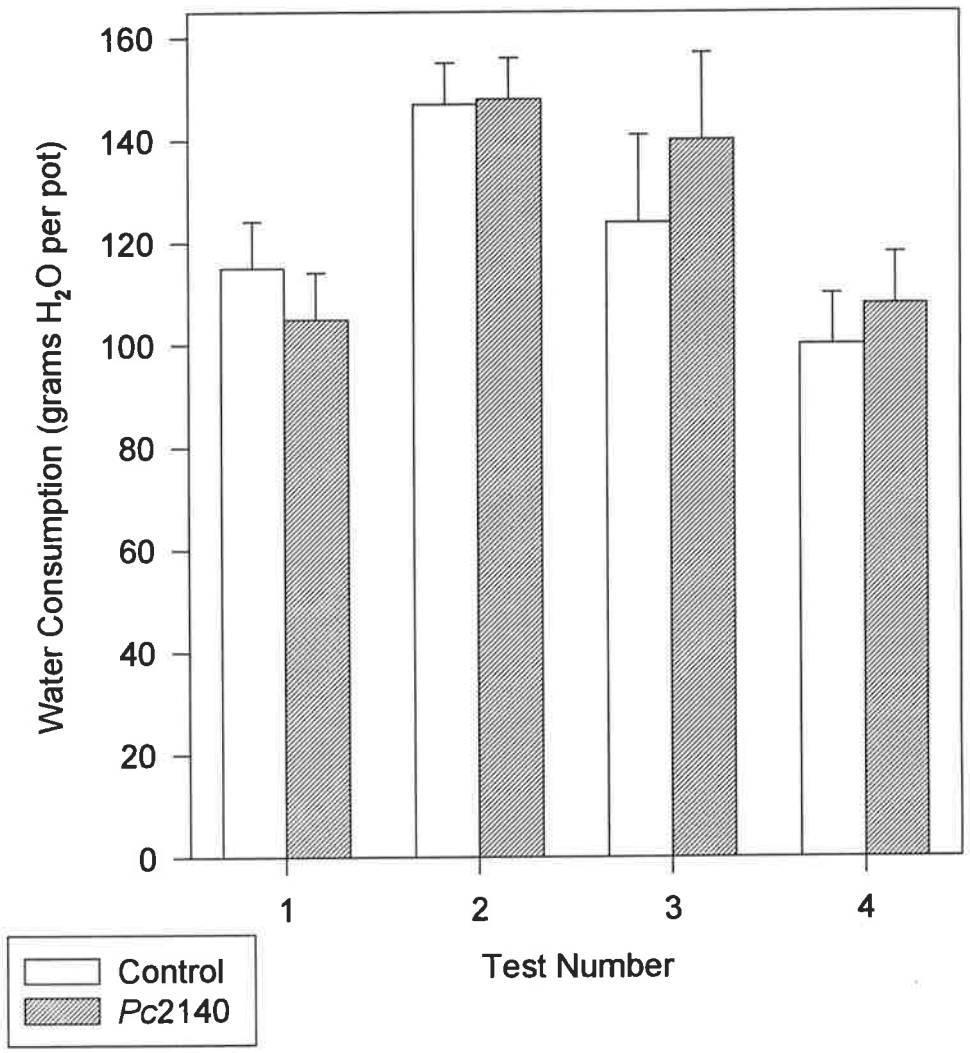


Figure 3.30: Plant growth-promotion by *P. corrugata* 2140 in the absence of *Ggt*: Comparison of water consumption between untreated wheat plants and plants inoculated with *P. corrugata* 2140. In the first trial water consumption by untreated plants was significantly greater than plants treated with *Pc*2140 ($P < 0.05$). In the other three trials there was no significant difference in water consumption between the two treatments.

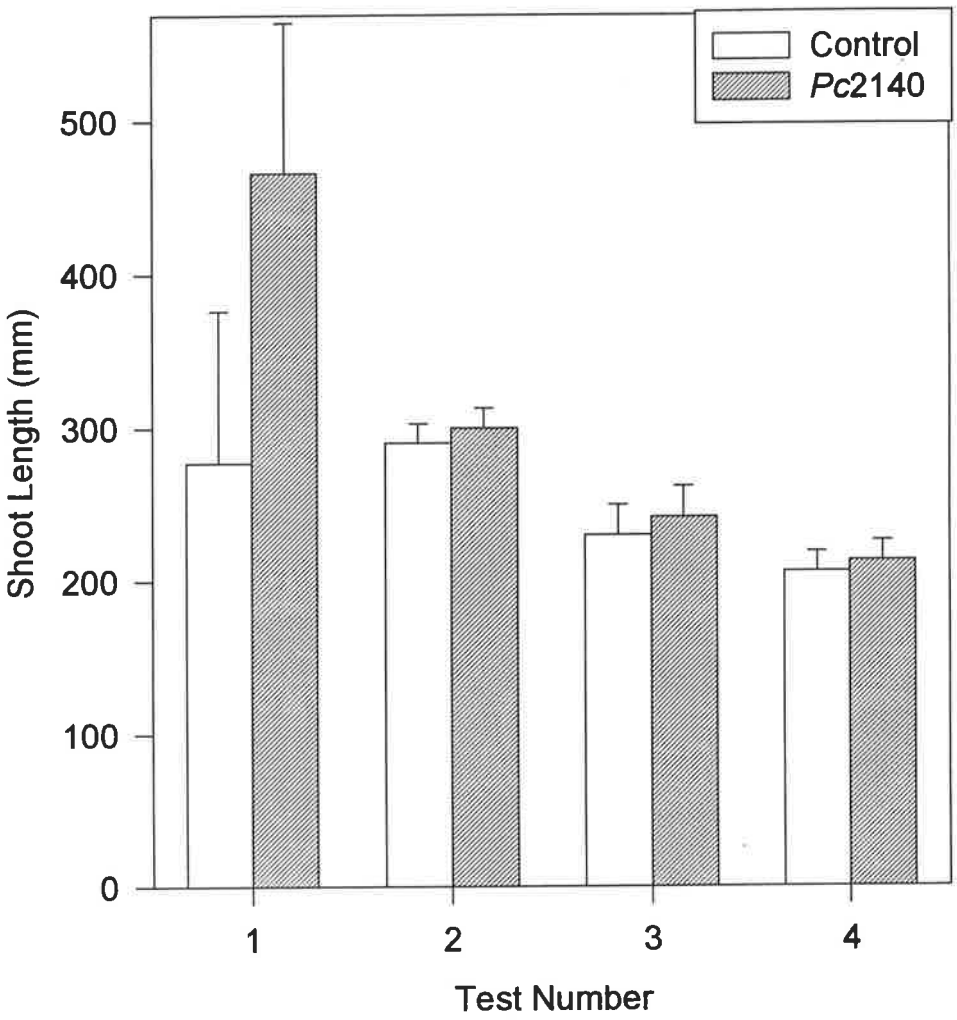


Figure 3.31: Plant growth-promotion by *P. corrugata* 2140 in the absence of *Ggt*: Comparison of shoot lengths between untreated wheat plants and plants inoculated with *P. corrugata* 2140. In all four trials shoot length of plants treated with *Pc*2140 was greater than untreated control plants. Only in the first trial however, was shoot length significantly different ($P < 0.05$).

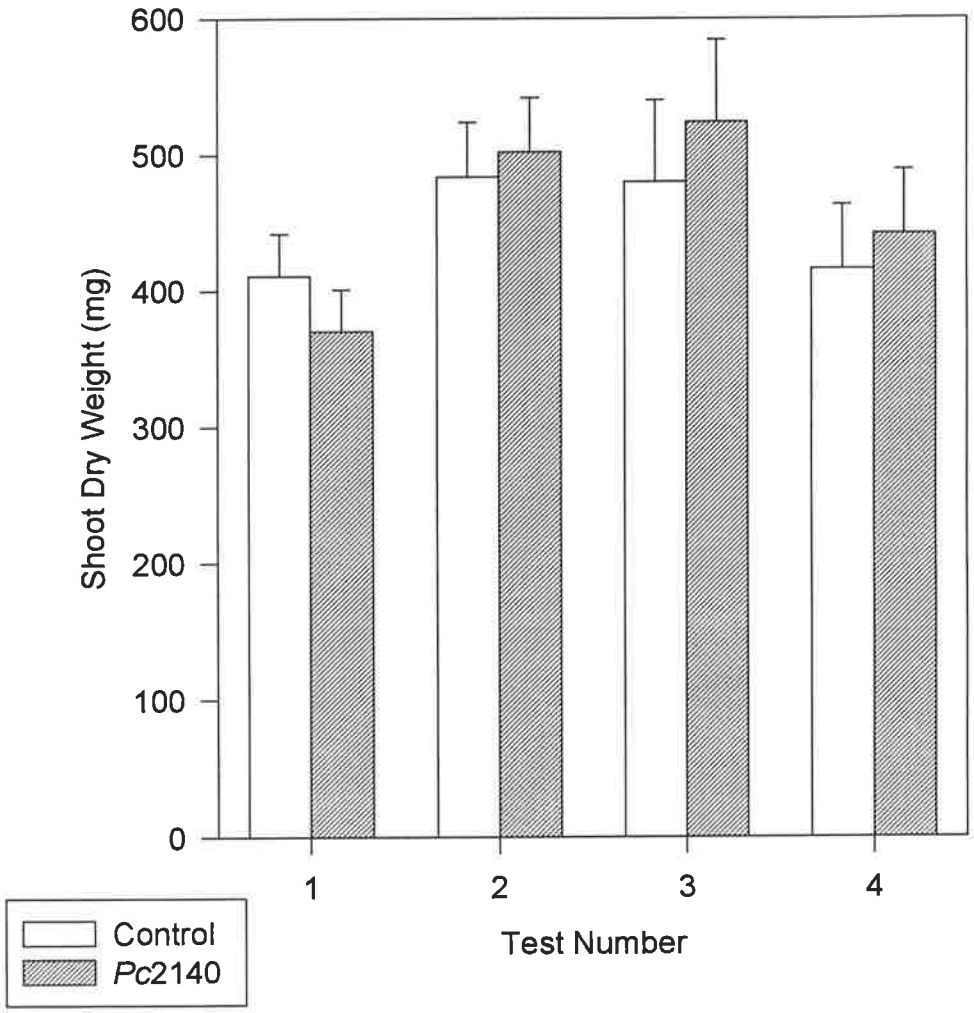


Figure 3.32: Plant growth-promotion by *P. corrugata* 2140 in the absence of *Ggt*: Comparison of shoot dry weight between untreated wheat plants and plants inoculated with *P. corrugata* 2140. In the first trial shoot dry weight of untreated plants was significantly greater than plants treated with *Pc*2140 ($P < 0.05$). In the other three trials there was no significant difference in shoot dry weights between the two treatments.

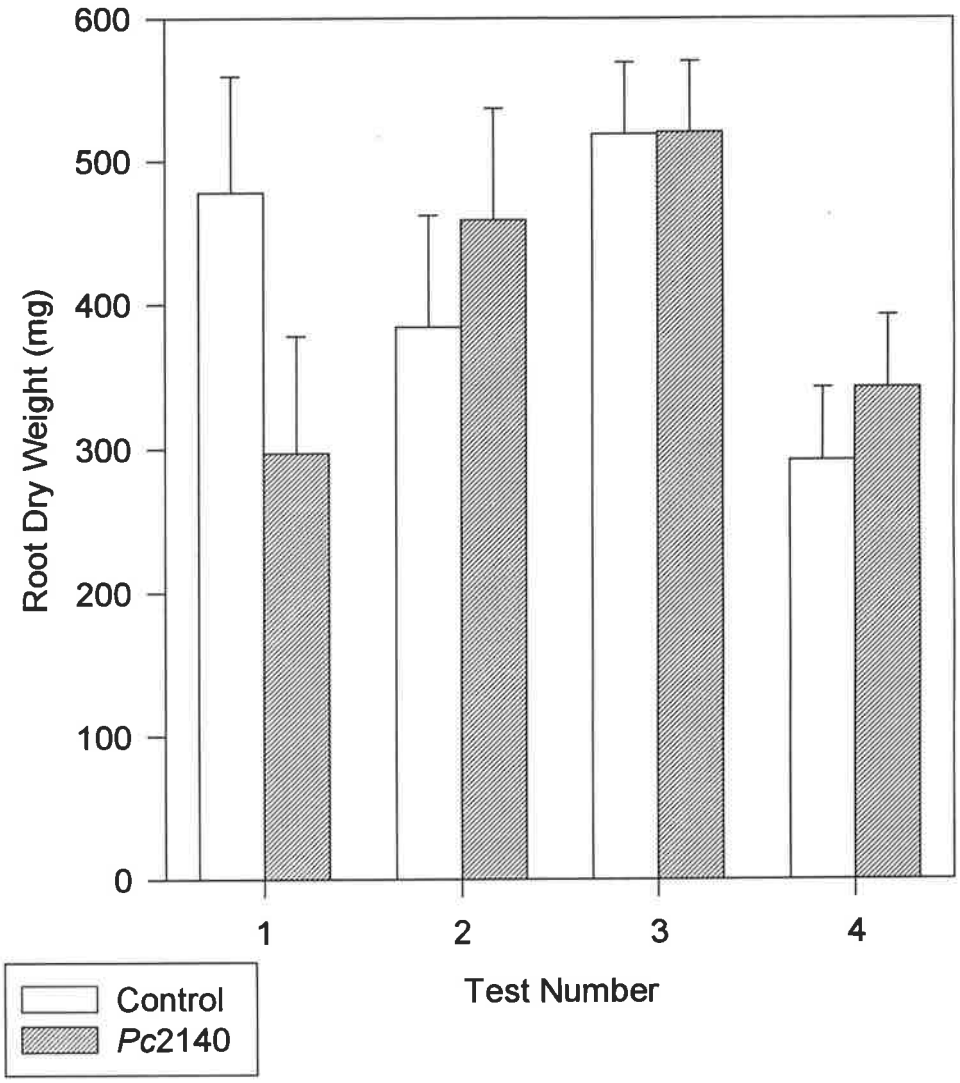


Figure 3.33: Plant growth-promotion by *P. corrugata* 2140 in the absence of *Ggt*: Comparison of root dry weight between untreated wheat plants and plants inoculated with *P. corrugata* 2140. In the first trial root dry weight of untreated plants was significantly greater than plants treated with *Pc*2140 ($P < 0.05$). In the other three trials there was no significant difference in root dry weights between the two treatments.

Strain	Log_{10} total *P. corrugata* per gram fresh root weight
*Pc*2140	3.92^{a}
TM300	3.98^{a}
TM692	3.20^{a}
TM1120	3.62^{a}

Table 3.4: Log_{10} CFU *P. corrugata* per gram fresh root weight (5 replicates per treatment). No significant difference was found in the root colonisation ability of the four strains examined ($P = 0.05$) after 4 weeks incubation in pot trials. No colonies showing typical *P. corrugata* type morphology on TZCA medium were detected in control pots (no added *P. corrugata* inoculum).

chloramphenicol did not exceed 50μg.ml^{-1}. Above this concentration (75μg.ml^{-1}) growth became retarded; an extra day's incubation was required for the colonies on the plate to reach the same size as colonies growing in the presence of 50μg.ml^{-1} chloramphenicol. Growth of *P. corrugata* was detectable up to 200μg.ml^{-1}; above this concentration visible growth was inhibited. Based on these results it was decided to add 50μg.ml^{-1} chloramphenicol to media for the reisolation of *P. corrugata* strains from wheat roots.

3.4 DISCUSSION

The aim of this phase of the project was to generate mutants of *P. corrugata* 2140 that had reduced *in vitro* antagonism of *Ggt* and reduced ability to suppress take-all symptoms on wheat in pot trials. By doing so, the mechanisms by which *Pc*2140 suppresses take-all may then be established through determining phenotype differences between the mutants and the wild-type strain. Successful mutagenesis of the biocontrol agent was achieved by the transposition of single copies of the Tn*5*::*luxAB*Tc^r transposon (deLorenzo *et al.* 1990) from the suicide vector pUT carried by *E. coli* S17-1 λ_{pir}. Five mutants with varying levels of reduced *in vitro* antagonism of *Ggt* were isolated and subsequently examined for loss of *in situ* take-all suppression. Of these five mutants, strain TM1120 did not antagonise *Ggt in vitro* and was found to have lost approximately 95% of its disease suppressive capabilities when tested in three independent pot trials.

Attempts to mutate *P. corrugata* 2140 with other vector/transposon cassette systems (Table 3.2) proved unsuccessful. Mutagenesis with each particular vector/transposon system was conducted as stringently as possible in accordance with the appropriate literature In most cases mutagenesis was unsuccessful as the appropriate antibiotic marker on each transposon was either not expressed or not expressed sufficiently well in *P. corrugata* 2140 for the mutants to grow on selective media. There are number of

possible reasons why mutagenesis with these systems was unsuccessful. They include failure of conjugation, ie, conjugation did not occur between donor and recipient, transposon instability in the *P. corrugata* background or antibiotic marker expression was insufficient for the recipient to grow on a selective medium. Potential problems associated with transposon insertion and expression in a recipient chromosome including those described above have been reviewed by Mills (1985). The actual reasons why each of the unsuccessful matings did not generate antibiotic-resistant mutants was not tested in the context of this project.

When Tn*5*::Km^r (Simon *et al.* 1983) was introduced into *Pc*2140, the transposon was successfully inserted into the chromosome as transconjugants of *P. corrugata* resistant to kanamycin were isolated. These mutants were initially screened for metabolic characteristics such as siderophore production and auxotrophy. It was noticed after a period of time that the phenotype characteristic lost as a result of the transposon insertion would be regained but the strain would also retain kanamycin resistance. For example, a siderophore-negative mutant was isolated during screening of mutants generated after insertion of Tn*5*::Km^r. After 3 - 4 months the strain remained kanamycin resistant but reverted to being siderophore positive on a medium specifically prepared for siderophore detection (refer Chapter 5). During this time period the strain had been stored at -70^{o}C as described in Section 2.2. This observation suggests that the transposon was undergoing secondary transposition within the *P. corrugata* chromosome. Consequently the mutant would retain kanamycin resistance but lose the mutation phenotype (in this case loss of siderophore production). The possibility of secondary transposition of Tn*5*::Km^r in *Pc*2140 was not investigated in the course of this work. The phenomenon of secondary transposition of transposons to genomic "hot spots" in a recipient's chromosome has been reviewed by Berg (1989).

The problem of unsuccessful mutagenesis was overcome by altering the conditions of mating and utilising a vector/transposon which carries the transposase gene externally

but *cis* to the transposon. Firstly, cells were stringently washed before mating to remove any extracellular material which may have formed a barrier to conjugation. The donor to recipient ratio was increased to approximately 10:1 (based on initial growth periods and volumes added to millipore filters) to increase the likelihood of a recipient cell lying adjacent to a donor cell. Matings was undertaken overnight (approximately 16 hours) to increase the period in which conjugation may occur. Furthermore, by using the Tn*5*::*luxAB*Tc^r cassette with the transposase gene adjacent to but outside the transposable element (Figure 3.3), stable mutations of strain 2140 were generated.

The *lux* genes of the transposon may have a number of roles when introduced into the genome. These two genes are promoterless in the construct used, requiring a *cis*-acting promoter from the recipient genome for expression. Consequently the transposon has specific insertion prerequisites for *lux* expression. Firstly, if the insertion is inverted, ie the I end of the transposon is upstream, then the *lux* genes will not be expressed. Secondly, if the insertion is not near a suitable promoter then expression will not occur, or will be significantly reduced. The requirement for an active host promoter for *lux* expression explains the variation in bioluminescence observed with the five mutants (Table 3.3).

The requirement for an external promoter makes this transposon a suitable reporter cassette as reviewed by Loper *et al.* (1994). The *lux* genes may also be used to track and identify isolates from soil (Shaw *et al.* 1992), identification being based on bioluminescence (and tetracycline resistance with the *tet* gene).

In this study we utilised the promoterless *lux* genes to assist in identifying different mutants with the Tn*5* insertion. This was useful in distinguishing TM692 (bioluminescent positive) and TM1120 (bioluminescent negative) as separate mutants as both strains did not antagonise *Ggt in vitro* and, when cut with *Pst*I, showed similar band sizes when hybridised (Figure 3.4). The *tet* gene on the transposon was utilised to

reisolate mutants from the rhizosphere when determining root colonisation (Section 2.11). The lack of bioluminescence activity by TM1120 (Table 3.3) meant that the *lux* genes could not be utilised to identify this strain when reisolated from the rhizosphere. Bioluminescence activity in association with tetracycline resistance was utilised to identify mutant strains TM300 and TM692 when reisolated from the rhizosphere.

One of the basic premises for mutating bacteria with transposons rather than with UV light or chemical mutagens is that each mutant isolated will carry only a single copy of the transposon in its genome thus be subjected to only a single mutational event. Consequently it is important to check mutants for single insertion events by hybridisation with a suitable probe. Digestion of DNA from the selected *P. corrugata* strains with *Pst*I produced a series of cuts within the transposon including one fragment containing most of the transposon and *P. corrugata* chromosomal DNA flanking the I end of the transposon to the first *Pst*I site in the genomic DNA (Figure 3.4). A unique band would be produced for each insertion event; the size of the band being dependent upon the location of the first *Pst*I site in the flanking genomic DNA. The probe, derived from the transposon and including the region between the *Pst*I site in *luxA* and the I end, would thus hybridise separately to each transposon inserted in the chromosome.

It is acknowledged that the probe used to detect the transposon would also hybridise to transposon DNA from the *Pst*I site in *luxA* to the O end. This in effect would result in three bands per mutant (presuming only one copy of the transposon was present in the chromosome). The bands would be a 0.4kb fragment (part of *luxA*), a 1.0kb fragment (*luxA* to the O end of the transposon) and the main band discussed above. As the minimum size of the main band would greater than 3.5kb (the distance from the *Pst*I site in *luxA* to the I end is approximately 3.5kb), the two extra bands on the membrane are easily distinguished from the main bands through electrophoresis separation before blotting.

An alternate method of ensuring only bands consisting of the I end of the transposon plus flanking genomic DNA would hybridise to the probe could have been adopted. The 3.2kb *Sal*I probe from the transposon may have been further digested with *Pst*I prior to labelling and hybridisation. This new probe (1.8kb in length) would only hybridise to the main bands as the remaining 1.4kb *luxA* region had been removed.

It is important to thoroughly examine the phenotypic changes in a mutant as a result of transposon insertions. Two scenarios may occur even when a single insertion event has been occurred by DNA hybridisation. The strain's basic metabolic functions may have been affected leading, for example, to possible reductions in its ability to survive in the rhizosphere. Secondly, the mutation may affect more than one mechanism involved in biocontrol. For example, disruption of the *gac*A gene in *P. fluorescens* CHA0 was found to affect both antibiotic and cyanide production in that strain (Voisard *et al.* 1989). A regulatory region responsible for production of antifungal compounds including chitinase, cyanide and the antibiotic pyrrolnitrin in *P. fluorescens* BL915 was inserted into spontaneous mutants which did not produce those compounds, thus restoring activity against *Rhizoctonia solani* (Gaffney *et al.* 1994). Like strain CHA0, this result suggested that HCN production was regulated by the same gene or gene region as antibiotics and other antifungal compounds. Consequently the loss of HCN activity in mutants with disrupted antibiotic production through the mutation of regulatory genes must be considered when screening mutants. Secondary effects of transposon insertions has been reviewed by Berg *et al.* (1989).

Mutant TM1120 grew in a minimal medium at a similar rate as the parent strain. This has indicated that the metabolic ability of TM1120 was not compromised in any way aside from loss of biocontrol activity. The production of volatiles by TM1120 which inhibit *Ggt* growth *in vitro* was compared with the parent strain. It was found that production of antagonistic volatiles appeared unaffected by the transposon insertion. Furthermore, quantification of HCN produced by the two strains (Chapter 5) found near

identical levels of HCN being produced *in vitro*. A similar level of root colonisation on wheat roots to *Pc*2140 is further evidence that the mutation of TM1120 did not produce any significant detrimental pleiotropic effects. It was therefore concluded that TM1120, with its loss of *in vitro* antagonism of *Ggt* and apparent lack of detrimental pleiotropic effects, would be a suitable candidate for further examination.

The reason for the more rapid production of extracellular mucoid material by TM1120 and TM692 on TZCA agar medium is unclear. These two strains produced mucoid colonies on TZCA medium after 2 days incubation at 25^{o}C while the parent strain and other transconjugants took 4-5 days to become mucoid to the same extent. This phenomenon only occurred on TZCA medium; on other complex media such as NA and LB agar, *P. corrugata* 2140 and all derivatives were equally mucoid in appearance after 2-3 days growth. Enhanced EPS production causing a mucoid colony appearance was observed in transposon-generated mutants of *Pseudomonas* strain AN5 (Nayudu *et al.* 1994). These mutants were found to be deficient in biocontrol activity against *Ggt*. It was postulated that enhanced EPS production formed a physical barrier to antibiotic secretion by AN5 thereby reducing biocontrol activity.

This effect may have been be a partial explanation for the results presented here. Strain TM692 showed no antagonism to *Ggt in vitro* but was found to have at least partial fungal antagonism in supernatants suggesting that the production of extracellular mucoid material mutant prevented or slowed diffusion of antagonistic compounds into the medium. Conversely, the production and release of siderophores and volatiles by these mutants did not appear to be affected by their mucoid nature (Chapter 5). The mucoid layer therefore, could not be considered a barrier to diffusion or secretion of a range of bacterial metabolites. Furthermore, results from the pot trials with TM692 where significant disease suppression compared to diseased controls was still observed indicate that *in vitro* production of extracellular material is not an important indicator of biocontrol activity *in situ*.

Overall the pot trial results confirmed that all five mutants that displayed reduced *in vitro* antagonism of *Ggt* also showed reduced levels of biocontrol protection on wheat against take-all symptoms *in situ*. Mutant strain TM1120 displayed no *in vitro* antagonism towards *Ggt in vitro* and very poor or no disease suppression *in situ*. It is likely therefore that the mutation in TM1120 has disrupted the synthesis of most or possibly all of the antagonistic compounds responsible for disease suppression. It is possible that a regulatory rather than a structural gene has been disrupted by the transposon insertion. The other four mutants showed varying degrees of disease suppression *in situ*. These results suggest that more than one compound is involved in the biocontrol of take-all by *Pc*2140 and that multiple loci are involved in their synthesis. Further characterisation of these mutants including locating the site of transposon insertion in the chromosome and analysis of disruption of metabolite production would be required before these results can be fully understood.

The slight reduction in root disease in two tests on plants treated with TM1120 including a small but significant level of activity (test #3) suggests that other factors such as induced resistance and/or metabolites still being produced by the mutant may play a role, albeit minor, in biocontrol of take-all. The possible role of induced resistance in disease control will be addressed in the General Discussion (Chapter 6).

Data obtained from this series of trials verify that the root disease rating is the most reliable parameter to measure take-all on wheat seedlings. The black vascular lesions on the wheat roots develop during initial infection by the fungus therefore they are the first macroscopic symptom of take-all to appear. The time period of the trials (4 weeks) often does not allow sufficient time for other symptoms of disease such as plant growth parameters to appear. Longer incubation periods of the wheat plants (eg to maturity and

head ripening) may be required for the other parameters to show consistant significantly different results.

Water consumption by plants grown in non-draining pots may be a suitable parameter to measure in addition to the root ratings. This parameter only becomes relevant well after the initial infection takes place as the *Ggt* hyphae infest and obstruct vascular tissue, leading to reduced water movement. For this reason the water consumption of the plants was only measured for the final two weeks of the four week trials. Although not tested it would be expected that water consumption would be a more reliable parameter to measure disease severity if the trials were allowed to continue for a longer period. As the hyphae penetrate further into the vascular tissue there would be an expected continual decrease in water consumption by affected plants compared to disease-free treatments.

The data obtained from the shoot lengths, shoot dry weights and root dry weights of the wheat seedlings were less consistent than both the root ratings and water consumption measurements. This suggests that these parameters are not suitable as a measure of take-all for this type of trial unless root ratings are taken in conjunction with this data. Like water consumption these parameters may be more relevant if the trial was to be conducted over a longer period. Restricted water movement due to hyphal penetration would result in limited nutrients being distributed throughout the plant. This would result in chlorosis and stunting of growth. Chlorotic symptoms in diseased plants were found to occur late in the four week period of the trial, consequently growth may not be suitably affected after four weeks. As a result of these findings, pot trials of complemented mutants (Chapter 4) were based on root ratings and water consumption rates only.

Data from the pot trials comparing growth of healthy wheat plants with and without added *Pc*2140 inoculum indicate that this biocontrol strain has limited value as a plant

growth-promoting rhizobacterium. In only one trial was shoot length of wheat plants (Figure 3.1) inoculated with the bacterium significantly greater ($P < 0.05$). In the other three trials there was a small but insignificant increase in the shoot weights of inoculated plants. Water consumption, shoot and root dry weights showed a similar trend; ie, a small but insignificant increase in the inoculated wheat plants compared to the controls. These results are similar to earlier studies of the plant growth promotion properties of *Pc*2140 whereby small but insignificant increases in wheat size were observed in *Pc*2140-inoculated plants in *Ggt*-free soil (Ryder and Rovira, 1993).

CHAPTER 4

COMPLEMENTATION OF STRAIN TM1120 TO RESTORE BIOCONTROL ACTIVITY

4.1 INTRODUCTION

The next stage of the project was to complement selected mutants with a cosmid library to restore biocontrol activity. This work had two objectives. First, complementation of a mutant with wild-type genes corresponding to the region of mutation on the chromosome, resulting in restoration of biocontrol activity both *in vitro* and *in situ*, would confirm that loss of biocontrol activity observed in Chapter 3 was due to the transposon insertion disrupting gene activity. Second, isolation and subsequent characterisation of a genomic region contained within the cosmid that restored activity. This would lead to a better understanding of the genetics of functions involved in biocontrol of take-all by *P. corrugata* 2140. Strain TM1120 was selected for the initial complementation study due to its nearly complete loss of biocontrol activity.

4.2 MATERIALS AND METHODS

4.2.1 Overview

Mutant strain TM1120 was complemented with *Pc*2140 genomic DNA contained within a cosmid library constructed from the broad host range cosmid pLA2917 (Allen and Hanson, 1985) and maintained in *E. coli* S17-1. Matings were undertaken with the assistance of helper plasmid pRK2013 (Figurski and Helinski, 1979) maintained in *E. coli* strain DF3046. Complemented mutants were tested for restoration of *in vitro* antagonism of *Ggt* and suppression of take-all symptoms on wheat. The complemented mutants which exhibited *in vitro* antagonism towards *Ggt* were also examined for growth

in a glucose/minimal salts broth and stability and expression of the cosmid in the *P. corrugata* background.

A probe comprising *P. corrugata* DNA flanking the I end of the Tn*5*::*luxAB*Tc^{r} insertion in TM1120 was hybridised with DNA from the cosmid library to confirm that sequences in the cosmid library restoring biocontrol activity were homologous to the genomic region disrupted by the transposon insertion. Screening for homology between the probe and candidate cosmid inserts was undertaken by dot-blot analysis. Cosmid DNA was spotted onto a nylon membrane and hybridised with a DIG-labelled probe. Autoradiography detection of hybridised DNA was undertaken utilising CSPD (see Section 4.2.7) as the chemoluminescent indicator.

4.2.2 Strains

All strains used for the complementation and subsequent screening of strain TM1120 are listed in Table 4.1. Unless otherwise stated all strains were stored and cultured by the methods described in Chapter 2. Cosmids and plasmids, including constructs generated during this phase of the project, are described in Table 4.2.

4.2.3 Construction of the Cosmid Library

A cosmid library of *P. corrugata* 2140 was prepared by E. Carey and B.W. Holloway at Monash University (Melbourne, Victoria). To construct the library *P. corrugata* DNA was isolated and partially digested with *Hind*III. The fragments were then cloned into pLA2917 (Allen and Hanson, 1985) before transformation into *E. coli* S17-1. Approximately 2,000 cosmid clones were isolated and inserts of foreign DNA in the cosmid were confirmed by *Eco*RI, *Hind*III and *Eco*RI/*Hind*III digests of cosmid DNA (E. Carey, pers. comm.). Clones were stored at -70°C in 8.8% v/v glycerol solution.

Strain	Phenotype/Characteristics	Source/Reference
Pseudomonas corrugata 2140	Tp^r, Cm^r, Ap^r, Tc^S, Km^S	Ryder and Rovira (1993)
P. corrugata TM1120	*Pc*2140::Tn*5* Tc^r reduced take-all suppression	This study
P. corrugata TM1120α1	TM1120 containing cosmid pMO142614	This study
P. corrugata TM1120α2	TM1120 containing cosmid pMO142812	This study
P. corrugata TM1120α3	TM1120 containing cosmid pMO142218	This study
P. corrugata TM1120α6	TM1120 containing cosmid pMO142439	This study
P. corrugata TM1120α7	TM1120 containing cosmid pMO141930	This study
P. corrugata TM1120α8	TM1120 containing cosmid pMO142133	This study
P. corrugata TM1120α12	TM1120 containing cosmid pMO142610	This study
E. coli JM83	*pro*, *str*A, *thi*, ϕ80d, *lac*ZΔM15	
E. coli JM83	*E. coli* JM83 containing pBluescript M13	This study
E. coli JM83	*E. coli* JM83 containing pBSM13-FF	This study
E. coli JM83	*E. coli* JM83 containing pBSM13-PD	This study
E. coli DF3046	contains pRK2013	Figurski and Helinski (1979)
E. coli S17-1 λpir	contains pUT::Tn5::*luxABTc*^{r}	deLorenzo *et al* (1990)
E. coli HB101	contains cosmid pLA2917	Bruce Holloway
E. coli S17-1	contains cosmid pMO141930	Bruce Holloway
E. coli S17-1	contains cosmid pMO142133	Bruce Holloway
E. coli S17-1	contains cosmid pMO142218	Bruce Holloway
E. coli S17-1	contains cosmid pMO142439	Bruce Holloway
E. coli S17-1	contains cosmid pMO142614	Bruce Holloway
E. coli S17-1	contains cosmid pMO142812	Bruce Holloway
Escherichia coli DH5α	*rec*A1, Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB ,*lac*I^qZΔM15]λ^-	Hanrahan (1983)
E. coli DH5α	contains pUC18	This study
E. coli DH5α	contains pUC18-TM1120A	This study

Table 4.1: Bacterial strains used in the complementation of mutant strain TM1120.

Plasmid	Characteristics	Reference/Source
pUT::Tn*5*	Tn*5*::*luxAB*Tc^r, *amp*, *ori*	deLorenzo *et al* (1990)
PBluescript M13	Ap^r, *ori*	Short *et al*, 1988
pBluescript M13-FF	pBluescript M13 containing TM1120 genomic DNA + *tet*	This study
pBluescript M13-PD	pBluescript M13 containing TM1120 genomic DNA	This study
pRK2013	ColEI, *ori*, *kam*	Figurski and Helinski (1979)
pLA2917	Km^r, Tc^r, contains RK2-derived cloning vector	Allen and Hanson (1985)
pMO141930	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142133	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142218	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142439	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142610	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142614	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pMO142812	pLA2917 + *Pc*2140 genomic DNA, *kam*, *tet*	B. Holloway
pUC18	*lac*$^-$, Ap^r	Norrander *et al*, (1983)
pUC18-TM1120A	pUC18 containing *Pst*I-digested TM1120 genomic DNA + *tet*	This study

Table 4.2: Cosmids and plasmids used for the complementation of mutant strain TM1120. The cosmid library was constructed from partial *Hind*III digest of *P. corrugata* 2140 genomic DNA ligated into pLA2917.

4.2.4 Complementation of Strain TM1120

4.2.4(i) Preparation of Cells

Cosmid clones were grown for 24 hours at 37°C on NA in 96-well microtitre plates. *E. coli* strain DF3046 containing helper plasmid pRK2013 (Figurski and Helinski, 1979) was grown overnight at 37°C in NB:Km^{25} on a rotary shaker (200 RPM). After growing the culture was spun to pellet cells (Sorvall SS34, 10K, 15 mins.) and then resuspended in an equal volume of 0.45% w/v sterile saline solution. Strain TM1120 was grown for 24 hours at 25°C in NB:Tc^{10} on a rotary shaker. After growing for 24 hours 0.5 ml of TM1120 culture was transferred to 500 mls fresh NB:Tc^{10} and grown as before until the absorbance of the culture broth, A_{550} = 0.3 approx. The TM1120 culture was then centrifuged to pellet the cells and resuspended in a 1/10th. volume of the saline solution.

4.2.4(ii) Triparental Mating of TM1120

Immediately prior to mating, TM1120 and *E. coli* DF3046 cell suspensions were pooled in equal volumes and vortexed briefly to mix the cells. After mixing, 50µl of this cell suspension was added to each well of the microtitre plates containing a growing cosmid clone. The added cell suspension was allowed to partially evaporate allowing the three bacterial strains to interact on the surface of the NA. The plates were then covered and incubated at 25°C for 3 days.

TM1120 mutants containing cosmids pLA2917 were subsequently isolated by patching cells from the NA onto PSM:$Km^{25}Cm^{40}Tp^{100}$ agar after the 3 day mating incubation. The patched cultures were then incubated at 25°C until growth appeared. *P. corrugata* 2140, strain TM1120, *E. coli* strain HB101 containing pLA2917 and *E. coli* strain

DF3046 containing helper plasmid pRK2103 were also streaked onto the selection medium as controls.

4.2.5 Screening of Transconjugant Mutants for Restored *In vitro* Inhibition of *Ggt*

All isolates of TM1120 containing cosmid pLA2917 with *Pc*2140 gDNA inserts from the cosmid library were screened for *in vitro* inhibition of *Ggt* on PDA medium by the method described in Section 2.7.1. Those transconjugants which showed antagonism towards *Ggt* were then restreaked onto TZCA:$Km^{25}Cm^{40}Tp^{100}$ and incubated for 48 hours at 25°C. Single colonies (approximately 6 per transconjugant) were then retested for *Ggt* antagonism. Strains which still displayed *in vitro* antagonism were stored (refer Section 2.2) for further testing.

As well as the controls described in Section 4.2.4(ii), *E. coli* strain S17-1 from the cosmid library containing the corresponding cosmids from the cosmid library were streaked onto the selective medium described in Section 4.2.4(ii) and incubated at 37°C for 48 hours to ensure that only TM1120 cells carrying the corresponding cosmids grew on the selective medium.

4.2.6 Stability of Cosmids in TM1120

Complemented strains of TM1120 that exhibited *in vitro* antagonism of *Ggt* were tested for stability of the cosmid insertion. The method of screening was almost identical to the protocol for testing transposon retention described in Section 3.2.5 except that 25ug.ml^{-1} kanamycin was used in the medium instead of tetracycline. Each strain of TM1120 with a separate cosmid insert were screened over a 7 day period at 25°C. Stability was calculated as a percentage of CFU's growing on LB agar supplemented with kanamycin compared to CFU's on LB agar medium only.

4.2.7 Probing Cosmids for Homologous Sequences

4.2.7(i) General Overview

Cosmids that restored *in vitro* antagonism of *Ggt* by complementing strains of TM1120 were probed for regions of homology with a *P. corrugata* genomic DNA fragment flanking the I end of Tn5::*luxAB*Tc^r inserted into TM1120 (Figure 3.4). Plasmid pBluescript M13 (pBSM13) (Short *et al.* 1988) contained in *E. coli* strain JM83 was used as the cloning vector for the probe DNA.

Total genomic DNA was isolated from strain TM1120 by the method described in Section 3.2.9(i). The DNA was then digested with *Not*I by the method described in Section 3.2.9(ii) The digested DNA was then ligated into pBSM13 and the vector transformed into *E. coli* JM83. Digestion of TM1120 genomic DNA with *Not*I gave rise to a fragment containing the *tet* gene from the transposon and a genomic DNA fragment flanking the I end of the Tn*5* insertion (Figure 3.4). *E. coli* JM83 cells carrying the plasmid with this fragment were isolated on a medium containing 10μg.ml^{-1} tetracycline. The tetracycline gene was then removed from the plasmid by digestion with *Eco*RI (refer Figure 3.2) and the plasmid religated and transformed back into *E. coli* JM83. The resultant plasmid contained a 2.1kb *Eco*RI/*Not*I fragment of *P. corrugata* genomic DNA suitable to use as a probe for homology between the transposon insertion site in TM1120 and cosmids containing complementary sequences.

4.2.7(ii) Treatment of pBluescript M13 with Calf Intestinal Phosphatase

Plasmid pBSM13 was isolated from *E. coli* JM83-PB by the method described in Section 3.2.10(i). After the 70% wash and subsequent drying the vector was resuspended in Tris, pH8.3 (Sambrook *et al.* 1989). Purified plasmid was then digested with *Not*I by the method described in Section 3.29(ii). The purified vector was then treated with calf

intestine phosphatase (CIP) by the method of Sambrook *et al.* (1989). CIP-treated plasmid DNA was stored at -20°C until required for ligation.

4.2.7(iii) Ligation of Genomic DNA into pBluescript M13

The concentrations of the treated vector DNA and *Not*I digested TM1120 genomic DNA were adjusted to 1μg.ml^{-1} approx. with SDDW before setting up the ligations. Ligation was undertaken by adding 1μl vector DNA to 10μl TM1120 digested DNA, then adding 4μl 10x buffer and 1μl T4 DNA ligase (Boehringer Mannheim). The volume was taken up to 40μl with SDDW and the solution was then mixed gently and spun briefly. The solution was incubated overnight at 15°C.

4.2.7(iv) Transformation of Cloned DNA into *E. coli* JM83

Plasmid pBluescript M13 containing cloned fragments of *P. corrugata* genomic DNA were introduced into competent *E. coli* strain JM83 cells. Plasmids were transformed into strain JM83 by heat shock treatment.

(a) Preparation of Competent *E. coli* JM83 Cells

E. coli JM83 was streaked onto LB agar and grown overnight at 37°C. A single colony was then transferred to 10mls LB broth and grown on a shaker overnight at 37°C. Ten microlitres of this culture was then added to 250mls fresh LB broth and grown on a shaker as before until the absorbance of the culture broth, A_{550} = 0.3 approx. (about 5 hours incubation time). After incubation the culture was transferred to a centrifuge tube and stored on ice for 5 minutes. The culture was then centrifuged (Sorvall SS34, 10,000 RPM, 20 minutes, 4°C) to pellet cells and the supernatant was discarded. The pellet was resuspended in a half volume of ice-cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes. The cells were repelletted as before, the supernatant was discarded and the cells were resuspended in a 1/10th original volume of ice-cold 0.1M $CaCl_2$. The cell

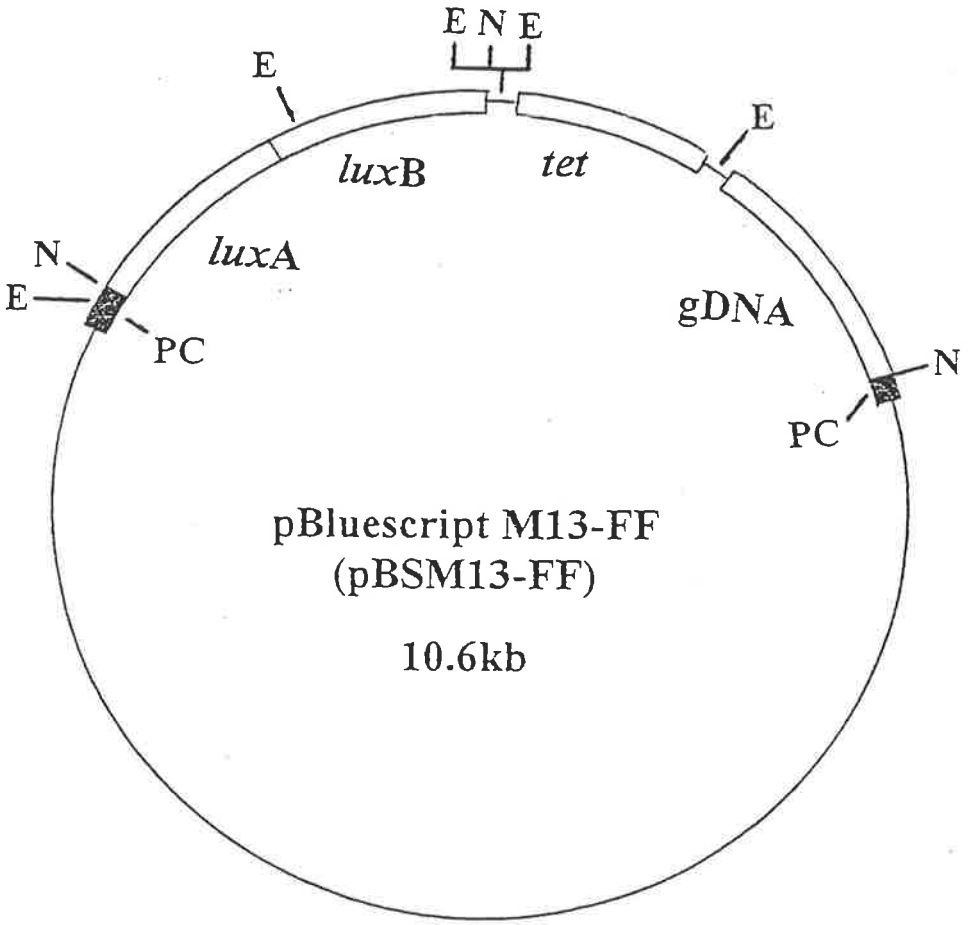


Figure 4.1: Construction of plasmids pBluescript M13-FF (pBSM13-FF) and pBluescript M13-PD (pBSM13-PD). Genomic DNA from mutant strain TM1120 was digested with *Not*I and ligated into pBluescript M13 to create pBSM13-FF (illustrated). The plasmid was transformed into *E. coli* JM83 and selection was made on LB:Tc^{10} medium (refer text). This plasmid was then digested with *Eco*RI to remove transposon DNA and religated to form pBSM13-PD before transforming back into strain JM83. Selection was based on ampicillin resistance and α-complementation (refer text). Plasmid pBSM13-PD was determined to be a 5.1kb plasmid containing the 3.0kb pBluescript plasmid and a 2.1kb *Not*I/*Eco*RI *P. corrugata* genomic fragment flanking the I end of the transposon insertion in TM1120. The 2.1kb fragment was subsequently excised from the plasmid and DIG-labelled for Southern hybridization. PC; polycloning site of pBSM13 and relevant restriction sites shown are E; *Eco*RI and N; *Not*I.

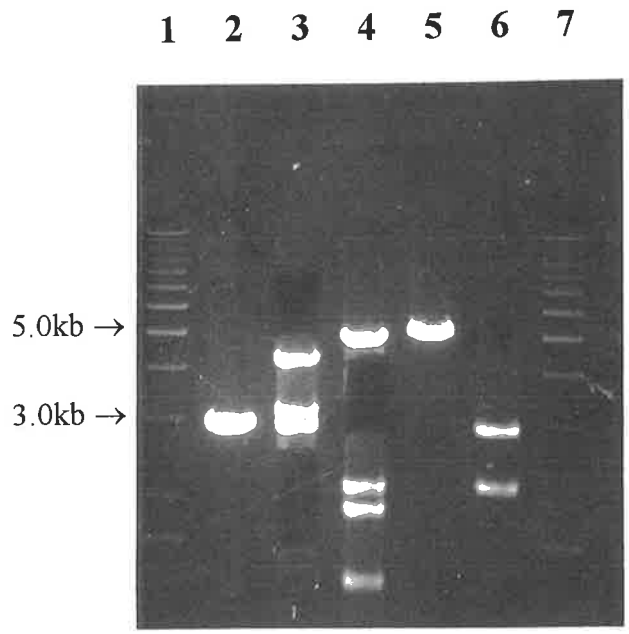


Figure 4.2: Gel electrophoresis of digested plasmids pBSM13, pBSM13-FF and pBSM13-PD. Lane 1 and 7, Molecular Marker X (Boehringer Mannheim). Lane 2, PBSM13 digested with *Eco*RI. Lane 3, *Not*I digestion of PBSM13-FF showing the 3.0kb plasmid, the 3.2kb *lux* genes and the 4.5kb fragment containing *tet* and a flanking region of *P. corrugata* genomic DNA. Lane 4, *Eco*RI digest showing the 5.1kb pBSM13 plasmid/2.1kb genomic DNA fragment plus other *Eco*RI fragments from the transposon (Figure 3.2). Lane 5, pBSM13-PD digested with *Eco*RI showing a single restriction site and lane 6, *Eco*RI/*Not*I double digestion of pBSM13-PD separating the genomic fragment from the 3.0kb pBSM13 plasmid.

suspension was stored on ice for immediate transformation. Cells not immediately required were stored for up to one week at -70°C. Fresh cells were prepared beyond one week.

(b) Transformation of Competent *E. coli* JM83 Cells

The ligated vector/gDNA plasmid was added to 200µl competent *E. coli* JM83 cells in 0.1M $CaCl_2$, mixed and placed on ice for 30 minutes. The tube was then placed in a 42°C water bath for 2 minutes and then returned to ice for a further 10 minutes. The volume in the tube was then taken to 1ml with LB broth, the tube shaken briefly and then incubated at 37°C for 1 hour. A control was also prepared containing uncut vector and competent cells to test cell competency.

Cells were then plated on LB agar containing Ap^{50} and Tc^{10}. IPTG and X-gal were added to the surface of the LB medium prior to spread-plating cells by the method of Sambrook *et al.* (1989). 50µl was also plated on LB:Ap^{50} with IPTG and X-gal to check success of ligations. 10µl of control transformation cells were plated on LB only to ensure cell viability.

Strains which grew on LB containing tetracycline and ampicillin were subsequently grown overnight in LB:Tc^{10} broth and plasmid DNA extracted by the method described in Section 3.2.9(i). The DNA was digested overnight with *Not*I (Section 3.2.9(ii)) and the digested DNA run on agarose gel (Section 3.29(iii)). A sample of plasmid pBSM13 digested with *Not*I was also run on the gel.

4.2.7(v) Separation of Genomic DNA from Tetracycline Gene

The resultant 7.6kb *Not*I fragment of TM1120 DNA ligated into pBSM13 contained both the 2.2kb *tet* gene from the transposon and a flanking piece of *P. corrugata*

genomic DNA. This plasmid, containing the required insertion, was designated pBSM13-FF (Figure 4.1). The next stage was to remove the transposon DNA from pBSM13-FF.

A 5.1kb DNA fragment comprising plasmid PBSM13 DNA plus the flanking *Pc*2140 genomic DNA was generated by digesting pBSM13-FF with *Eco*RI. Digested DNA was separated by gel electrophoresis (Figure 4.2) and the 5.1kb fragment excised from the gel, religated and transformed into *E. coli* JM83. The new plasmid containing the genomic DNA to be used as the cosmid probe was designated pBSM13-PD. The genomic DNA was separated from the *tet* gene by cutting plasmid pBSM13-FF with *Eco*RI and religating the plasmid and transforming back into *E. coli* JM83. All procedures were carried out as previously described.

Plasmid pBSM13-FF was also digested with either *Bam*HI and *Hind*III as well as *Eco*RI to separate transposon DNA from the plasmid (Figure 3.2). Plasmid pBSM13-FF was also cut with *Not*I as a control. The digested DNA was size fractionated by gel electrophoresis.

4.2.7(vi) DIG-Labelling the Probe for Dot-blot Hybridisation

(a) Isolation and Purification of the 2.1kb DNA Fragment

The 2.1kb genomic fragment was isolated and purified by growing *E. coli* JM83 containing plasmid pBSM13-PD overnight in NB:Ap^{50} at 37°C. Plasmid DNA was isolated as previously described (Section 3.2.10(i)). The plasmid was double digested with *Eco*RI and *Not*I overnight (Section 3.2.9(ii)), separated by gel electrophoresis (Section 3.2.9(iii)) and the 2.1kb band extracted and purified (Section 3.2.10(iv)).

(b) Labelling the Probe with Digoxygenin

The purified DNA was boiled for 10 minutes at 95°C and placed on ice to prevent renaturation. The fragment was labelled by adding to 30 µl of DNA in TE buffer the following: 5 µl hexanucleotide mix, 5 µl dNTL labelling mix and 2.5 µl Klenow enzyme. The volume was taken up to 50 µl with SDDW. The solution was spun briefly and incubated at 37°C overnight.

The reaction was halted by adding 4 µl 0.25M EDTA to the mixture. The labelled DNA was precipitated by adding 6 µl 10mg.ml^{-1} tRNA (prepared as per Sambrook *et al.* 1989), 5 µl 3M sodium acetate solution (pH 5.2) and 150 µl 100% ethanol. This solution was incubated at -20°C for two hours. The mixture was then spun at 4°C for 30 minutes and the supernatant discarded. The pellet was washed once in 70% ethanol, respun and then allowed to dry. The probe was then dissolved in 50 µl TE + 0.1% SDS at 37°C for 10 minutes and stored at 4°C.

4.2.7(vii) Dot-Blot Hybridisation

(a) Purification of DNA

Cosmid DNA from a range of selected cosmids was tested for homology to the probe. Total genomic DNA from both *Pc*2140 and TM1120 were included as positive controls. Cosmid pLA2917 from *E. coli* HB101 was also included as a negative control. All cosmid DNA was isolated from *E. coli* by the method described in Section 3.2.10(i). Total genomic DNA from the two control strains was isolated by the method described in Section 3.2.9(i).

(b) Fixing DNA Samples to a Membrane

Ten microlitres of 20x SSC solution was added to an equal volume of DNA in TE8 buffer. The solution was then boiled for ten minutes to denature the DNA then placed immediately on ice to prevent renaturation. The DNA solutions were then individually spotted onto a nylon membrane (Amersham) in 2µl aliquots and allowed to dry. After drying the membrane was soaked in 1.5m NaCl/0.5M NaOH solution for 5 minutes and then in 1.5m NaCl/0.5M Tris-Cl (pH 7.5) solution for 60 seconds. DNA was fixed on the membrane by the heat/alkali treatment described in Section 3.2.9(v).

(c) Prehybridisation of the Membrane

The membrane was prepared for hybridisation by incubating in 30 mls hybridisation buffer for 2 hours. The buffer comprised per 50 mls, 25 mls formamide, 12.5 mls 20x SSC solution, 10 mls 10% w/v skim milk powder solution, 1 ml 10% aqueous sarkosyl and 1 ml 10% w/v SDS and 0.5 mls H_2O. The buffer was then poured from the membrane immediately prior to hybridisation.

(d) Hybridisation

The labelled probe prepared in Section 4.6.5 was boiled at 95°C for 10 minutes to denature the DNA, pulse spun and then placed on ice to prevent renaturation. The denatured probe was then added to 15 mls of hybridisation buffer (see (c) above) prewarmed to 42°C. The probe/buffer solution was added to the membrane and the membrane was incubated overnight at 42°C.

After incubation the membrane was washed twice with 2x SSC, 0.1% SDS at room temperature for 5 minutes and twice with 0.1x SSC, 0.1% SDS at 65°C for 15 minutes.

The membrane was then washed in washing buffer (300 µl polyoxyethylenesorbitan monolaurate in 300 mls DIG buffer 1 (0.1M maleic acid, 0.15M NaCl, pH 7.5). The membrane was then incubated at room temperature for 1 hour in DIG buffer 2 (90% v/v DIG buffer 1, 10% v/v 10% skim milk). It was then incubated for 30 minutes in anti-DIG-AP-conjugate solution (Boehringer Mannheim) (3 µl conjugate stock solution in 30 mls DIG buffer 2). The membrane was then washed twice for 15 minutes in washing buffer before equilibrating for 5 minutes in DIG buffer 3 (0.1M Tris, 0.1M NaCl, 50mM $MgCl_2$, pH 9.5). Disodium 3-(4-methoxyspiro{1,2-dixetane-3,2'- (5'-chlor) tricyclo-[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD) stock solution was diluted 1:100 in DIG buffer 3 and the membrane soaked in this solution for 5 minutes in the dark. The membrane was then blotted to remove excess moisture and sealed in a plastic bag. The membrane was incubated for 1 hour at 37°C in the dark prior to autoradiography.

Autoradiography and development of the X-Ray film was carried out according to the protocol described in Section 3.2.11(iv) except that film exposure was conducted for 1 - 2 hours at 37°C.

4.2.8 Pot Trials for Complemented Mutants

Derivatives of TM1120 complemented with the cosmid library that displayed *in vitro* antagonism to *Ggt*, a high level of cosmid retention and cosmid insert homology to the probe were tested for restoration of take-all suppression activity in pot trials.

Two independent pot trials were prepared and conducted according to the conditions described in Chapter 2. Both *Pc*2140 and TM1120 were included as controls. *Pc*2140 inoculum was grown in NB, TM1120 was grown in NB:Tc^{10} and complemented strains of TM1120 were grown in NB:Km^{25}. Root disease ratings and water consumption were used to analyse disease suppression by the various strains and controls (Section 2.9). Statistical analysis of the resulting data was carried in accordance with Section 2.12.

4.3 RESULTS

4.3.1 Complementation Mating

With appropriate preparation of donor and recipient cells and under suitable conditions for mating, *P. corrugata* strain TM1120 accepted cosmid pLA2917 from *E. coli* S17-1. Approximately 40-50% of the total cosmid library (2,000 clones) was conjugated into and expressed in TM1120 based on the number of clones isolated on the selective medium.

None of the control strains grew on the selective medium at either 25°C or 37°C. This signifies that the colonies patched from the selective medium and subsequently screened for biological control activity were strain TM1120 containing various cosmid clones.

4.3.2 *In vitro* Antagonism of *Ggt* by Complemented Mutants

All cosmid-containing mutants were screened for restoration of *in vitro* antagonism against *Ggt*. A number of cosmid-containing mutants appeared to inhibit the fungus within 24-48 hours but zones of inhibition usually disappeared after further incubation for a day or two. Six strains showed varying degrees of persistent fungal inhibition. These strains were given a strain designation of TM1120α plus a number: The six strains are listed in Table 4.1. Of these complemented mutants TM1120α6 (pMO142439) and TM1120α8 (pMO142133) (Figure 4.3) showed the largest and most persistent zones of inhibition. When each strain was restreaked for single colonies and the single colonies retested for fungal inhibition, similar results to the initial screening were observed for all 6 strains. The level of *in vitro Ggt* inhibition of each complemented mutant is summarised in Table 4.3.

The characterisation of each complemented mutant is summarised in Table 4.3.

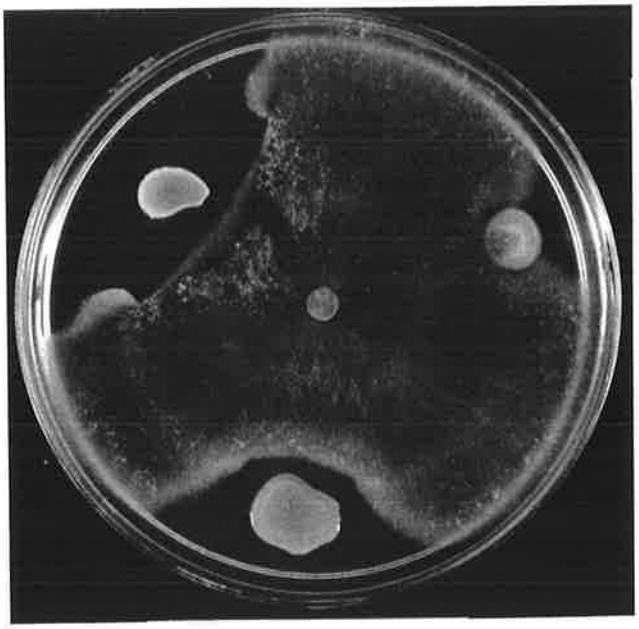


Figure 4.3: *In vitro* inhibition of *Ggt* by *Pc*2140, TM1120 and TM1120α8 containing cosmid pMO142133. Inhibition trials were carried out on PDA medium at 25°C as described in Chapter 2. This photo was taken three days after addition of the bacterial cultures; antagonism by *Pc*2140 and the complemented mutant was still evident after seven days incubation.

Strain	Cosmid	*In vitro* inhibition of *Ggt*[a]	Growth[b] on Km[25]	Homology to Probe[c]
TM1120α1	pMO142614	+	++	±
TM1120α2	pMO142812	+	++	+
TM1120α3	pMO142218	+	+	+
TM1120α6	pMO142439	++	++	+
TM1120α7	pMO141930	+	++	±
TM1120α8	pMO142133	++	++	++

a Inhibition based on size and persistency of zone of fungal antagonism around bacterial colony. ++ strong and persistent inhibition, + smaller but persistent inhibition.

b Strains grown on TZCA medium supplemented with 25μg.ml^{-1} kanamycin. Growth compared to growth of TM1120 on TZCA:Tc[10]. Refer text for details.

c Relative homology to probe between each cosmid. ++ strongest homology to ± weakest homology.

Table 4.3: Summary of characterisation of complemented TM1120 strains.

4.3.3 Stability of the Cosmid in *P. corrugata*

Four of the complemented mutants described in Table 4.1 were tested for the stability of the cosmid in the *P. corrugata* background. These four strains were selected based on *Ggt* inhibition and growth on an agar medium. Of the four strains tested, TM1120α8 containing cosmid pMO142133 showed the greatest degree of stability. Strains TM1120 α2 containing cosmid pMO142812 and TM1120α6 containing cosmid pMO142439 both showed a high level of cosmid retention for the first 5 days but retention was reduced to approximately 50 - 60% by day 7. Strain TM1120α3 containing cosmid pMO142218 displayed poor cosmid retention, counts on agar containing Km^{25} were less than 1% of the counts on the kanamycin-free medium after 3-4 days. Figure 4.4 summarises the cosmid retention rates of the four strains for each day over the 7 day period of the trial.

4.3.4 Dot-blot Hybridisation

4.3.4(i) Construction of the Probe

A previous attempt to construct a probe for the cosmid library using TM1120 genomic fragments digested with *Pst*I and ligated into pUC18 was not successful. While a plasmid conferring tetracycline resistance was detected after transformation into *E. coli* DH5α, subsequent isolation and digestion of the plasmid failed to isolate a fragment of genomic DNA (data not shown). It was concluded that a *Pst*I site was present in the flanking region of genomic DNA in very close proximity to the transposon insertion and that a flanking piece of genomic DNA, if present, may be too small for detection and utilisation as a probe.

Digestion of TM1120 genomic DNA with *Not*I and subsequent ligation into pBSM13 revealed an insert of 7.6kb in length. After recutting the plasmid with *Not*I and

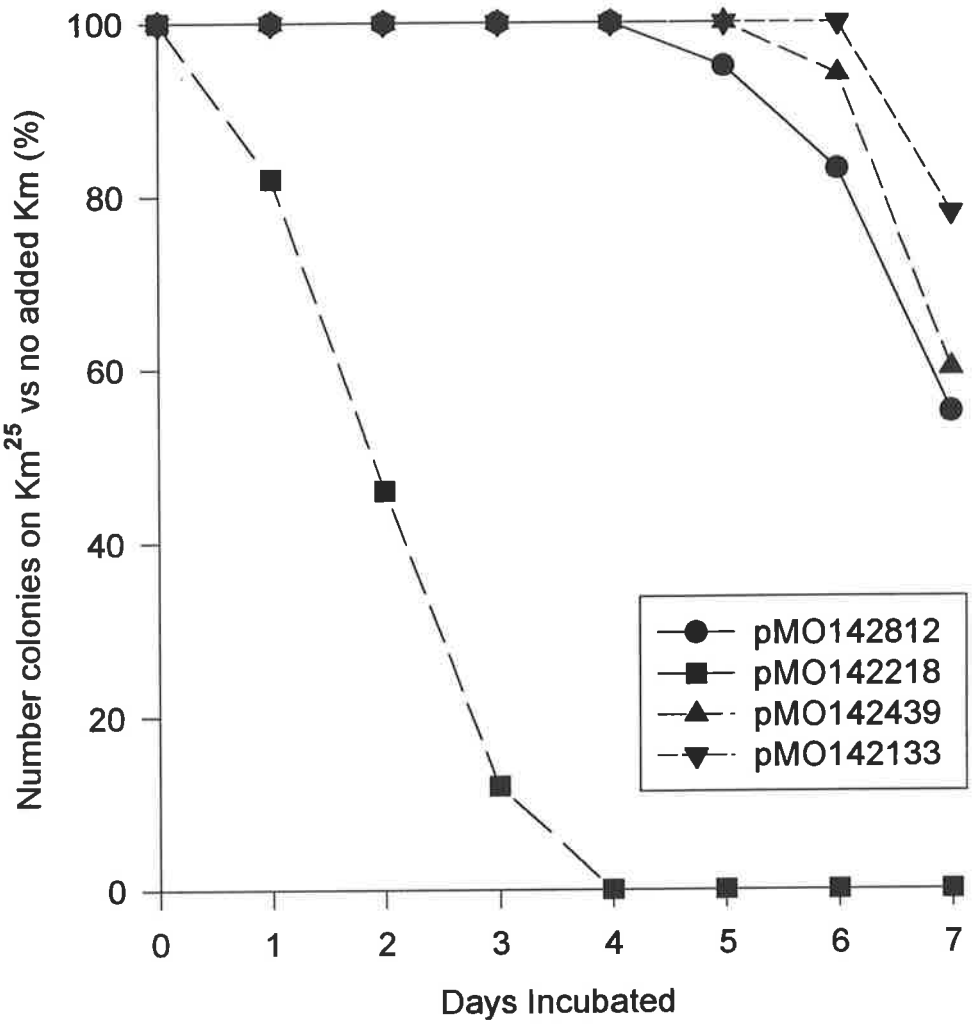


Figure 4.4: Stability of cosmids in *P. corrugata*. Strains were grown in MMBE broth with no antibiotics for seven days at 25°C. Each day a sample was taken from the cultures, serially diluted and plated onto media with and without 25µg.ml^{-1} kanamycin. Colony forming units were counted and the number of colonies growing in the presence of kanamycin were compared to the number on the kanamycin-free medium. The result was expressed as a percentage. Of the four strains tested, TM1120α8 containing pMO142133 was the most stable and TM1120α3 containing pMO142218 was the least stable.

separating the fragments on a gel it was revealed that there were two fragments derived from TM1120 inserted into the plasmid (Figure 4.2). These two fragments were 4.5kb and 3.2kb in length. Digestion with either *Eco*RI, *Bam*HI, or *Hind*III (Figure 4.5) indicated that the 3.2kb fragment consisted of the *luxAB* genes from the transposon (Figures 3.1 and 3.2). This signifies that the *Not*I digestion of TM1120 genomic DNA was not complete and that the *Not*I site between *luxAB* and *tet* on the transposon (Figure 3.2) was not cut in the fragment ligated into pBSM13. *E. coli* JM83 containing pBSM13-FF was tetracycline resistant, therefore it was concluded that the 4.5kb fragment was the *tet* gene from Tn*5*::*luxAB*Tc^r plus genomic DNA from *P. corrugata*.

Digestion and agarose gel electrophoresis of pBSM13-FF with *Eco*RI, *Bam*HI, or *Hind*III did not produce a unique fragment of *P. corrugata* genomic DNA separate from both the *tet* gene and the plasmid (Figure 4.5). In each case the flanking genomic DNA was cut from the *tet* gene (refer Figure 3.2) but could not be cut from the plasmid. This indicated that there were no restriction sites for these three endonucleases in the *P. corrugata* genomic fragment. It was therefore decided to cut pBSM13-FF with *Eco*RI to remove transposon DNA and religate the remaining 5.1kb fragment. This produced a new plasmid, designated pBSM13-PD, containing a 2.1kb *Eco*RI/*Not*I genomic fragment to be used as the probe. *Eco*RI was used as this endonuclease functions under identical conditions to *Not*I, allowing for a double digestion of the plasmid to be performed, rather than two separate digestions as would be the case with *Bam*HI or *Hind*III.

4.3.4(ii) Dot-blot Hybridisation

The labelled 2.1kb probe (Section 4.2.7) hybridised very strongly to both *Pc*2140 and TM1120 genomic DNA. Hybridisation to cosmid pLA2917 was either not detected or appeared as background hybridisation. Of the cosmids which restored *in vitro* antagonism of *Ggt* by TM1120, cosmid pMO142133 (TM1120α8) showed the strongest hybridisation to the probe. Of the other cosmids from the library (Table 4.2)

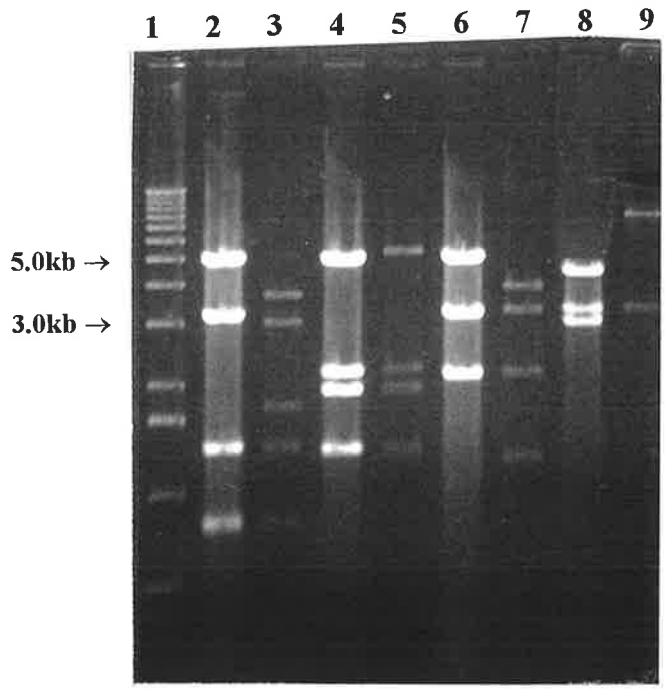


Figure 4.5: Digestion of plasmids pBSM13-FF and pUT::Tn*5* with *Eco*RI, *Bam*HI, *Hind*III and *Not*I. Lane **1** (Marker X); **2**, pBSM13-FF/*Eco*RI; **3**, pUT::Tn*5*/*Eco*RI; **4**, pBSM13-FF/*Bam*HI; **5**, pUT::Tn*5*/*Bam*HI; **6**, pBSM13-FF/*Hind*III; **7**, pUT::Tn*5*/*Hind*III; **8**, pBSM13-FF/*Not*I; **9**, pUT::Tn*5*/*Not*I. None of the enzymes used produced a separate fragment of *P. corrugata* genomic DNA with identical ends which could be religated back into pBSM13 or pUC18. A 5.1kb fragment comprising pBSM13 (3.0kb) and gDNA (2.1kb) was produced when pBSM13-FF was cut with either *Bam*HI, *Eco*RI or *Hind*III. The 5.1kb fragment produced with the *Eco*RI digest was subsequently religated to produce pBSM13-PD (refer text). Digestion of pBSM13-FF with either *Eco*RI or *Hind*III produces a 2.2kb fragment corresponding to the *tet* gene from pUT::Tn*5* when cut with the same restriction enzymes. Digestion of both plasmids with *Bam*HI produced two smaller fragments of 0.8kb and 1.4kb (lanes 2 and 3). It is suspected that there is a possible *Bam*HI site within the *tet* gene (refer discussion in text).

pMO142610, pMO142439, pMO142812 and pMO142218 exhibited strong levels of hybridisation to the probe. The remaining cosmids displayed a level of hybridisation less than the other cosmids but generally greater than pLA2917. Purification of the cosmids by electrophoresis suggested that similar amounts of DNA were added to the membrane for each cosmid (data not shown). It was therefore concluded that the levels of hybridisation observed were indicative of the degree of homology between the probe and the genomic inserts in the cosmids.

4.3.5 Pot Trials of Complemented Mutants

Strain TM1120α8 was selected for testing in a series of *in situ* pot trials for restoration of disease suppression due to its *in vitro* inhibition of *Ggt*, cosmid stability and the high degree of homology between cosmid pMO142133 and the 2.1kb probe.

In the two independent pot trials conducted there was a significant increase in disease suppression on wheat plants by TM1120α8 compared to TM1120. In both trials the incidence of root lesions on the wheat plants was significantly reduced ($P < 0.05$) when the plants were treated with TM1120α8 compared to TM1120-inoculated and control plants (Figure 4.6). Restoration of disease suppression activity by the complemented mutant was not 100%; in the first trial there was a small but significant difference in incidence of Ggt-induced root lesions between the *Pc*2140 and TM1120α8 treatments. In the second trial there was no significant difference between *Pc*2140-inoculated and TM1120α8-inoculated roots although, like the first trial, root lesions on *Pc*2140-inoculated plants was less than on TM1120α8-inoculated plants. No root lesions were observed on the roots of control plants with no added *Ggt* inoculum in both trials (data not shown).

Water consumption rates by the four treatments showed a similar pattern to that observed when trialing mutants of *Pc*2140 for loss of disease suppression discussed in

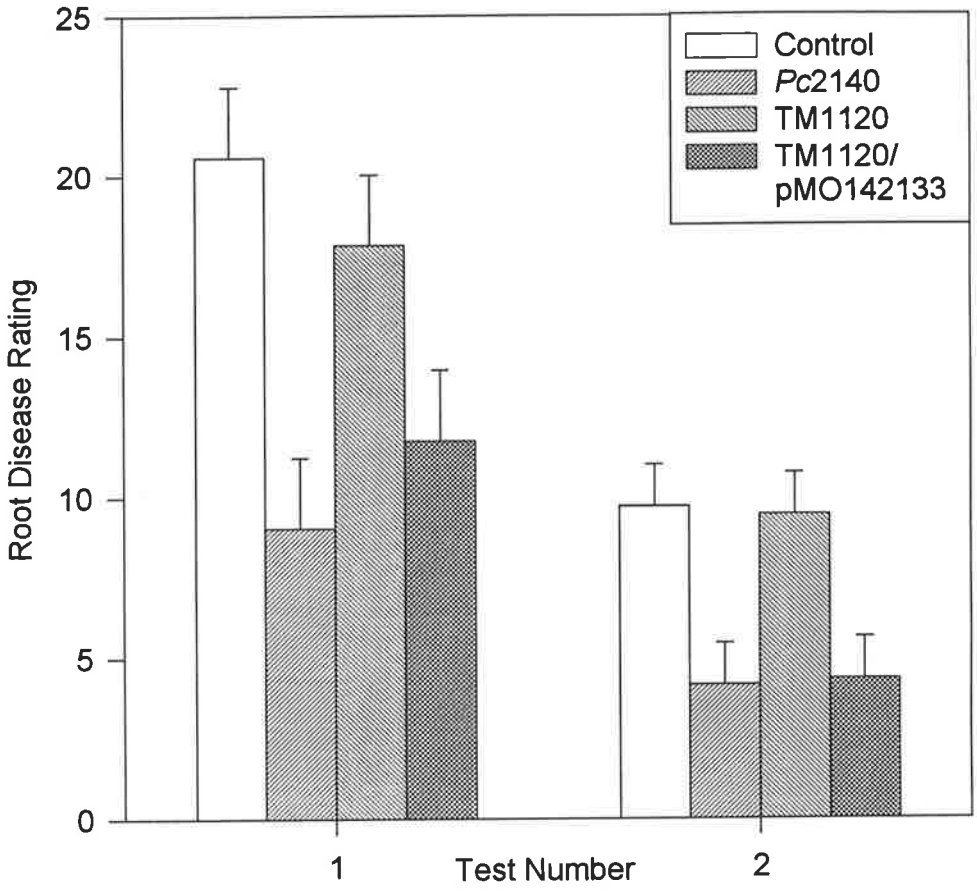


Figure 4.6: Comparison of disease suppression by *Pc*2140, TM1120 and TM1120 α8 (TM1120/pMO142133) by examination of lesions on wheat roots. In both trials strains *Pc*2140 and TM1120α8 offered significantly greater protection than strain TM1120 ($P < 0.05$). Restoration of disease suppressiveness in TM1120α8 was not 100%. In the first trial, incidence of root lesions on plants inoculated with *Pc*2140 was significantly less than TM1120α8-inoculated plants ($P < 0.05$). In the second trial there was no significant difference between the two treatments although the incidence of lesions on *Pc*2140-inoculated roots was slightly less than TM1120α8-treated plants. The overall reduction of take-all lesions observed in the second trial will be addressed in the discussion. Error bars represent LSD between means ($P = 0.05$). Legend codes: Control, *Ggt* only; *Pc*2140, *Ggt* plus inoculation with *Pc*2140; TM1120, *Ggt* plus inoculation with TM1120; TM1120/pMO142133, *Ggt* plus inoculation with TM1120α8.

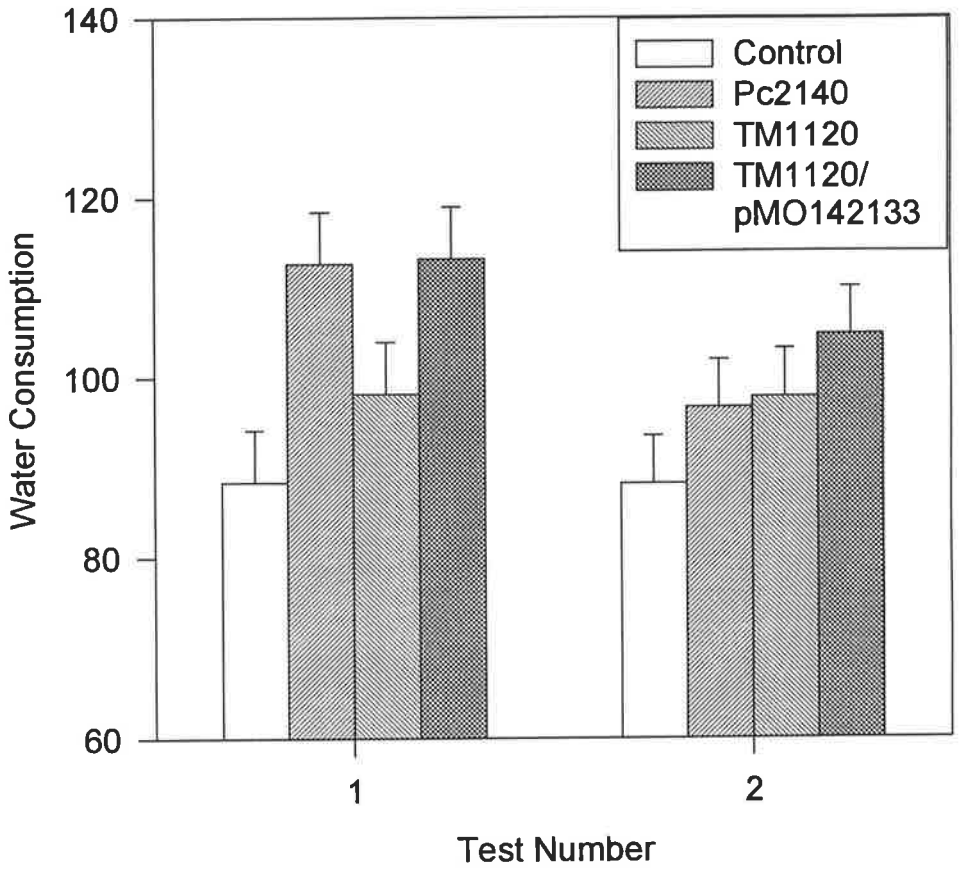


Figure 4.7: Comparison of disease suppression by *Pc*2140, TM1120 and TM1120 α8 (TM1120/pMO142133) by measurement of water consumption by wheat plants. In both trials water consumption by control plants with no added bacterial inoculum was significantly less than the other three treatments ($P < 0.05$). In the first trial, water consumption by both Pc2140-inoculated and TM1120-inoculated plants was significantly greater than TM1120-treated plants. In the second trial there was no significant difference between the *Pc*2140 and TM1120 treatments but water consumption by these plants was significantly less than TM1120α8-inoculated plants. Error bars represent LSD between means ($P = 0.05$). Legend codes: Control, *Ggt* only; *Pc*2140, *Ggt* plus inoculation with *Pc*2140; TM1120, *Ggt* plus inoculation with TM1120; TM1120/pMo142133, *Ggt* plus inoculation with TM1120α8.

Chapter 3. In the first trial, water consumption by plants inoculated with either *Pc*2140 or TM1120α8 was significantly greater than control plants and plants inoculated with TM1120. (Figure 4.7) ($P < 0.05$). In this trial water consumption by TM1120-inoculated plants was also significantly greater than control plants. In the second trial water consumption by control plants was significantly less than the other three treatments ($P < 0.05$) and water consumption by TM1120α8-inoculated plants was significantly greater than either *Pc*2140-inoculated or TM1120-inoculated plants.

4.4 DISCUSSION

Complementation of *P. corrugata* strain TM1120 with *Pc*2140 cosmid library clones generated 6 derivatives of the mutant with varying degrees of increased fungal antagonism. Cosmid pMO142133, containing homologous sequences to the region of the Tn*5* insertion in TM1120, was introduced into the mutant, generating strain TM1120 α8. This complemented strain showed persistent *in vitro* antagonism towards *Ggt* at levels equivalent to *Pc*2140, high cosmid stability and restored disease suppression >80% in 2 independent pot trials. The results obtained from this strain confirms that loss of *in vitro* antagonism of *Ggt* and disease suppression in pot trials by TM1120 was due to the transposon insertion disrupting DNA sequences associated with biocontrol.

Complementation of mutants to restore gene activity may be achieved by two approaches. Firstly, total DNA from the cosmid library fixed on a nylon membrane and a probe as described in this chapter can be used to identify cosmids with homologous sequences. These cosmids can then be conjugated into the mutant and the cosmid-containing strains be screened for restored activity. As an example this approach has been used to identify genes involved in the production of 2,4-diacetylphloroglucinol by *P. aureofaciens* Q2-87 (Vincent *et al.* 1991). The second approach, as described in this chapter, is to mate the cosmid library *en masse* with the mutant, isolate mutants with the cosmid on a selective medium then screen for restored activity and DNA homology.

Complementation by this approach has been used to identify genes involved in biocontrol including 2,4-diacetylphloroglucinol (Fenton *et al.* 1992; Keel, *et al.* 1992) and phenazines (Thomashow and Weller, 1988). This second approach is more useful than the first method where *in vitro* identification of restored activity is easily distinguishable from mutant activity, for example, pigment production on a specific medium or, in the case described in this chapter, restoration of fungal antagonism where no antagonism was observed in the mutant. In the case of complementation of mutants TM27, TM129 and TM300 (Chapter 3) where reduction of *in vitro* antagonism of *Ggt* is less readily discernible, it is suggested that the first approach whereby a probe is used to identify homologous sequences before mating takes place be adopted.

The conditions of triparental mating of TM1120 for complementation appeared to be a factor in the frequency of conjugation. Of the 2,000 cosmid clones in the library, approximately 800 successfully conjugated into TM1120 based on the number of isolates on the selective medium. A prior check of the viability of the cosmid library when grown on NA:$Km^{25}Tc^{10}$ indicated that over 98% of the clones grew on this medium (data not shown). All efforts were made to reproduce the conditions that were used to mutate *Pc*2140 in Chapter 3 including cell preparation; ie growth conditions and cell washes, to conditions of mating; ie media and temperature. It was noted that the successful matings generally occurred in wells close to the perimeter of the microtitre dishes. This suggests that aerobic conditions may play a role in successful matings and that a build up of volatiles (including HCN) in the central wells may have reduced the possibility of conjugation taking place. This hypothesis may have been tested by undertaking matings under the same conditions but in microtitre dishes with the lids removed and incubated in a sterile incubator. This method would eliminate the build up of volatiles in the central wells. As the original mating experiment produced complemented mutants with restored biocontrol activity this hypothesis was not tested within the context of this project.

Results from the pot trial indicate that biocontrol activity by pMO142133 in TM1120α8 was not fully restored. In the first trial the reduction in the incidence of root lesions on plants inoculated with TM1120α8 was approximately 75% compared to *Pc*2140-inoculated plants. In the second trial disease suppression by TM1120α8 was almost identical to the parent strain. These results are also reflected in the relative zones of *Ggt* inhibition (Figure 4.3) where the zone around *Pc*2140 is slightly larger than that around TM1120α8. Full restoration of both *in vitro* antagonism and *in situ* disease suppression is not often achieved with this methodology. For example, mutagenesis and complementation of *P. aureofaciens* Q2-87 for the disruption of DAP production and biological activity resulted in complemented mutants that antagonised *Ggt in vitro* to the same level as Q2-87 but only restored disease suppression (measured by root lesions) to 75-80% of that of the parent strain (Vincent *et al.* 1991). Other examples where complementation did not restore 100% gene activity both *in vitro* and *in situ* are cited; DAP production by *Pseudomonas* strains (Fenton *et al.* 1992; Keel, *et al.* 1992) and phenazine production by *P. fluorescens* 2-79 (Thomashow and Weller, 1988).

There are a number of possible explanations for these results. Firstly, the stability of the cosmid must be considered. If the cosmid is not 100% stable then the loss of the cosmid which restored activity through successive generations would result in an overall reduction of gene expression by the bacterial population both *in vitro* and in the wheat rhizosphere. This problem would manifest itself in two different ways when testing either *in vitro* or in pot trials. Under *in vitro* conditions on a suitable medium, the rapid growth and mean generation time of the strain would result in a loss of the cosmid within the population of the colony through cell division and cell death. In the wheat rhizosphere growth is less rapid than with the "premium" conditions of *in vitro* growth but the length of time for the pot trials to take place (4 weeks) suggests that loss of the cosmid may still occur within the bacterial population. As an example partial instability of cosmid pME3128 in *P. fluorescens* CHA625, a Tn*5*-induced mutant of CHA0, that

was unable to produce DAP was suggested as a likely reason why DAP production was not fully restored in the complemented mutant (Keel *et al.* 1992).

Another factor that may influence the level of restoration of biocontrol activity in the complemented mutant is metabolic stress on the cell induced by the presence of the cosmid. Insertion of a cosmid which may be up to 50kb in length and containing numerous genes being expressed may reduce the overall metabolic ability of the cell, thus reducing its growth rate along with other functions which may have a role, albeit indirect, in biocontrol. Growth of the complemented TM1120 strains on solid agar media containing kanamycin appeared to be slower than growth of TM1120 on the same medium supplemented with tetracycline instead of kanamycin. This may be due to metabolic stress and/or cosmid stability over time reducing the number of viable cells that can grow in the presence of the antibiotic. In media not containing any antibiotics, TM1120α8 grew as rapidly as TM1120 in the same medium as observed during the trials for cosmid stability (Section 4.3.3). Based on these observation it is concluded that cosmid stability, rather than metabolic stress, was an important factor influencing the degree of restoration of biocontrol activity in TM1120α8.

Molecular factors also may influence the biocontrol activity of complemented mutants. Many factors involved in biocontrol including antibiotics such as DAP (Fenton *et al.* 1992) and siderophores (Dowling and O'Gara, 1994) are often the products of more than one gene sequence and siderophores. Production of the biocontrol factor can therefore depend on the expression of at least one operon of genes. Introduction of wild-type genes into a mutant strain via a cosmid results in biocontrol genes functioning in *trans*. If the genes in the parent strain were *cis*-operating then the overall expression of the operon involved in biocontrol will be reduced due to spatial separation of the expressed genes within the one operon. This phenomenon would result in a reduced level of product formation and therefore reduce the biocontrol activity of the complemented mutant. Measurement of the amount of gene product produced by the complemented

mutant compared to the parent strain by such techniques as spectrophotometry, either with purified product or HPLC analysis of supernatants of both strains may give an indication of the degree of operon expression in the complemented mutant. Another method would be to introduce reporter genes into the operon in the parent strain and into the cosmid (which would then be introduced back into the mutant) and measure the levels of activity of expression of these genes in both strains.

A second molecular factor that may influence the expression of cosmids in a mutant is the presence of parent strain "negative" genes contained within the cosmid insert. For example, it was suggested that as-yet uncharacterised negative genes reduced expression of biocontrol genes when mutants of *P. aureofaciens* Q2-87 were complemented for restoration of DAP production (Vincent *et al.* 1991). Two cosmids, designated pMON5117 and pMON5118 restored DAP production in a DAP-negative transposon-induced mutant of the biocontrol strain. Cosmid pMON5119 showed homology to the genomic DNA present in the other two cosmids yet DAP production was not detected in a mutant carrying this cosmid. It was suggested by the authors that pMON5119 contained genes that repressed the synthesis of DAP.

No cosmids with homologous sequences to pMO142133 (ie, cosmids that hybridised to the 2.1kb probe) were identified that did not restore biocontrol activity by strain TM1120. To identify such cosmids would require the probing of total DNA from the cosmid library and undertake matings and screenings as described in this chapter. This work however, was not considered to be within the primary aims of this project. Furthermore, presence of so-called negative genes repressing gene expression and biocontrol activity appear to be an all-or-nothing response; ie, no restored biocontrol activity detected. Therefore it is not considered that the presence or otherwise of these genes explain why restoration of biocontrol activity in complemented mutants is often not 100%.

In the case of TM1120α8, cosmid stability was completely 100% over the seven day trial (Figure 4.4). In contrast, growth of the complemented mutant in the absence of kanamycin did not appear to be compromised when compared to TM1120. It is concluded that cosmid stability together with the possibility that gene expression was not 100% due to *trans*-acting regions of the operon disrupted by the transposon insertion accounted for the reduced biocontrol activity in TM1120α8.

CHAPTER 5

METABOLITES OF *P. CORRUGATA* 2140 AND BIOCONTROL OF *GGT*

5.1 INTRODUCTION

The final stage of the project was to screen *P. corrugata* 2140 for a range of metabolites antagonistic to *Ggt* which were involved in the suppression of take-all symptoms in pot trials. Biocontrol agents of plant pathogens, including species of the genus *Pseudomonas*, usually release one or more metabolites that directly antagonise the pathogen and may therefore result in a reduction in plant infection and incidence of disease symptoms. These compounds include antibiotics, siderophores, volatiles such as hydrogen cyanide and ammonia, peptides, and cell wall-degrading enzymes including chitinase and glucanase. The role of various metabolites in disease suppression has been discussed in Chapter 1.

This stage of the project was divided into two parts. Firstly, *P. corrugata* 2140 was extensively screened for the *in vitro* production of compounds found to be produced by other microbial biocontrol agents that suppress fungal plant pathogens. The screening process concentrated on those groups of compounds known to be produced by *Pseudomonas* strains to determine what metabolites produced by *Pc*2140 may be potentially antagonistic to *Ggt*. Once a list of candidate compounds was compiled comparisons were made between *Pc*2140 wild-type and mutant strain TM1120.

Mutant strain TM1120, because of its almost complete loss of biocontrol activity, was selected as the primary comparison with *Pc*2140. TM1120 was selected because candidate compounds produced in similar amounts by both *Pc*2140 and TM1120 can be eliminated as the primary mechanism of biocontrol. The argument follows that

compounds produced by a strain of bacteria that has little or no activity against *Ggt* do not have a role in the biocontrol of take-all of wheat. Antagonistic compounds produced by *Pc*2140 but not by TM1120 may then be considered for further investigation for the role in biocontrol.

5.2 MATERIALS AND METHODS

5.2.1 Strains and Culture Conditions

P. corrugata 2140 and the five Tn*5*-induced mutants described in Table 3.2 were used to determine the role of various metabolites of the biocontrol strain in take-all control. Strains used as controls in various bioassays are described where appropriate. Unless otherwise stated all strains were maintained and cultured as described in Chapter 2.

5.2.2 Siderophores

5.2.2(i) Detection of Siderophores

Production of siderophores by *P. corrugata* strains was detected on a siderophore detection (SID) medium (Appendix 1) prepared by the method of Schwyn and Neilands (1987). Strains were grown overnight on TZCA medium with appropriate antibiotics and a single colony was stabbed into the SID medium with a sterilised toothpick and incubated at 25°C. Plates were inspected daily for orange/yellow zones around the colonies signifying siderophore activity.

Production of fluorescent siderophores by *P. corrugata* 2140 was undertaken on Kings B agar medium (King *et al*, 1954). A medium comprising Kings B agar supplemented with 100μM $FeCl_3$ was used as a negative control. *P. fluorescens* strain 2-79, which produces the fluorescent siderophore pyoverdine (Hamdan *et al*. 1991) was included in the test as a positive control.

Strains were grown overnight at 25°C and a single colony stabbed into the media as previously described. Plates were incubated for 36-48 hours and then examined under UV_{366} for fluorescent halos surrounding the colonies.

5.2.2(ii) Quantification of Siderophore Production

Production of siderophores by *P. corrugata* strains was quantified by measuring the zone of iron chelation surrounding each colony after 24 and 48 hours' incubation on the SID medium at 25°C. Four measurements at right angles to each other were taken from the edge of the colony to the outer edge of the chelation zone (Figure 2.1) and the average for each set of four measurements calculated. Ten replicates were prepared for each strain.

5.2.3 Volatile Production and Antagonism of *Ggt*

The *in vitro* production of volatiles antagonistic to *Ggt* by *P. corrugata* strains was examined. Production of volatile compounds by *Pc*2140 and mutants described in Chapter 3 was measured by the split-plate assay described in Section 2.7.2. A control treatment with no added bacteria was also prepared. There were ten replicates for each treatment and plates were incubated for 5 days at 25°C. Plates were examined daily for *Ggt* growth which was measured by the method described in Section 2.6.

5.2.4 Hydrogen Cyanide

5.2.4(i) Detection of Hydrogen Cyanide

Production of HCN was detected by the Prussian Blue method (Gettler and Goldbaum, 1947). Strains were inoculated as a single streak on a 2% PP3 agar slope. A disc of Whatman filter paper #50 presoaked firstly in $FeSO_4$ (5.0g per 50mls water) and then

20% w/v NaOH was placed inside the lid of the slope vial so that the disc did not come in contact with the medium or bacteria. The lids were tightly sealed and the cultures incubated at 25°C for 2 days. After incubation the discs were removed with forceps and placed in 2M HCl until a blue colour signifying HCN production appeared.

5.2.4(ii) Quantification of HCN

The production of HCN was quantified by the method of Guilbault and Kramer (1966) as modified by Keel *et al.* (1989). *P. corrugata* strains were grown overnight on TZCA with appropriate antibiotics and single colony was suspended in 0.9% w/v saline, vortexed and pelleted. The supernatant was discarded and the pellet resuspended in 1ml saline solution. 100µl of the cell suspension was placed in 50 mls CME broth (modified after Keel *et al.* (1989) (Appendix 1) in 250ml flasks capped with a subaseal. Incubation was for 60 hours at 25°C at 200 RPM. Ten replicates per strain were prepared.

After incubation 10 mls of 4.2M NaOH was added with a hypodermic syringe through the seal to ionise the HCN. The flask was shaken vigorously for 5 - 10 seconds and allowed to stand for 5 minutes before reshaking. A 0.2 ml culture sample was removed and added to 1.0 ml of 0.088M NaOH and mixed. A 0.6 ml volume of this solution was added to a mixture of 1.0 ml of 0.1M *o*-dinitrobenzene (Sigma) and 1.0 ml of 0.2M 4-nitrobenzaldehyde (Sigma) in ethylene glycol monomethyl ether (Sigma). The sample was incubated at 20°C for 30 minutes without mixing. Absorbance was measured with a spectrophotometer at 578nm. Samples requiring dilution before absorbance readings were diluted with 0.1M NaOH prior to addition to reagents.

A standard curve for calculated HCN concentrations was prepared (Appendix IIIA). Potassium cyanide at varying concentrations was prepared in 0.1M NaOH before adding to the reagents. A blank containing NaOH solution only also was prepared.

5.2.4(iii) Sensitivity of *Ggt* to HCN

A series of tenfold dilutions of a solution of KCN was prepared in 10^{-4}M NaOH. The KCN stock solutions were added to half strength PDA medium (Difco, Detroit). Five replicates of each KCN strength medium were prepared. An equivalent volume of 10^{-4}M NaOH only was added to each control. Agar plugs from the growing edge of a *Ggt* inoculum were placed in the centre of each plate and the plates were sealed with plastic film. The plates were incubated at 25°C and inspected daily for growth. Hyphal growth was measured from the edge of the initial plug inoculum to the leading edge of the hyphal mat by the method described in Figure 2.1.

5.2.5 Ammonia

5.2.5(i) Culture Conditions

P. corrugata 2140 and TM1120 were grown overnight on TZCA and TZCA:Tc[10] respectively. A single colony was suspended in 0.9% w/v saline solution, vortexed and centrifuged to pellet cells. The supernatant was discarded and the cells resuspended in 0.5mls saline.

The culture apparatus for gas collection was prepared as follows: Air was bubbled through 100mls 1M H_2SO_4 to remove any ammonia present. The ammonia-free air was then bubbled through 100mls Medium 'N' (refer Appendix 1) inoculated with 100µl cell suspension described above. Gases from the culture vessels were then passed through a gas trap containing 1ml 1M H_2SO_4. The cultures were incubated at room temperature with constant stirring for 3 days.

5.2.5(ii) Detection of Ammonia

Ammonia production was detected by the ninhydrin reaction as described by Amato and Ladd (1988). After 3 days incubation of the cultures, the 1ml of H_2SO_4 in the gas trap was removed and diluted with 4 volumes of double-distilled water. The samples were then treated with the ninhydrin reagent solutions as described by Amato and Ladd (1988). Samples were then analysed colourmetrically on a spectrophotometer at A_{570}. Three replicates for each strain were prepared.

5.2.5(iii) Standard Curve

A standard curve was prepared by dissolving $(NH_4)_2SO_4$ in 0.2M H_2SO_4 to the following concentrations: 0.05mM, 0.2mM, 0.4mM and 1.0mM. The solutions were developed with ninhydrin and diluted with ethanol by the method described above. Five replicates of each concentration were prepared and a standard curve prepared (Appendix IIIB).

5.2.6 Manganese Reductase

Production of manganese reductase (MR) by *P. corrugata* strains was detected by the method of Marschner *et al.* (1991). Manganese reductase production was detected on a MR detection medium modified after Marschner *et al.* (1991) (Appendix 1). Manganese (as Mn^{4+}) was added as a sterile stock solution to a final concentration of 0.54g $KMnO_4$ per litre. Addition of permanganate to the medium converted the purple Mn^{7+} to brown Mn^{4+}.

Strains were stab-inoculated with toothpicks into the MR detection medium and incubated at 15 and 25°C for 3 days. Clear zones in the brown medium surrounding the colonies signified MR activity. Production was quantified by the method described by

Marschner *et al.* (1991). *P. fluorescens* 2-79 (Marschner *et al.* 1991) was used as a control.

An alternative medium and incubation protocol was used to confirm manganese reductase activity by *P. corrugata* strains. Strains were grown on an agar medium as described by Rogers (1969). MnO_2 was added as a sterile powder after the other components were autoclaved and cooled to 50°C.

A single colony of *P. corrugata* growing on TZCA medium was streaked down the centre of a plate containing the Mn-reductase agar medium. Plates were then incubated at either 15 or 25°C for 4 weeks and then inspected for clear zones surrounding the bacterial streak (Elmer, 1995).

5.2.7 Proteases

Production of extracellular proteases by *Pseudomonas corrugata* was detected on half-strength PDA medium supplemented with 1% skim milk (PDA:SM) (Hopwood, 1970). Skim milk solution was prepared as a 10% stock solution and filter sterilised through 0.22µm millipore paper, then added to autoclaved PDA to be produce an opaque medium.

PDA:SM plates were inoculated with *P. corrugata* strains by stabbing the agar medium with toothpicks containing bacterial inoculum. Plates were incubated for 2 - 5 days at 15°C. A clear halo in the medium surrounding the bacterial colony indicated the presence of protease.

Protease activity was also detected by the method of Hankin and Anagnostakis (1975) as described by Lacy *et al.* (1990). Briefly, 4.0g of commercial grade gelatine was hydrated in 50 mls distilled water then added to near-boiling NA and then autoclaved. Plates were inoculated by stabbing bacterial strains into the medium with toothpicks. Plates were incubated for 2 days at 15 and 25°C. Plates were washed with saturated $(NH_4)_2SO_4$

solution to precipitate unhydrolysed gelatine. Clear zones surrounding bacterial colonies indicated protease activity.

5.2.8 Chitinase

Chitinase production was determined on a chitinase medium (Hankin and Anagnostakis, 1975) (Appendix I) by the presence of halos surrounding a colony grown on agar containing colloidal chitin. The chitinase medium was modified by substituting 0.5% glucose for the yeast extract. A similar medium not containing glucose was also prepared. Chitin (Sigma) was prepared as a sterile colloidal suspension by the method of Campbell and Williams (1951) and added to the remaining ingredients after autoclaving. Plates were prepared as 2mm thick overlays on water agar.

P. corrugata strains were prepared by growing overnight on TZCA with appropriate antibiotics at 25°C. A chitinase-producing bacterium isolated from soil beneath a domestic compost heap (P. Brisbane, pers. comm.) was used as a positive control. The control strain was maintained on chitinase medium at 25°C and picked directly from the plate. Strains were inoculated onto the chitinase medium by stab culture and incubated at 15 and 25°C for 7 days. Plates were inspected for the appearance of clear halos surrounding the colonies where colloidal chitin was catabolised.

5.2.9 Cellulase

All *P. corrugata* strains were grown overnight on TZCA with appropriate antibiotics at 25°C. *P. fluorescens* strain 2-79 was used as a positive control and grown on NA at 25°C. A sterile toothpick was used to stab each strain into the cellulase detection medium (modified after Wollum, 1982). Strains were incubated on inoculated agar plates for 5 days at either 15 or 25°C.

The staining method of Gupta and Roper (1994) was used to detect cellulase activity on the medium. An aqueous solution of 0.1g/100mls congo red was poured onto each plate. Plates were incubated at room temperature for 15 minutes with gentle agitation. The congo red was removed, rinsed twice with SDW and replaced with a 1M solution of NaCl. The plates were incubated as before for a further 30 minutes, then the NaCl solution was poured off. Yellow zones around the bacterial colonies in the now red medium indicated cellulase activity.

5.2.10 β - 1,3 Glucanase

β-1,3 glucanase was detected by the glucose oxidase reaction (Sigma) as described by Fridlender *et al.* (1993). *P. corrugata* strains were grown on a β-1,3 glucanase detection medium as described by Elad *et al.* 1982). Laminarin (Sigma) was added to a final concentration of 0.2% w/v and the medium was filter sterilised through a 0.2μm millipore filter (Whatman) before inoculation with bacteria. Cultures were grown as 20ml batches on a rotary shaker at 25°C for 7 days.

After incubation powdered $(NH_4)_2SO_4$ was slowly added to the cultures to saturation point to precipitate proteins. The samples were then centrifuged at 18,000g (4°C, 15 mins) and the supernatant discarded. The pellet was then redissolved in 0.5 volumes DDW. The samples were then dialysed for 48 hours in DDW with gentle agitation at 4°C (10kDa dialysis tubing (Sigma)). Water was changed regularly to ensure complete dialysis. Dialysis tubing was prepared by the method of Sambrook *et al.* (1989).

Dialysis was completed when the water surrounding the dialysis tubes was negative for ammonia as detected by Nessler's reagent. After dialysis was complete a 1ml sample (negative control) from each strain was removed and boiled for 10 minutes to destroy enzyme activity. The glucose oxidase reactions were prepared as per the manufacturer's instructions and the final reaction cocktail was read spectrophotometrically (A_{500}).

5.2.11 Water-soluble Antibiotics

5.2.11(i) Production and Initial Isolation

A single colony of *P. corrugata* grown overnight on TZCA with appropriate antibiotics was removed from the plate and resuspended in saline by the method described in Section 5.5.1. 100µl of the cell suspension was then spread-plated onto sucrose-asparagine medium (SAFe) (modified after Scher and Baker, 1982) (Appendix I). The agar concentration was 0.375% w/v. Plates were then incubated lid-side up for 8 days at 15°C before harvesting.

After the incubation period the agar was removed from the plates and broken up into pieces 1cm^2 approx. The agar was then centrifuged at 15°C to separate the agar and cells from the aqueous phase (12,000g, 20 mins.). The aqueous supernatant was decanted and replaced with an equal volume of acetone and the agar was resuspended before recentrifugation as above. The acetone phase was then decanted and the remaining agar/cell pellet was discarded.

The acetone was then evaporated by passing dry N_2 over the surface with mild heating (30°C). During the evaporation the aqueous phase was slowly added to the acetone and the drying phase continued until no acetone could be detected by smell. The pH of the pooled supernatants was then adjusted to 7.0 ±0.1 with 0.1M HCl and the volume then readjusted to the original volume of the aqueous supernatant with DDW. The final supernatant was stored at -20°C.

5.2.11(ii) Partial Purification of Antifungal Compounds

Antifungal compounds in the supernatant prepared in Section 5.2.11(i) were partially purified by partitioning into ethyl acetate as follows: The supernatant (pH 7.0) was added to an equal volume of ethyl acetate in a separation funnel, mixed, then allowed to

stand until the two phases separated. The aqueous phase was removed, leaving the organic phase plus any material accumulating at the interface of the two phases. The pH of the aqueous phase was then adjusted to 6.5 ± 0.05, with 0.1M HCl, mixed with an equal volume of ethyl acetate as before and then separated. This process of sequentially decreasing the pH of the aqueous phase by 0.5 pH units for each ethyl acetate extraction continued until pH 2.0 was reached. After ethyl acetate extraction at pH 2.0, the pH of the remaining aqueous supernatant was readjusted to pH 7.0 with 2.0M NaOH.

A one-tenth volume of DDW was then added to each of the ethyl acetate extractions and were then reduced in volume with dry N_2 to remove the ethyl acetate. The pH of each sample was then readjusted to pH 7.0 with 0.1M NaOH before testing for antifungal activity.

5.2.11(iii) Screening Samples for Antifungal Activity

One ml of each sample prepared in Section 5.2.11(ii) was spotted onto a 1 cm diameter Whatman #50 filter paper disc and allowed to dry. The discs were then sterilised overnight in a chamber filled with fumes from a 1:1 chloroform/methanol source. The discs were then placed "upside-down" on a PDA containing growing *Ggt* hyphae. The discs were placed approximately 5mm from the leading edge of the hyphal mat. The plates were allowed to dry and then incubated at 25^{o}C. Plates were inspected daily for inhibition zones.

5.211(iv) Thin-Layer Chromatography

Samples that displayed antifungal activity were further purified on thin-layer chromatography plates (TLC). Three mls of samples was reduced with dry N_2 to an oily residue. This residue was then streaked onto a silica gel 60 F_{254} TLC plate (Merck). The plates were developed with either a methanol/chloroform (9:1) or butanol/glacial acetic acid/H_2O (3:1:1) running solvents. Running solvent was added to the

chromatography tanks at least 1 hour before the plates were added to ensure atmospheric equilibration.

After development the plate was dried and inspected for UV absorbing and fluorescing zones at λ_{254} and λ_{366} respectively. The silica was then scraped from the plate in R_f 0.1 increments and then the silica eluted first with methanol and then with ethyl acetate. The silica matrix was allowed to dry before the ethyl acetate elution. The elutants were then reduced with dry N_2 and tested for antifungal activity as described above. UV absorbing and fluorescing zones were scraped and eluted separately from the R_f=0.1 increments.

5.2.12 Peptides

Production of peptides by *P. corrugata* 2140 was determined by the Michler's base-ninhydrin reaction as described by Von Arx *et al.* (1976). Detection of peptides was undertaken by developing supernatants of *Pc*2140 on thin layer chromatography sheets as described in Section 5.2.11(iv) and spraying with appropriate reagents as described below.

Supernatants of *Pc*2140 were prepared as described in Section 5.2.11. A sample of pooled crude supernatant was reduced tenfold with dry N_2 and streaked on a TLC plate (silica gel 60 F_{254} TLC plate (Merck)). The plate was developed in butanol:glacial acetic acid (3:1:1) (Leifert *et al.* 1995). After developing, the plate was dried in a stream of warm air. Once dry the plate was sprayed with a sodium hypochlorite solution (Commercial grade sodium hypochlorite solution containing $125g.l^{-1}$ active chlorine diluted 1 in 5) after Von Arx *et al.* (1976). The plate was sprayed to dampness with the hypochlorite solution. The plate was then dried as described above.

After drying, the plate was resprayed with the Michler's base-ninhydrin reagent. The reagent consisted of 5g KI in 100mls H_2O, 0.3g ninhydrin in 90 mls H_2O plus 10mls

glacial acetic acid, and 1.5 mls Michler's base. The Michler's base solution was prepared by dissolving 2.5g 4,4'-tetramethyldiamino-diphenylmethane (TDM) (Sigma-Aldrich) in 10 mls glacial acetic acid. Upon dissolving, 50mls H_2O was slowly added to the solution while continually stirring. The TLC plate was sprayed with the reagent was described above and allowed to dry under a warm air stream.

Peptides were detected by the appearance of blue-green spots which turned to blue-black after a short time. The plates were also examined under UV light to determine whether absorbing or fluorescing zones corresponded to peptides.

5.3 RESULTS

5.3.1 Siderophore Production by *P. corrugata* 2140

P. corrugata strain 2140 produced a distinct orange halo in the blue SID medium surrounding the colony after 24 hour incubation at 25°C. The orange halo, indicating sequestering of iron from the dye complex by bacterial siderophores, displayed a distinct boundary with the blue medium. The radius of the halo from the edge of the colony to the edge of the halo was 3.2mm. After a further 24 hours growth the radius of the halo had increased to 8.4mm. The five mutants described in Chapter 3 were also tested for siderophore activity. All strains showed a similar sized zone of iron chelation as the parent strain except TM1120 which had a slightly larger zone (Table 5.1).

On Kings B medium, *P. corrugata* 2140 did not display any fluorescence in the surrounding medium when examined under UV light at 366nm. The control strain, *P. fluorescens* 2-79, did exhibit strong fluorescence when examined under the UV light. No fluorescence was observed for either strain under UV light when grown on Kings B medium supplemented with ferric chloride.

	INCUBATION TIME	
STRAIN	**24 Hours**	**48 Hours**
P. corrugata 2140	3.2 *a*	8.4 *a*
TM27	3.1 *a*	8.2 *a*
TM129	3.1 *a*	8.4 *a*
TM300	3.1 *a*	8.3 *a*
TM692	3.0 *a*	8.2 *a*
TM1120	3.5 *b*	8.9 *b*

Table 5.1: Siderophore production by *P. corrugata* 2140 and five transposon-induced mutants on SID medium (Schwyn and Neilands, 1987). Siderophore production was determined by measuring the radius of the orange halo from the edge of the bacterial colony to the outer edge of the halo (mm) (refer text for full description). Letters after figures refer to homologous groups with no significant difference between means (P = 0.05). Four of the mutants showed no significant variation in siderophore production when compared to the parent strain. Mutant TM1120 exhibited a small but significant increase in siderophore production.

5.3.2 Inhibition of *Ggt* Growth by Volatile Production

Hyphae of *Ggt* on the control plates (no added bacteria) grew to a radius of 13mm after 5 days incubation at 25°C. Growth on split plates inoculated with *Pc*2140 was restricted to 3.2mm. Hyphal growth of *Ggt* on plates inoculated with the mutants was not significantly different to the plates inoculated with *Pc*2140 ($P \geq 0.05$). Table 5.2 depicts *Ggt* hyphal growth in the presence of the bacterial volatiles.

5.3.3 Production of Hydrogen Cyanide

Treated filter paper exposed to volatiles of *P. corrugata* 2140 turned blue in hydrochloric acid, signifying HCN production. All five mutants tested positive for HCN production as well. Quantification of HCN produced by *Pc*2140 showed a concentration of 236μM. Two mutants, TM300 and TM1120 were also quantified by the same method. The resultant concentrations of HCN produced by each mutant were 233μM and 239μM respectively. Neither concentration was significantly different to that produced by the parent strain.

Radial growth of *Ggt* on a medium supplemented with different concentrations of KCN was significantly reduced at a CN^- concentration of 100μM. ($P < 0.05$). At 10μM growth was not significantly affected. At 10mM, growth of *Ggt* was severely affected (Figure 5.1). The results from these trials suggest that *P. corrugata* 2140 produces sufficient HCN under *in vitro* conditions to antagonise growth of *Ggt*. The implications of these results will be addressed in the discussion.

5.3.4 Ammonia Production

Production of ammonia by *P. corrugata* strain 2140 was not detected. Spectrophotometric analysis of gases trapped during culturing did not reveal any

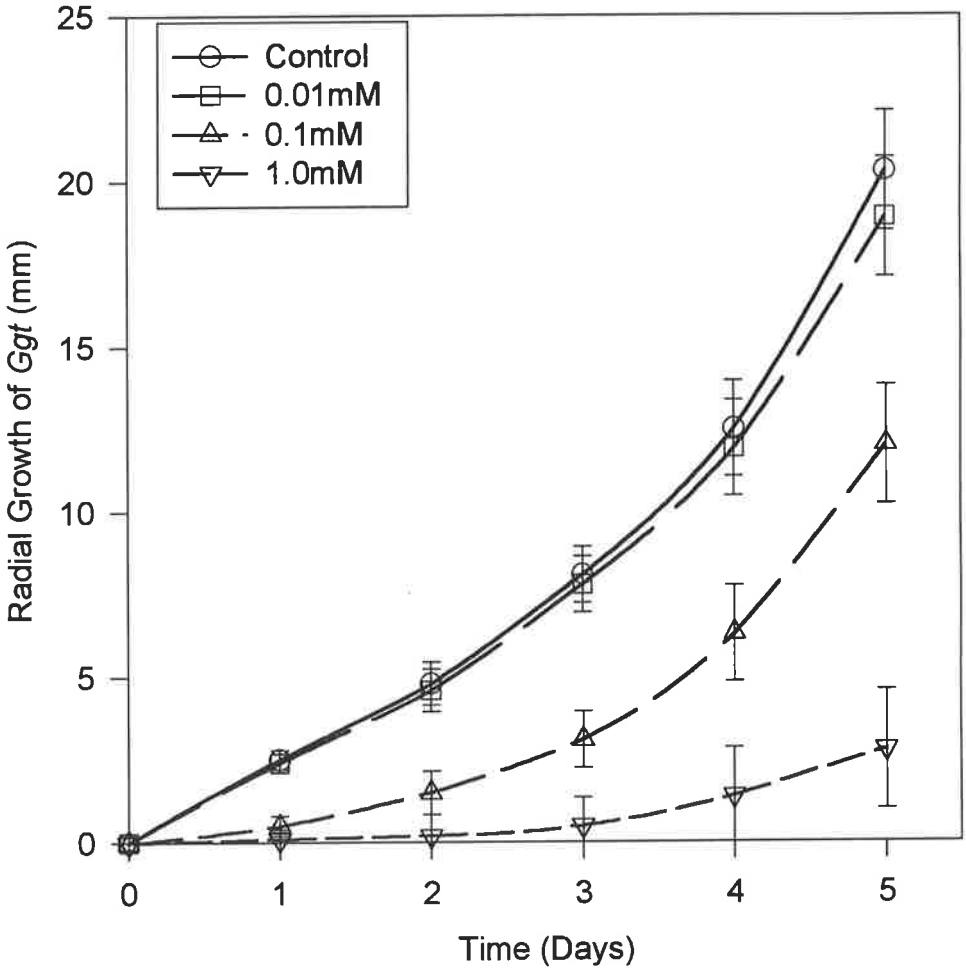


Figure 5.1: Inhibition of radial growth of *Ggt* on half-strength PDA medium supplemented with varying concentrations of cyanide. Cyanide was added as KCN in a tenfold series of dilutions. *Ggt* inoculum was added to each plate as described in Chapter 2. The plates were sealed and inspected daily for radial growth of *Ggt*. Legend: Control, no added KCN; concentrations refer to final [CN^-] of plates prior to adding *Ggt* inoculum. Error bars represent standard error at $P = 0.05$.

STRAIN	Day 1	Day 2	Day 3	Day 4	Day 5
Control	1.42 *a*	3.51 *a*	5.55 *a*	8.60 *a*	12.95 *a*
Pc2140	0.40 *b*	0.45 *b*	0.46 *c*	1.04 *c*	3.19 *bc*
TM27	0.49 *b*	0.49 *b*	0.51 *bc*	0.98 *c*	2.70 *c*
TM129	0.50 *b*	0.50 *b*	0.68 *b*	1.70 *bc*	4.10 *b*
TM300	0.46 *b*	0.48 *b*	0.50 *bc*	1.25 *bc*	4.02 *bc*
TM692	0.37 *b*	0.38 *b*	0.42 *c*	0.90 *c*	2.78 *bc*
TM1120	0.45 *b*	0.50 *b*	0.53 *bc*	0.98 *c*	3.31 *bc*

Table 5.2: *In vitro* inhibition of *Ggt* growth by volatiles of *P. corrugata* strains. Radial growth (mm) of *Ggt* growing on split plates was measured daily for five days. *P. corrugata* 2140 and the five mutants all displayed significant inhibition of *Ggt* through production of volatiles. After five days incubation no significant variation in *Ggt* inhibition was observed between *P. corrugata* 2140 and the five mutants. Letters after figures refer to homologous groups with no significant difference between means (P = 0.05).

ammonia present in the ninhydrin solution. The five mutants of *Pc*2140 were not tested for ammonia production.

5.3.5 Manganese Reductase

On the medium modified after Marschner *et al* (1991) *P. fluorescens* 2-79 produced a distinct clear halo extending from the edge of the bacterial colony for approximately 1mm after 3 days incubation at both 15 and 25°C. No zone of clearing was observed for *Pc*2140 even after scraping the bacterial colonies off the plate. The five mutants were tested in conjunction with *Pc*2140 on the same medium. No zones of manganese reduction were observed for any of the mutants.

On the medium described by Rogers (1969) *P. fluorescens* 2-79 showed poor but detectable reduction of MnO_2. No reduction of the manganese was noted for *Pc*2140 or the five mutants.

5.3.6 Proteases

On both the skim milk medium (Hopwood, 1970) and the gelatine medium (Hankin and Anagnostakis, 1975) *P. corrugata* 2140 produced distinct zones of clearing, indicating protease production. Protease activity appeared to be greater at 15°C than at 25°C but the zones of clearing on both media were not significantly different at $P = 0.05$.

The five mutants were tested in conjunction with *Pc*2140. All five strains showed near identical zones of protease activity as the parent strain on both media and at both temperatures.

5.3.7 Cell Wall-Lytic Enzymes

On the chitinase medium the fluorescent pseudomonad used as a positive control produced a distinct halo in the surrounding medium generated by the degradation of colloidal chitin by chitinase. Although this halo could be visually detected with the unaided eye, the halo was much more apparent when observed under low magnification (16x) with a dissecting microscope. Growth of the control strain appeared quite strong on both the chitin medium and the same medium supplemented with glucose.

P. corrugata 2140 grew steadily on the chitin medium supplemented with glucose. No zone of clearing by *P. corrugata* 2140 was observed on this medium, either visually or under the dissecting microscope. *P. corrugata* 2140 grew extremely poorly on the glucose-free chitin medium. Again, no zone was observed around the colony. The five mutant strains were not tested for chitinase activity.

When tested for β-1,3 glucanase production, *P. corrugata* grew poorly in detection medium described by Elad *et al.* (1982). As a consequence, β-1,3 glucanase activity was not detected in the supernatants of *Pc*2140. Production of a standard curve and detection of enzyme activity by binucleate *Rhizoctonia* strains by the same methodology run in conjunction with the assay for *Pc*2140 are further indications that *Pc*2140 does not produce β-1,3 glucanase. The five mutants were not tested for β-1,3 glucanase production.

P. fluorescens 2-79 produced a distinct halo on the cellulase detection medium when stained with congo red. The zone extended approximately 5mm beyond the edge of the colony. This zone was evident when plates were incubated at both 15 and 25°C. *P. corrugata* 2140 produced a very poor zone of clearing, barely extending 1mm beyond the point of the stab culture. Growth of *P. corrugata* 2140 on this medium was noted to be extremely slow compared to *P. fluorescens* 2-79.

5.3.8 Water-soluble Antibiotics

Partitioning of crude supernatants of *P. corrugata* 2140 into ethyl acetate at different pH values showed several peaks of antifungal activity, suggesting that *Pc*2140 produces more than one compound antagonistic to *Ggt*. When the extracts were tested for *Ggt* antagonism, some extracts produced distinct and persistent zones of fungal inhibition around the paper discs. The results of the ethyl acetate extractions and *Ggt* inhibition tests are summarised in Table 5.3. No inhibition zones were observed when supernatants of mutant strain TM1120 were tested in the same manner. Supernatants of the other four mutants were not tested.

When the pH of the supernatants was adjusted with NaOH from pH7.5 to pH12 and extracted with ethyl acetate, no fraction exhibited antagonism to *Ggt* when tested as described above (data not shown).

Thin layer chromatography of supernatants of both *Pc*2140 and TM1120 were performed in two separate solvent systems. When supernatants were developed in the methanol/chloroform solvent and the plates examined under UV light at both 254 and 366nm, zones of absorbance (254nm) and fluorescence (366nm) were observed. The zones were observed both near the origin and towards an R_f value of 1.0. The bands showed poor resolution and tended to smear across the plate suggesting poor separation of compounds.

When developed in the butanol/glacial acetic acid/water solvent high resolution separation of fluorescing and absorbing bands was achieved. Crude supernatants of *Pc*2140 and TM1120 were subsequently run on the same plate as a comparison. The results of the chromatography trial for these two strains is summarised in Table 5.4.

A number of fluorescing bands with low R_f values were present in the supernatants of both strains. These bands exhibited near identical intensity of fluorescence suggesting that the two supernatants were virtually identical in the concentrations of compounds

pH	Ggt Inhibition *
7.0	++
6.5	++
6.0	+
5.5	++
5.0	±
4.5	-
4.0	++
3.5	-
3.0	±
2.5	++
2.0	+
Residue	-

Table 5.3: *Ggt* inhibition by supernatants of *P. corrugata* 2140 partitioned into ethyl acetate at different pH values. The aqueous supernatant was sequentially partitioned in 0.5 pH unit increments from pH 7.0 to 2.0. Ethyl acetate fractions were partitioned back into water and the pH readjusted to 7.0 before testing for *Ggt* inhibition (refer text for details).

Key for Symbols

++	Strong inhibition of *Ggt* after 3 days incubation
+	Some inhibition of *Ggt* detectable after 3 days
±	Little or no inhibition of *Ggt* detectable after 48 hour inhibition
-	No inhibition of *Ggt* detected at any stage of incubation

R_f value	Band Observed *	Pc2140	TM1120
0.07	strong yellow fluorescence	present	present
0.18	weak blue fluorescence	present	present
0.21	weak blue fluorescence	present	present
0.25	blue-black spot indicating a peptide	present	present
0.29	strong bluish-white fluorescence	present	present
0.57	very strong white fluorescence corresponding to a visible yellow pigment	present	not detected
0.59	weak absorbance	present	not detected
0.63	reasonably strong yellow fluorescence	present	present
0.67	blue-black spot indicating a peptide	not detected	present
0.86	blue-black spot indicating a peptide corresponding to very strong absorbance	present	v. weak spot
0.91	strong yellow fluorescence	present	not detected

* Absorbing bands obseved under UV_{254} light. Fluorescing bands observed under UV_{366} light. Peptide bands observed after spraying with Michler's reagent (refer text for details)

Table 5.4: Absorbing and fluorescing zones plus detection of peptides on TLC plates containing crude supernatants of Pc2140 and TM1120. Plates were developed in a butanol/glacial acetic acid/water (3:1:1) solvent system. For details of individual fluorescing and absorbing bands refer Section 5.19; for details of bands corresponding to peptides, Sections 5.20.

produced by both strains not affected by the Tn*5* insertion. Four bands that either fluoresced or absorbed UV light were present in the supernatants of *Pc*2140 but were either absent or present in very low concentrations in the supernatants of TM1120. These bands are described in Table 5.4. The strong fluorescing band at $R_f = 0.57$ in the supernatants of *Pc*2140 corresponded precisely to a yellow pigment visible to the unaided eye. No corresponding pigment or fluorescence was detected for TM1120 suggesting that the yellow compound was the probable cause of the fluorescence. The absorbance noted at $R_f = 0.86$ for *Pc*2140 was very weak in TM1120 suggesting that production of this compound was severely limited but not eliminated in the mutant. The other two bands observed in *Pc*2140 at R_f 0.63 and 0.91 were not observed in TM1120.

5.3.9 Peptides

Crude supernatants of both *P. corrugata* 2140 and TM1120 were developed on thin layer chromatography plates using a butanol/glacial acetic acid/water solvent system as described above. When sprayed with the Michler's base reagent, two distinct blue-black bands appear on lanes containing the supernatant of *Pc*2140. These bands appeared at R_f values of 0.25 and 0.86. The corresponding bands appear on lanes containing supernatants of TM1120. However the peptide band at R_f =0.86 was much fainter than the corresponding band on the *Pc*2140 lane. This band also corresponded to a region of absorbance of UV light (refer Table 5.4). An extra band at R_f =0.67 was present in TM1120 supernatants that was not present in *Pc*2140. The results of the peptide spraying of chromatography plates containing fractionated *Pc*2140 and TM1120 supernatants are summarised in Table 5.4.

5.4 DISCUSSION

Biochemical assays were performed on *P. corrugata* strain 2140 and TM1120 to determine what metabolites produced by the biocontrol strain may have a role in the

biocontrol of take-all. Compounds produced by both strains were eliminated as likely candidates in biocontrol of take-all. Conversely, those metabolites antagonistic to *Ggt* that are produced by *Pc*2140 but absent in TM1120 may be considered the mechanism of biocontrol of take-all.

P. corrugata 2140 was assayed for a number of compounds found to be produced by other biocontrol strains of *Pseudomonas* and linked to biocontrol activity against a range of phytopathogens. Of these compounds, *Pc*2140 produced siderophores, volatiles including hydrogen cyanide, proteases, water-soluble antibiotics and peptides which may have a role in biocontrol of take-all (Table 5.5). Production of ammonia, manganese reductase, β-1,3 glucanase and chitinase by *Pc*2140 was not detected and production of cellulase by *Pc*2140 was detected in very low quantities. Of these compounds produced by *Pc*2140, it was found that mutant strain TM1120 was deficient in the production of the water-soluble antibiotics and the production of at least one peptide (Table 5.5).

Antibiotics have been found to be a primary mechanism of biocontrol of take-all by a number of *Pseudomonas* strains as detailed in Table 1.2. These earlier observations where a relationship between the direct antagonism of *Ggt* by antibiotics produced by *Pseudomonas* strains and suppression of disease symptoms on wheat provides further support to the observations made regarding control of take-all by *P. corrugata* 2140 by the production of antibiotics.

Results of the ethyl acetate extractions suggest that *P. corrugata* 2140 produces more than one water-soluble antibiotic antagonistic to *Ggt*. Three or four peaks of antifungal activity at different pH values (Table 5.3) indicate that more than one antagonistic compound is produced by the biocontrol agent. These compounds may be closely related to each other and are produced by a single gene cluster or is more likely that more than one gene cluster is responsible for antifungal activity by *Pc*2140. Production of more than one antifungal compound by a biocontrol strain is not unique. *P. fluorescens* 2-79 produces PCA and anthranilic acid which antagonise *Ggt* (Thomashow

METABOLITE	*Pc*2140	TM1120	COMMENTS
Water-soluble Antibiotics	++	nd	no detectable antagonism of *Ggt* by TM1120 supernatants.
Siderophores	++	++	small but significant increase in siderophore production by TM1120
Volatiles inhibitory to *Ggt*	++	++	no significant difference between both strains
Hydrogen Cyanide	++	++	no significant difference between both strains
Ammonia	nd	nt	not detected after culturing *Pc*2140 for 3 days
Manganese Reductase	nd	nd	not detected in any of the 6 *P. corrugata* strains
Proteases	++	++	no significant difference between both strains
Chitinase	nd	nt	not detected for *Pc*2140: no mutants tested
β-1,3 Glucanase	nd	nt	not detected for *Pc*2140: no mutants tested
Cellulase	±	nt	very low level of activity observed with *Pc*2140
Peptides	++	+	some variation in peptide production detected on TLC plates

nd not detected
nt not tested

++ production of metabolite detected
+ production detected but appeared reduced in mutant stain TM1120
± production detected but at very low levels compared to control strain

Table 5.5: Comparison of metabolite production of Pc2140 and TM1120. Where metabolite production was not observed for Pc2140, mutants were generally not tested except where time and facilities permitted. Of all the metabolites examined, loss of production of water-soluble antibiotics antagonistic to *Ggt* and variations in the production of peptides were the only significant changes in metabolite production in strain TM1120.

and Weller, 1990) and *P. fluorescens* CHA0 produces DAP and pyoluteorin which suppresses *Pythium ultimum* (Maurhofer *et al.* 1992).

Variations in UV fluorescing and absorbing bands on thin-layer chromatography plates also suggest more than one compound is involved in biocontrol of *Ggt* by *Pc*2140. Four bands (including one band signifying a peptide) visible on the *Pc*2140 sample were not detected in supernatants of TM1120. Further to these results it is possible that an antagonistic compound produced by *Pc*2140 is not optically active under UV light and therefore remained undetected on the plate. The ramifications of these results will be addressed in more detail in the General Discussion (Chapter 6).

A correlation between peptide production by *Pseudomonas* strains and the control of take-all has yet to be established. Peptides have been previously isolated from a *P. corrugata* strain (Gustine *et al.* 1995) which were detected by a hypersensitive response in tobacco leaves. It was suggested that these peptides may have a potential role in biocontrol of plant diseases. Peptide production by two strains of *Bacillus* also were found to have role in the *in vitro* antagonism of *Borytis cinerea* (Leifert *et al*, 1995) although their role in *in situ* suppression of disease was not established.

Bioassays of *P. corrugata* 2140 grown on the SID medium demonstrates that this strain produces at least one siderophore. Furthermore, the lack of fluorescence under UV light surrounding the bacterial colony grown on Kings B medium indicates that the siderophore(s) produced by *Pc*2140 is non-fluorescent. On the SID medium designed specifically for the detection and quantification of siderophores it was found that TM1120 was more active than the parent strain, ie TM1120 produced significantly larger zones of iron chelation than the parent strain. The reason for this observation (aside from being simply described as a pleiotropic effect) has not been determined. It was originally thought that the slightly more mucoid nature of this strain (refer Chapter 3) may have restricted the uptake of the siderophore/Fe^{3+} complex by TM1120 creating an iron stress. This would result in the continual production of siderophores in an attempt

to obtain iron. However, strain TM692 was found to produce near identical zones of chelation as the parent strain despite its similar appearance (Table 5.1). Furthermore strain TM1120 produced colonies of similar dimensions to the parent strain, suggesting that the growth of this strain was not compromised on this medium. Although TM1120 appears to have a slight increase in siderophore production *in vitro* the strain still exhibited poor biocontrol activity in pot trials. Furthermore, the four other mutants with reduced biocontrol activity all were found to produce siderophores to the same extent as the parent strain. This data suggests that siderophore production by *P. corrugata* 2140 is not an important factor in control of take-all. This conclusion is in line with other investigators' results when determining the role of siderophores produced by *Pseudomonas* strains in biocontrol of take-all where specific siderophore-negative mutants were tested *in situ* (Keel and Défago, 1991; Hamdan *et al.* 1991).

Results from the split-plate trials and detection and quantification of HCN suggested that these compounds have a role in biocontrol of take-all. Furthermore, *in vitro* production of HCN by *Pc*2140 appeared to be in excess of the level of CN^- required to inhibit *Ggt* (Figure 5.1 and Table 5.2) implying that HCN production by *Pc*2140 was sufficient to antagonise *Ggt*. However, both the production of hydrogen cyanide and the *in vitro* antagonism of *Ggt* by volatiles produced by TM1120 were similar to the parent strain. The results obtained with the mutant strain indicate that volatiles including HCN do not play a significant role in the suppression of take-all. Although HCN has been found to play a role in biocontrol of some plant diseases (Voisard *et al.* 1989) no evidence is available that HCN produced by other biocontrol strains suppresses take-all in pot or field trials (Maurhofer *et al.* 1994). The results obtained with strain 2140 tend to agree with these earlier findings that HCN is an insignificant factor in the suppression of take-all. Furthermore, these results are conclusive evidence that the *in vitro* production of a compound which antagonises a pathogen does not mean that the compound has a role in the suppression of disease *in situ*.

In vitro production of manganese reductase by *P. corrugata* 2140 or the five mutants was not detected when grown on the two media described in Section 5.4. Although manganese reductase activity is not considered to be strong for the control strain *P. fluorescens* 2-79 (Marschner *et al.* 1991) clear zones signifying reduction of Mn^{4+} to Mn^{2+} were detectable on both media for *Pc*2140 strain, indicating that the medium was suitable to detect low levels of manganese reductase activity. A recent study of 1,114 bacterial strains isolated from feeder roots of asparagus indicated that fluorescent pseudomonads were more likely to be manganese reductase-positive than non-fluorescent strains (Elmer, 1995). In spite of this, one non-fluorescent strain which was identified as *P. corrugata* was found to be manganese reductase-positive and also controlled *Fusarium* crown and root rot of asparagus. It was hypothesised that the manganese reductase activity of this strain made manganese more available to the plant, increasing its resistance to the disease rather than direct antagonism of the pathogen by the biocontrol agent.

Protease activity was detected for both *P. corrugata* 2140 and the five transposon-induced mutants on two separate media. Although there is no evidence that protease activity by a biocontrol agent directly antagonises a phytopathogen, proteases may have a role in the suppression of take-all symptoms on wheat. It has been suggested that proteases released by a biocontrol agent catabolise cell wall-lytic degrading enzymes such as pectases and cellulases released by a pathogen as a prelude to infection (Borowicz and Pietr, 1994). Inactivation of these compounds by protease activity would subsequently result in reduced plant infection and presence of disease symptoms. The results for *Pc*2140 and TM1120 which have similar *in vitro* protease activities suggest that, although *P. corrugata* 2140 produces proteases, their role in suppression of take-all symptoms may be limited.

Although the production of fungal cell wall-degrading enzymes as a mechanism of biocontrol is often attributed to parasitic fungi such as *Trichoderma*, a number of

bacterial strains including *Pseudomonas* produce cell wall-lytic enzymes (Robbins *et al.* 1988; Lim *et al.* 1991; Fridlender *et al.* 1993). *Ggt*, having a cell wall composition containing chitin and glucan, would therefore be susceptible to biocontrol agents that produce chitinase and/or β-1,3 glucanase. Neither of these two enzymes were detected as metabolites of *P. corrugata* 2140. Furthermore, when the chitinase-positive strain (identified as a fluorescent pseudomonad by GC-FAME analysis (D. Kluepfel, pers. comm.)) was tested for *in vitro* antagonism of *Ggt* on PDA medium, no antagonism was observed (data not shown). It was therefore concluded that cell wall degrading enzymes play no part in the biocontrol of *Ggt* by *P. corrugata* 2140.

It is acknowledged that the lack of growth of *Pc*2140 in a minimal broth containing laminarin as the sole carbon source is not conclusive evidence that β-1,3 glucanase is not produced by this bacterium. To confirm that *Pc*2140 does not produce this enzyme the experiment could be repeated by growing the bacterium in a broth (or semi-solid medium) containing a second carbon compound, one that can be utilised by *Pc*2140 as a source of energy. This compound could supplement the medium in low concentrations to provide enough energy for the bacterium to generate β-1,3 glucanase to catabolise the laminarin to simpler sugars.

Although cellulose is not a constituent of the cell wall of *Gaeumannomyces*, it was tested due to its implications in biocontrol in general. Cellulase activity by a biocontrol agent may be considered a likely mechanism in the biocontrol of other phytopathogens such as *Phytophthora* which contain cellulose in their cell walls. Furthermore the production of cellulase may have a role in the ability of the producing bacterium to colonise the epidermal layer of the roots of the host plants, increasing their root colonisation efficiency and subsequent biocontrol activity. Low levels of cellulase activity exhibited by *P. corrugata* 2140 suggest that although this enzyme is detectable in *Pc*2140 cultures, it is not likely to be a factor in the biocontrol of take-all. Its possible role in the colonisation of roots has not been determined.

In conclusion, comparisons in metabolite production between *P. corrugata* 2140 and mutant strain TM1120 were made to determine the role of various groups of compounds in biocontrol of take-all. The mutant strain was found to be deficient in the production of antibiotics and compromised in peptide production. Production of other potentially antagonistic compounds by TM1120 were found to be similar to the parent strain. Based on these results it has been determined that the primary mechanism of biocontrol of take-all by *P. corrugata* 2140 is through the production of antibiotics and peptides which directly antagonise the fungus. These results have also demonstrated that the *in vitro* production of compounds antagonistic to a pathogen (eg HCN) do not necessarily mean that these compounds have a role in the *in situ* suppression of disease.

CHAPTER 6

GENERAL DISCUSSION

Pseudomonas corrugata strain 2140, a non-fluorescent pseudomonad isolated from wheat field soil in New South Wales, antagonised the take-all fungus *Ggt in vitro* and suppressed take-all symptoms on wheat in glasshouse and field trials. To develop an understanding of the mechanisms of biocontrol, *Pc*2140 was mutated by the random insertion of the Tn*5*::*luxAB*Tc^r transposon into the chromosome. Of 2,500 mutants tested, five non-auxotrophic Tn*5* mutants of *Pc*2140 were found to have reduced *in vitro* antagonism of *Ggt* and reduced ability to suppress take-all symptoms on wheat in a series of pot trials. One of these mutants, designated TM1120, showed poor inhibition of inhibition *Ggt* growth *in vitro* and had lost >95% of its ability to suppress disease in three independent pot trials. The mutant was complemented with a clone from a cosmid library of *Pc*2140 genomic DNA integrated into pLA2917. Complementation with a cosmid containing *Pc*2140 genomic DNA homologous to the region in the chromosome of TM1120 disrupted by the Tn*5* insertion restored both *in vitro* antagonism and disease suppression in TM1120. Biochemical analysis of *Pc*2140 indicated that the strain produced a number of compounds which may potentially antagonise *Ggt* and have a role in biocontrol. These compounds included siderophores, water-soluble antibiotics, peptides, proteases and volatiles including hydrogen cyanide. Lack of production of water-soluble antibiotics and compromised peptide production by TM1120 indicated that these two groups of compounds are the primary mechanism of biocontrol of take-all by *P. corrugata* 2140.

Transposon mutagenesis is often used to eliminate production of one particular metabolite such as a siderophore or antibiotic per mutant. The mutant, deficient in the production of a single compound, is then tested for biocontrol activity to determine the

degree of contribution of that compound in disease suppression. As most biocontrol agents produce a number of compounds with potential disease-control capabilities, there is a requirement to produce a collection of "single-metabolite" mutants, deficient in the production of single compounds but covering the range of potential metabolites to determine which ones have a role in disease control. In this study a mutant, generated by a single transposon insertion, had lost >95% its biocontrol activity *in situ*. This mutant was then compared to the parent strain for the production of the potentially antagonistic compounds. By doing so, a number of metabolites that may have a possible role in biocontrol of take-all have been eliminated as possible mechanisms since they were still produced by the mutant strain at levels comparable to the parent strain. These compounds included siderophores, volatiles including HCN, proteases and common peptides and UV absorbing and/or fluorescing compounds detected by TLC/UV analysis as described in Table 5.4. This approach provided a more rapid process for the identification of the primary mechanism of biocontrol by eliminating the time consuming process of examining each metabolite separately with different mutants.

The nature of the gene which was mutated in TM1120 has not been elucidated. The number of *Ggt*-antagonistic compounds produced by *Pc*2140, combined with the extent of loss of biocontrol activity in TM1120, suggests that a regulatory gene that controls the expression of a range of antagonistic factors rather than a structural gene was disrupted by the transposon insertion. The role of regulatory genes in biocontrol has only recently been investigated compared to the study of actual primary mechanisms. A feature of many regulatory genes is their control over a number of different metabolites, the production of which appears to have no apparent common genetic base. For example, transposon mutagenesis of the *gacA* gene in *P. fluorescens* CHA0 disrupted the production of the low molecular-weight antibiotics 2,4-diaceltylphloroglucinol and pyoluteorin, and the volatile HCN (Laville *et al.* 1992), secondary metabolites which have been shown to control a range of phytopathogens (Table 1.2). In another example

of a link between regulatory genes and biocontrol activity, spontaneous mutants of *P. fluorescens* BL915, a biocontrol strain that suppresses damping-off disease caused by *Rhizoctonia solani*, were found to be deficient in the production of the cell wall-degrading enzyme chitinase, the volatile HCN, and the antibiotic pyrrolnitrin (Gaffney *et al.* 1994). An 11kb *Eco*RI genomic fragment from the wild type strain integrated into pRK290 and introduced into the spontaneous mutants restored all wild-type functions including biocontrol activity against damping-off. In both examples, a genomic region of the biocontrol strain appeared to promote the synthesis of a range of chemically distinct and seemingly genetically unrelated compounds which were antagonistic to the targeted pathogen(s).

Biochemical analysis of both *Pc*2140 and TM1120 suggests that this biocontrol strain produces a number of compounds antagonistic to *Ggt* which are likely to be involved in disease suppression. Ethyl acetate partitioning of compounds at different pH values (Table 5.3) and TLC analysis of supernatants of both the parent strain and the mutant (Table 5.4) suggest that there are 3 or possibly 4 compounds produced by *Pc*2140 that are inhibitory to *Ggt in vitro* and may therefore be involved in take-all suppression. Furthermore, intermediate levels of disease control by the other four Tn*5* mutants studied support the conclusion that more than one metabolite is involved in biocontrol. The production of these compounds by TM1120 has been disrupted by a single insertion of the transposon into the chromosome. If in fact the region in question contains a regulatory gene whose expression has been disrupted by the transposon, the regulatory gene itself is likely to be dissimilar to the regulatory genes described in the above examples insofar as HCN production by TM1120 was not affected by the mutation. However, as these water-soluble compounds have yet to be characterised it would be premature to assume that the structural genes for the synthesis of these compounds are not contained within a single gene operon and that the gene disrupted by the Tn*5* insertion in TM1120 is regulatory in nature, controlling a number of separate gene sequences.

The data obtained from the study of volatile production (including HCN) by *Pc*2140 and their role in suppression of disease has important ramifications in biocontrol research. Split-plate trials indicated that volatiles produced by *Pc*2140 inhibited hyphal growth of *Ggt* (Table 5.2). Furthermore, it was demonstrated that *Ggt* is sensitive to cyanide (Figure 5.1) and that *Pc*2140, a cyanogenic bacterium, was able to produce sufficient HCN *in vitro* to inhibit *Ggt*. These results would suggest that volatiles, particularly HCN, played a role in the biocontrol of take-all. When TM1120 was compared to the parent strain it was found that HCN production by the mutant and inhibition of growth of *Ggt* by TM1120-generated volatiles were similar to *Pc*2140. These results, combined from the data from the three pot trials, indicates that these factors were not significant in take-all suppression. Consequently, concluding that any metabolite (or any other factor) has a role in biocontrol based only on *in vitro* observations can never be considered valid. Experiments based on molecular techniques and/or biochemical analysis of *in situ* trials such as detection of gene expression or metabolite production in the rhizosphere must be carried out before valid conclusions can be made about the role of any metabolite in biocontrol of pathogens and plant disease.

FUTURE WORK

The primary mechanism of biocontrol of take-all by *P. corrugata* 2140 has been identified by molecular and biochemical means to be through the direct antagonism of the pathogen by metabolites produced by the biocontrol agent. While this has answered one question it has created more questions as well as forming the basis for further development of this strain as a biocontrol agent. The direction of research into *Pc*2140 and the biocontrol of wheat take-all must now concentrate on metabolite and gene isolation and identification. In addition to further research into the genetics and biochemistry of fungal antagonism and biocontrol of take-all, the mechanism of induced resistance in take-all suppression, and possible phytotoxic effects of *Pc*2140 must also be addressed.

The next stage of the project is the identification of the antagonistic compounds produced by *P. corrugata* 2140. From the results of the thin-layer chromatography it appears that there are three metabolites detectable by UV fluorescence/absorbance or by peptide detection that are produced by *Pc*2140 but not by TM1120 (Table 5.4). These bands may be eluted from the chromatography plate and retested for *Ggt* antagonism. The remainder of the material on the TLC plate may also be tested in the same manner to ensure antagonistic compounds not detected by the above methods are also recovered. Those fractions that antagonise *Ggt* which are not present in TM1120 may then be prepared for further purification and characterisation.

Identification of these compounds preferably requires pure samples of sufficient quantity for mass, U.V. and infra-red spectrophotometry or NMR analysis. Purification may be achieved by combining both the ethyl acetate extraction process and the TLC as used in this study. This would first require the extraction of crude supernatant in ethyl acetate as described in Chapter 5, then running samples of each fraction on a TLC plate to determine at what pH's each compound is partitioned into the ethyl acetate. Once this data is obtained, larger quantities of the now partially purified antibiotic may be loaded onto a TLC or PLC for purification. Higher yields on a TLC/PLC plate would facilitate identification of antagonistic compounds through bioassays against *Ggt* and identification of the compound. Further TLC purification using different solvent systems with varying pH's, polarities, etc can be utilised to ensure the purity of the metabolites. An alternative to TLC would be to develop a high performance liquid chromatography (HPLC) system for metabolite purification.

Some work in this area has already been undertaken. Although the identification of these compounds is still in its infancy, it has been determined that none of the metabolites produced by *Pc*2140 which are antagonistic to *Ggt* are phenazines (Ryder pers. comm.) or 2,4-diacetylphloroglucinol (Ross and Ryder, unpublished data). A sample of synthetic DAP (courtesy Christoph Keel) dissolved in methanol was spotted on a TLC plate

alongside *Pc*2140 crude supernatant. After development the plate was sprayed with a sulfanilic acid solution (C. Keel, pers. comm.). The synthetic DAP sample turned red after spraying and subsequent heating to 120°C but no similar colour change was detected with the *Pc*2140 supernatant.

Once the metabolites have been identified and suitable methods for their isolation and detection have been developed then the other four mutants of *Pc*2140 can be characterised. Mutant strain TM1120 appears to have lost the ability to produce any of the antagonistic compounds based on its lack of disease suppression in pot trials. The other four mutants, especially, TM27, TM129 and TM300 show intermediate levels of disease suppression suggesting that only one, perhaps two compounds have been affected by the Tn*5* insertions. Identification of which compounds have been lost in these mutants will assist in determining whether any one of the metabolites is more important in disease control.

Characterisation of the phenotypic changes relating to antibiotic/peptide production in the other four Tn*5* mutants can lead on to further molecular development of *P. corrugata* 2140 as a biocontrol agent. Complementation of these mutants to restore activity will lead to the identification of gene sequences involved in the synthesis of each metabolite. This will assist in determining the genetic relationship of each compound such as common regulatory and/or structural genes. Work has already begun on the subcloning of genomic fragments from cosmids which restore biocontrol activity in TM1120. A 6.0kb *Hind*III *Pc*2140 genomic fragment has already been identified by Southern hybridisation as being homologous to the 2.1kb probe used to originally identify the cosmids (A. Lucas, pers. comm.). Homologous fragments will be subcloned into suitable vectors and transformed into TM1120 which would then be retested for restoration of biocontrol activity. These DNA fragments can be base sequenced and compared to similar DNA sequences in various data banks.

Identification of genes or gene clusters involved in antifungal metabolite production may lead to work with reporter genes such as *lux* or ice nucleation to determine what conditions are conducive for biosynthetic or regulatory gene expression. These results will give a clearer understanding of the range of environmental conditions which *Pc*2140 is biologically active against *Ggt*. Identification of these gene sequences also has major implications for the development of a biocontrol agent(s) to function in a wider range of environmental conditions than those in which *P. corrugata* 2140 is active in.

One of the problems facing the development of *P. corrugata* 2140 as a biocontrol agent for take-all is the inconsistency of disease suppression in field trials. Factors influencing the inconsistency of biocontrol agents has already been discussed, citing the inability of *P. fluorescens* 2-79 to suppress take-all in peat soils in the United Kingdom (Capper and Higgins, 1993), the effect of zinc on biocontrol activity (Ownley *et al.* 1991) and the effect of pH on the activity of the antibiotic phenazine-1-carboxylic acid (Brisbane and Rovira, 1988; Ownley *et al.* 1992). Other factors influencing biocontrol activity include abiotic factors as discussed in the General Introduction, influence of plant species on biocontrol activity (Maurhofer *et al.* 1995) and sensitivity of different strains of *Ggt* to antagonistic antibiotics (Mazzola *et al.* 1995).

Although *P. corrugata* 2140 suppresses disease in a slightly acidic soil (Kapunda sodic clay), disease control is not consistent for every crop in subsequent trials (Rovira *et al.* 1992; Ryder *et al*, 1992). Furthermore *Pc*2140 does not suppress disease symptoms on wheat grown in a calcareous soil (Avon soil, pH8.2) or in trials with below average rainfall resulting in a low water matric potential (Ryder pers. comm.). It has yet to be established whether the lack of control is due to low levels of production of antagonistic metabolites and/or inactivity of antagonistic metabolites in these environments, or whether the biocontrol agent does not survive in conditions where it cannot successfully compete with the native microflora.

Integrated control, utilising more than one method to control take-all offers an alternative approach to the problem. Utilising more than one biocontrol agent on seed and/or combining applications of biocontrol agents with general farming methods including crop rotation, fertiliser applications and sowing techniques may improve the overall control of disease (Cook, 1994: Pierson and Weller, 1994). *P. corrugata* 2140 has been combined with a biocontrol strain of the fungus *Trichoderma koningii* and/or fungicides including triadmefon to improve suppression of take-all in field trials in Kapunda soil (Ryder *et al.* 1992). These integrated approaches may be successful, leading to decreased disease and increased crop yields, but they can only be applied to environments where each biocontrol agent (including fungicides) is active against *Ggt*.

If the problem with inconsistent results is one of survival and not the activity of the antagonistic metabolites, then genes from *Pc*2140 conferring antibiotic production may be transferred to strains isolated from these different ecological niches such as from calcareous soils and regions of low annual rainfall. Conferral of production of antagonistic compounds to non-biocontrol strains would increase the range of environments where these antagonistic compounds can control disease as discussed by Cook (1994). Genes conferring production of *Pc*2140-derived antibiotics may be introduced into strains which capable of efficient expression of the genes and are resistant to the antibiotics themselves.

Induced Resistance

The question of whether induced resistance plays a role in the suppression of take-all by *Pc*2140 of wheat must be addressed. In a recent review on induced resistance in monocots (Steiner and Schönbeck, 1995) a number of criteria for verification of induction of resistance were listed. Most important of these was the absence of antagonistic compounds produced by the potential inducer towards the pathogen. The reviewers also discuss the importance of the absence of a dose-response correlation known for antagonistic compounds, non-specificity of protection and a dependence on

plant genotype, resulting in different levels of protection by induced resistance in different cultivars under identical abiotic conditions.

Mutant strain TM1120 was shown to exhibit poor *in vitro* antagonism to *Ggt* and did not offer any protection to wheat plants in 2 of 3 independent pot trials and only slight protection in the third trial. This would suggest that induced resistance is not a factor in biocontrol of take-all by *P. corrugata* 2140 and that direct fungal antagonism was the only factor in disease suppression, unless the antagonistic compounds produced by *Pc*2140 induce resistance to disease, independent of their direct effect on *Ggt*. Antagonistic compounds have been suggested as possible inducers of host plant defence mechanisms. Hydrogen cyanide, which inhibits *in vitro* growth of *Ggt*, has been suggested as a potential inducer of resistance (Tuzun and Kloepper, 1994). Production of 2,4-diacetylphloroglucinol by *P. fluorescens* CHA0, an antibiotic antagonistic to a range of phytopathogenic fungi including *Ggt* has also been suggested as a possible inducer of plant defence responses (Keel *et al.* 1992). As yet however, no evidence is available proving that induction of resistance by these antagonistic compounds occurs.

The reduction in root lesions on TM1120-inoculated plants in test #3 (Figure 3.25) suggests that other factors aside from direct antagonism by water-soluble antibiotics may be involved in biocontrol of take-all, albeit to a much lesser degree. These other factors may include a minor level of antifungal activity provided by compounds already described which are produced by both *Pc*2140 and TM1120 (eg, HCN and siderophores), or it may include a level of induced resistance provided by the biocontrol agent through the production of inducing compounds which were not affected by the Tn*5* insertion in strain TM1120.

The results obtained from the pot trials involving mutant strain TM692 could be interpreted that a level of resistance induction in the wheat plants was occurring. Strain TM692 exhibited a low level of *in vitro* antagonism against *Ggt* yet significantly reduced take-all lesions on wheat plants in 2 of 3 pot trials (Figure 3.20). These results fit the

first criterion of induction of resistance as outlined in the review by Steiner and Schönbeck (1995), that is, an absence of antagonistic compounds produced by the inducer.

Unfortunately there is a general lack of knowledge on signalling mechanisms for induced resistance and how they induce plant defence responses. Very little is understood as to what compounds produced by microorganisms induce plant resistance to disease. Furthermore, the actual response(s) by the plant for resistance as reviewed by Tuzun and Kloepper (1994) must be identified on inducer-inoculated plants but not on inducer-free controls to conclude that plant defence systems have been activated by the inducer. These plant responses include peroxidase activity and enhanced cell wall lignification, production of phytoalexins and other low molecular weight compounds and increased chitinase and/or glucanase enzyme activity Not only must they be identified but a measured correlation between detection and host plant resistance in the absence of other factors must be made, just as a correlation between production of antagonistic compounds by molecular and biochemical methods and *in situ* inhibition of a pathogen are made.

To examine the possible role of induced resistance by *P. corrugata* 2140 would require a number of split-pot trials and a thorough examination of the biochemistry and physiology of the wheat plant for the identification of induction markers such as those described above. Split-pot trials as described by Zhou and Paulitz (1994) and Liu *et al.* (1995) would need to be constructed to allow for spatially separated root inoculations of inducer and pathogen to eliminate fungal antagonism in the rhizosphere and root surface. The Tn*5*::*lux* cassette could be used to track movement of the *P. corrugata* strains to ensure both inducer and pathogen remained spatially isolated throughout the trials. The complete *lux* operon (includes *luxCDE*), conferring autobioluminescence, has been utilised by Liu *et al.* (1995) to study induction of resistance in cucumber towards Fusarium wilt.

Spatial isolation of the pathogen and biocontrol agent will not necessarily eliminate the antagonism of the fungus by antagonistic compounds produced by *Pc*2140. The likelihood that antagonistic metabolites produced by *Pc*2140 may be transported through the plant and antagonise fungal hyphae which have infiltrated wheat vascular tissue must be considered. Detection of production and movement of antagonistic compounds by recovery from plant tissue must be undertaken to either eliminate or determine the level of effect of these compounds on disease suppression in the split-pot assay.

Phytotoxicity of *P. corrugata* 2140

The relationship between antagonistic compounds produced by *P. corrugata* 2140 and phytotoxicity requires investigation. *P. corrugata* was originally described as the causative agent of tomato pith necrosis in England (Scarlett *et al.* 1978). Symptoms of this disease on tomato plants include extensive longitudinal pith discolouration in the stem, chlorosis of leaves and formation of adventitious roots (Scarlett *et al.* 1978: Fletcher, 1984). Since the initial isolation, *P. corrugata* strains have been isolated from diseased plants world-wide including the United States (Jones, 1983; Lai *et al.* 1983: Wick and Rane, 1989), Argentina (Alippi *et al.* 1993) and Spain (Lopez *et al.* 1994). The strain may also be isolated from other sources such as the roots of symptomless alfalfa (Lukezic, 1979) asparagus (Elmer, 1995) cucumber (Rankin *et al.* 1992) and from wheat field soil (Ryder and Rovira, 1993).

Although *P. corrugata* strains have been isolated from diseased plants, little research has been carried out to the causative mechanism of disease. *P. corrugata* strain 0782-6 which induced necrotic symptoms in tomato was found to produce a phytotoxin which, when inoculated into tomato stems in a purified form, induced similar symptoms to the disease (Chun and Leary, 1988). This phytotoxic strain was mutated with ethylmethansulfonate to generate prototrophic Tox^- mutants which failed produce the toxin, suspected of being a peptide, or induce pith necrosis symptoms in inoculated plants (Chun and Leary, 1987). The nature of the phytotoxin produced by strain 0782-6

was not determined. Another strain of *P. corrugata* which induced pith necrosis symptoms on tomato and generate a hypersensitivity response on tobacco leaves was found to produce two peptides, designated HR1 and HR2, which were linked to pith necrosis (Gustine *et al.* 1995).

The phytotoxicity of compounds produced by *P. corrugata* strains has important implications to the development of *P. corrugata* 2140 as a biocontrol agent. Phytotoxicity of metabolites of biocontrol agents must be fully appreciated to prevent unwanted side-effects on non-target plants when the biocontrol agent is released. The phytotoxicity of antifungal compounds such as phenazines, 2,4-diacetylphloroglucinol and pyoluteorin have already been discussed (Section 1.3.8). Strain 2140 has been extensively tested for inducement of pith necrosis in glasshouse trials to ascertain the level of toxicity of the strain towards tomato and other crops. On tomatoes, inducement of pith necrosis by *Pc*2140 in a number of independent experiments was poor (Ryder, unpublished data). *Pc*2140 was inoculated into the stems of *Lycopersicon lycopersicum* c.v. Mountain Pride, a cultivar susceptible to pith necrosis. Pith necrosis symptoms did not appear in the stem of inoculated plants although a localised lesion indicative of possible hypersensitivity or other plant defence response restricted to the site of inoculation appeared. Necrotic symptoms were induced on lettuce leaves but no symptoms were induced on either carrot or onion (Ryder, unpublished data). These results suggest that *P. corrugata* strain 2140 is a benign parasite of tomatoes. If the peptides produced by *P. corrugata* 2140 function in a similar manner to the toxins isolated by Chun and Leary (1988) and Gustine *et al.* (1995) then alternate methods must be employed to introduce *Pc*2140 into the wheat field to avoid deleterious side-effects.

The relationship between biocontrol and phytotoxic effects may be investigated on both a biochemical and genetic basis. Purified antibiotics and peptides can be tested for hypersensitivity and pathogenic responses on tobacco leaves or tomato stems. *Pc*2140 and the mutants can be inoculated into tomatoes and compared for plant responses.

Genes from *Pc*2140 which are associated with biocontrol can be introduced into non-hypersensitive-inducing strains and tested for plant responses as described. This research will answer an important question: Is there a biochemical and/or genetic relationship between biocontrol activity, phytotoxicity and plant pathogenicity? If a relationship exists between these factors then the development of *Pc*2140 as a biocontrol agent must be reconsidered. If there is no relationship, then it may be possible to separate these functions and produce a non-phytopathogenic (safer) biocontrol agent. Alternatively, genes from *Pc*2140 which generate antagonistic compounds which are benign to tomatoes may be introduced into other strains, either to confer biocontrol activity or to increase their activity against take-all if biocontrol capabilities already exist.

SUMMARY

P. corrugata 2140, a non-fluorescent pseudomonad, antagonises the take-all fungus in *vitro* and suppresses disease symptoms on wheat in glasshouse and field trials. The bacterium produces a number of compounds potentially antagonistic to the fungus and which may have a role in disease suppression. To determine what the factors of biocontrol were, the strain was mutated by the random insertion of a transposon. Five non-auxotrophic mutants which exhibited varying levels of reduced fungal antagonism and disease suppression were isolated. One mutant, designated TM1120, did not show any *in vitro* antagonism and had lost >95% of its disease suppressiveness. The biochemistry of this mutant was compared with the parent strain to determine what metabolites were associated with biocontrol. The mutant was also complemented with a cosmid library to restore biocontrol activity.

Variations in metabolite production between the parent strain and the mutant as detected on TLC plates indicated that antibiotics including peptides were responsible for disease suppression. The structure and identification of these metabolites are yet to be elucidated. The development of ethyl acetate partitioning of bacterial supernatants combined with TLC separation of metabolites has resulted in a high degree of metabolite

purification which will facilitate rapid identification of the metabolites. Cosmid complementation has identified a region of the *Pc*2140 genome that restored biocontrol activity in TM1120. The precise nature of this region has not been determined but it is suspected that it contains regulatory sequences for metabolite production and biocontrol activity.

This work has completed the first stage of the genetic and biochemical study of *P. corrugata* strain 2140. Once the metabolites responsible for biocontrol have been chemically identified then the other four mutants may be characterised and the degree of activity of each metabolite may be determined. Further genetic analysis based on the work originated herein will provide further insight to the behaviour and ecology of the bacterium. This knowledge will be vital for the long-term development of *Pseudomonas corrugata* strain 2140 and/or its metabolites as biocontrol agents against take-all.

APPENDIX I: MEDIA AND OTHER FORMULAE

MEDIA

Notes on media preparation:

(i) All formula are per litre of medium
(ii) Unless otherwise indicated $FeCl_3$ was prepared as a 100 $mg.l^{-1}$ stock solution in 10mM HCl. The solution was filter sterilized through 0.2µm millipore paper and added to autoclaved media after cooling to 50°C
(iii) Unless stated in the text Difco agar was added at a rate of 15.0 $g.l^{-1}$

β - 1,3 Glucanase Detection Medium
(Elad *et al*, 1982)

Laminarin (Sigma)	0.2% w/v
NH_4NO_3	1.0g
K_2HPO_4	0.9g
$MgSO_4.7H_2O$	0.2g
KCl	0.2g
$FeSO_4$	2mg
$MnCl_2$	2mg
$ZnCl_2$	2mg

Cellulase Detection Medium
(modified after Wollum, 1981)

Carboxymethyl cellulose (CMC)	12g
$NaNO_3$	0.5g
K_2HPO_4	1g
$MgSO_4.7H_2O$	0.5g
$FeSO_4$	10mg

Chitinase Detection Medium
(modified after Hankin and Anagnostakis, 1975)

Glucose (optional)	5g
Chitin*	2.4% w/v (approx)
K_2HPO_4	1g
$MgSO_4.7H_2O$	0.5g
$FeCl_3$	1ml stock solution
Trace elements (see below)	1.5 ml stock solution

Plates were poured as a 2ml thick overlay on prepoured and set 1.5% w/v water agar
* chitin was prepared by the method of Campbell and Williams (1951)

CME Medium (HCN quantification)
(modified after Keel *et al*, 1989)

L-glutamic acid	2.94g
DL-methionine	0.745g
Glycine	0.94g
Tris-HCl	6.05g
K_2HPO_4	0.87g
$Na_2HPO_4.2H_20$	0.89g
$MgSO_4.7H_2O$	0.49g
$FeCl_3$	1 ml stock solution

pH7.5

Luria-Bertani Medium
(Sambrook et al, 1989)

Bacto Tryptone (Difco)	10g
Yeast Extract (Difco)	5.0g
NaCl	10g

pH 7.0

Manganese Reductase Detection Medium
(after Marschner *et al*, 1991)

Proteose Peptone	5g
Yeast Extract	1g
Glucose	2.5g
K_2HPO_4	1g
$MgSO_4.7H_20$	0.5g
$FeCl_3$	1 ml stock solution
Trace elements*	1.5 mls stock solution
$KMnO_4$	0.54g

* The trace element solution added was prepared without the addition of $MnSO_4.H_2O$ (refer trace elements recipe below).

Manganese Reductase Detection Medium
(after Rogers, 1969)

Sucrose	30g
Yeast Extract (Difco)	1g
MnO_2	5g

MnO_2 was added as a powdered suspension after the other components were autoclaved and cooled to 50°C

Medium 'N'

Glucose	20g
KNO_3	5g
KH_2PO_4	2g
K_2HPO_4	4g
$MgSO_4.7H_20$	1.0g
Na_2SO_4	0.2g
$CaCl_2$	0.1g
$FeCl_3$	1ml stock solution
Trace elements (see below)	1.5 mls stock solution

Mineral Base E Medium
(modified after Owens & Keddie, 1969)

Glucose	20g
K_2HPO_4	10.61g
KH_2PO_4	5.3g
$(NH_4)_2SO_4$	5g
NaCl	0.1g
$MgSO_4.7H_20$	0.2g
$CaCl_2$	0.1g
EDTA	15mg
$FeCl_3$	1 ml stock solution
Trace elements (see below)	1.5 mls stock solution

pH7.0

Nutrient Agar (NA) and Nutrient Broth (NB)

Prepared as per manufacturer's guide (Difco, Detroit, Michigan)

pH 7.5

Plant Nutrient Supplement (PNS) (Hoagland's Solution)
(Hoagland & Aaron, 1938)

$MgSO_4.7H_20$	0.492g
KH_2PO_4	0.136g
$Ca(NO_3)_2$	1.18g
KNO_3	0.506g
Ferric Citrate	5.0mg
H_3BO_3	2.5mg
$MnSO_4.4H_2O$	2.5mg
$ZnCl_2$	0.1mg
$CuCl_2.H_2O$	0.05mg
MoO_3	0.05mg

Potato Dextrose Agar (PDA)

Prepared as per manufacturer's guide (Difco, Detroit, Michigan)
pH 6.7

Protease Detection Medium (Gelatine)
(Hankin and Anagnostakis, 1975)

Nutrient Broth (Difco)	8g
Gelatine (Commercial grade)	4g

Protease Detection Medium (Skim Milk)
(Hopwood, 1970)

Potato Dextrose Agar (Difco)	20g
10% w/v skim milk solution	100mls

Proteose Peptone #3 (PP3)

20.0 g of Difco Proteose Peptone #3 per litre
pH 7.5

***Pseudomonas* Selective Medium (PSM)**
(modified after Gasson, 1980)

$NH_4H_2PO_4$	1.0g
KCl	0.2g
$MgSO_4.7H_2O$	0.2g
Glucose	2.0g
$FeCl_3$	1.0 ml stock solution

Mg^{2+} autoclaved separately and added to medium after cooling
pH 7.1

Siderophore Detection Medium (SID)
(Schwyn and Neilands, 1987)

CAS/HDTMA:Fe Solution:

Chromeazurol S (Sigma)	60.5 mg
$FeCl_3$ (dissolved in 10 mls 10mM HCl)	1.62 mg
Hexadecyltrimethylammonium bromide (HDTMA)	72.9 mg
DDW	90 mls

Salts/Buffer Solution):

KH_2PO_4	0.3 g
NaCl	0.5 g
NH_4Cl	1.0 g
$CaCl_2$	10.0 mg
$MgSO_4.7H_2O$	0.5 g
NaOH	6.0 g
Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)	30.24 g
DDW	860 mls

Other Constitiuents

Casamino Acid (Difco) (in 30 mls DDW)	3.0 g
Sucrose (in 10 mls DDW)	2.0 g

The pH of the salts/buffer solution was adjusted to 7.0 before autoclaving. All four solutions were autoclaved separately and mixed when cooled to 50°C. The pH of the complete medium was adjusted to 6.8 with filter-sterilized 1.0M HCl before pouring.

Sucrose-Asparagine Medium (SA)
(Scher and Baker, 1982)

Sucrose	20.0g
Asparagine	2.0g
K_2HPO_4	1.0g
$MgSO_4.7H_2O$	0.5g
$FeCl_3$	1.0 ml stock solution

Mg^{2+} prepared separately and added to medium before autoclaving
pH 7.2

Tetrazolium Medium (TZCA)
(modified after Kelman, 1954)

Casamino Acids (Difco, Detroit)	1.0g
Glucose	5.0g
Proteose Peptone #3 (Difco, Detroit)	10.0g
2,3,5 Triphenyl Tetrazolium Chloride (TZCA)	0.05g

TZCA was added as a filter-sterilized stock solution after cooling the autoclaved medium to 50°C
pH 7.1

Trace Elements Solution(modified after Hankin & Anagnostakis, 1975)

H_3BO_3	10mg
$MnSO_4.H_2O$	15mg
$ZnCl_2$	60mg
$CuSO_4.5H_2O$	80mg
$MoNa_2O_4$	15mg
$CoSO_4.7H_2O$	20mg

pH 6.5

After dissolving all consituents in DDW the solution was filter sterilized with 0.2µm Millipore paper and stored at -20°C in the dark. The trace elements solution was added to other autoclaved media after cooling to 50°C.

BUFFERS AND SOLUTIONS

Denhardt's III Solution

2% gelatin
2% ficoll
2% polyvinyl pyrollidone (PVP)
5% tetrasodium pyrophosphate

DIG Detection: Hybridization Buffer

(per 50 mls)	
Formamide	25mls
20x SSC (see below)	12.5mls
10% w/v skim milk solution	10mls
10% v/v Sarkosyl	1ml
10% SDS solution	1ml
SDDW	0.5ml

DIG Detection: Buffer #1

Maleic Acid	11.6g
NaCl	8.7g
NaOH	8g

pH adjusted to 7.5 with 2M NaOH

DIG Detection: Buffer #2

90mls Buffer #1 plus 10mls 10% w/V skim milk powder solution

DIG Detection: Buffer #3

Tris-HCl	12.1g
NaCl	5.8g
MgCl2	10.15g

pH9.5

DIG Detection: Washing Buffer

0.3ml Tween 20 in 100mls Buffer#1

Gel Loading Buffer
(Sambrook et al, 1989)

0.25% bromophenol blue
0.25% xylene cyanol FF
15% ficoll (Type 400, Pharmacia) in water

GTE Buffer
(Sambrook et al, 1989)

50mM glucose
25mM Tris-HCl
10mM EDTA

pH8.0

HSB Buffer (5x)

3M NaCl
100mM PIPES Buffer
25mM NaEDTA

pH 6.8 (Adjusted with 4M NaOH)

Saline Sodium Citrate (SSC) (20x)

3M NaCl
300mM trisodium citrate

Southern Hybridization: Gel Denaturation Solution

0.4M NaOH
0.6M NaCl

Southern Hybridization: Gel Neutralisation Solution

1.5M NaCl
0.5M Tris-HCl

pH7.5

Southern Hybridization: Depurination Solution

0.25M HCl

STE Buffer

0.1M NaCl
10mM Tris-HCl
1mM EDTA

pH8.0

TAE Buffer (per litre)
(Sambrook et al, 1989)

Tris-HCl	4.84g
Glacial Acetic Acid	1.48 ml
EDTA	0.372g

pH8.0

TE8 Buffer
(Sambrook et al, 1989)

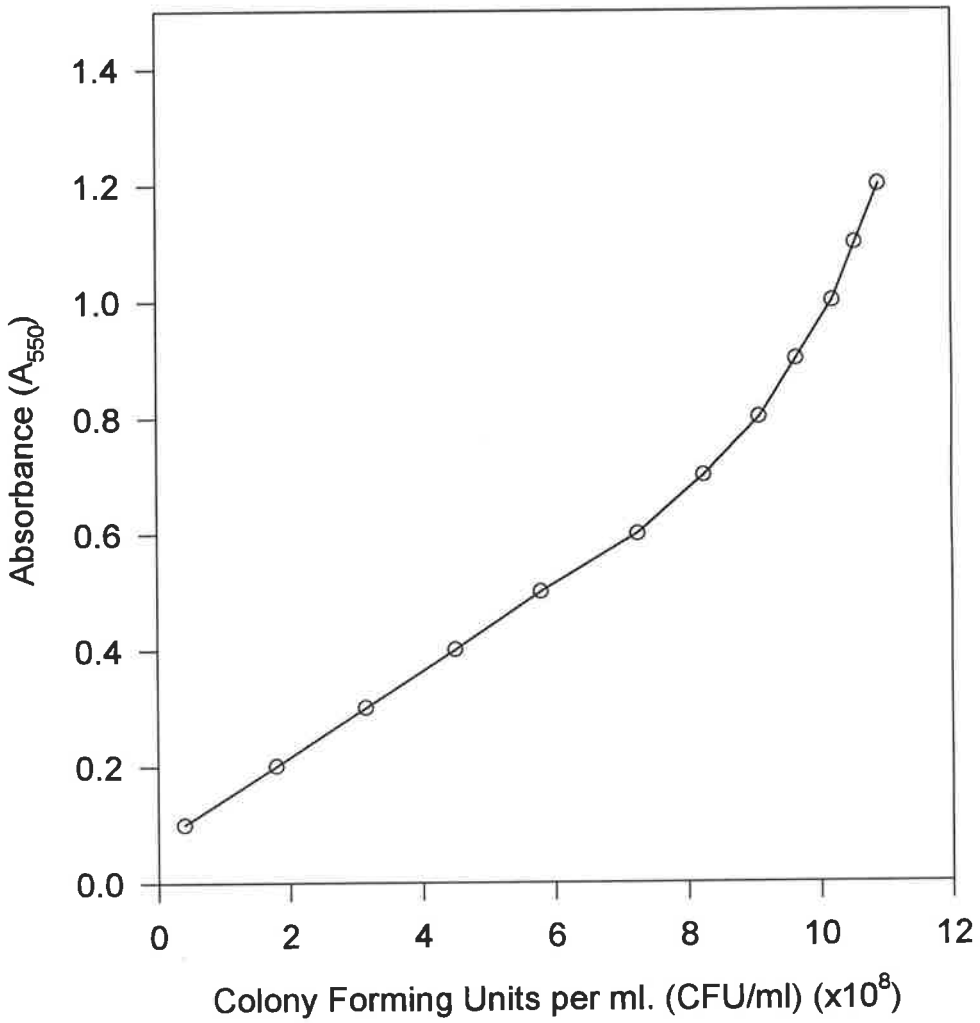
10mM Tris-HCl
1mM EDTA

pH8.0

APPENDIX II

Growth of *P. corrugata* in modified mineral base E medium:

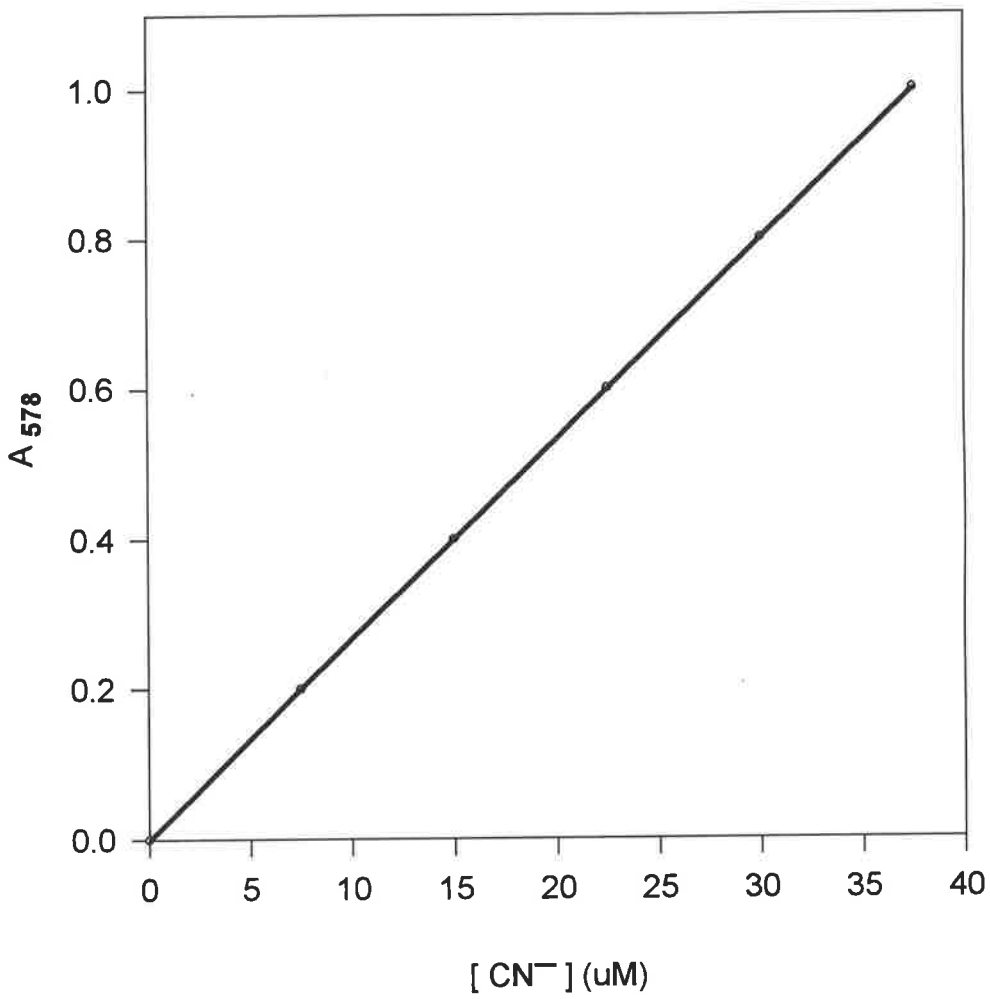
Relationship between A550 and CFU/ml.



P. corrugata 2140 was grown to late log phase (21-24 hours) in modified mineral base E medium (Owens and Keddie, 1969) at 25oC. Samples were taken every three hours for spectrophotometer analysis at λ=550nm. Sampler were also serially diluted and spread plated on TZCA medium and incubated for a further 48 hours and colonies counted.

APPENDIX IIIA

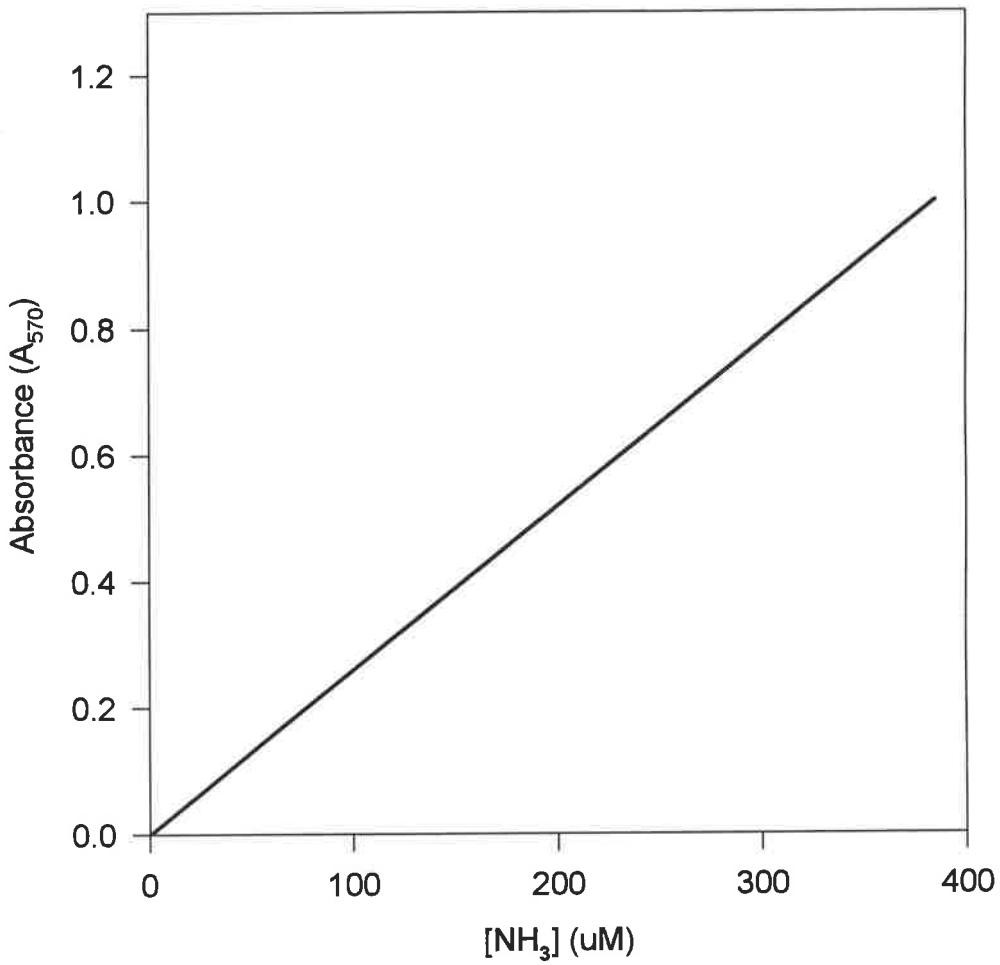
Standard Curve for the Quantification of Hydrogen Cyanide



The standard curve was prepared by dissolving KCN in 0.1M NaOH in varying concentrations. The two reagents, *o*-dinitrobenzene and 4-nitrobenzaldehyde (Guilbault and Kramer, 1966), were added and the solutions incubated for 30 minutes. A blank was also prepared comprising 0.1M NaOH solution only. Cyanide was then quantified at A_{578} for samples whose absorbance ranged between 0.1 and 1.0 and a standard curve prepared. Readings for the blank were <0.001.

APPENDIX IIIB

Standard Curve for the Quantification of Ammonia with Ninhydrin



The standard curve for ammonia was prepared by disolving $(NH_4)_2SO_4$ in 0.2M H_2SO_4 as described in Section 5.3.3. Blanks were prepared comprising 0.2M H_2SO_4 only. The samples were then treated with ninhydrin and 50% v/v aqueous ethanol by the method of Amato and Ladd (1988). Samples were read with a spectrophotometer and a standard curve prepared using samples whose absorbance value lay between the range 0.1 to 1.0.

APPENDIX IV: APPROVAL FOR TESTING GENETICALLY MODIFIED ORGANISMS IN SOIL

Genetic Manipulation Advisory Committee

5 East
111 Alinga Street
GPO Box 2183
CANBERRA ACT 2601
Telephone
06 275 3663
Facsimile
06 275 3967

Ref: 90/0435 (508)

Dr C Pankhurst
Secretary, Biosafety Committee
CSIRO Division of Soils

SCIENTIFIC SUB-COMMITTEE - PROPOSAL ADVICE AND RECOMMENDATIONS

GMAC REF NO: 1) 3037; 2) 3038; 3) 3039

IBC REF NO: 1) SOILS/93-1; 2) SOILS/93-3; 3) SOILS/93-2

TITLE OF PROPOSAL:

1) Growth cabinet testing of genetically engineered *Pseudomonas corrugata* containing Tn5 transposon insertions;
2) Interactions between *Pseudomonas corrugata* carrying chromosomal *lux* AB marker genes and soil fauna (earthworms, nematodes);
3) Mutagenesis and tracking of *Pseudomonas corrugata* using bioluminescence genes.

PRINCIPAL INVESTIGATORS:

1) Ross, Ryder
2) Ryder, Stephens, Doube
3) Ryder, Ross

GMAC SCIENTIFIC SUB-COMMITTEE ADVICE:

GMAC confirms that C1 physical containment, together with use of a quarantine growth cabinet and the procedures specified in the proposals, is appropriate for this work.

A Faragher
GMAC Secretariat

5 October 1993

APPENDIX V: SUMMARY OF CHARACTERISTICS OF MUTANTS OF *Pc*2140

PHENOTYPE	Pc 2140	TM27	TM129	TM300	TM692	TM1120
Colony morphology [i]	rough	rough	rough	rough	mucoid	mucoid
Bioluminescence	-	+	-	+	+	-
In vitro inhibition of *Ggt* (refer figure 2)	strong	slightly reduced	slightly reduced	slightly reduced	not detected	not detected
Growth in minimal medium						
Root colonisation [ii]	3.92^{a}	nt	nt	3.98^{a}	3.80^{a}	3.62^{a}
Siderophore production [iii]	8.4^{a}	8.2^{a}	8.4^{a}	8.3^{a}	8.2^{a}	8.9^{b}
HCN (μM)	236^{a}	nt	nt	233^{a}	nt	239^{a}
Volatile production and *in vitro* inhibition of *Ggt* [iv]	3.19^{bc}	2.7^{c}	4.1^{b}	4.02^{bc}	2.78^{bc}	3.31^{bc}
Ammonia	nd	nt	nt	nt	nt	nt
Manganese Reductase	nd	nd	nd	nd	nd	nd
Proteolytic Enzyme(s)	+	+	+	+	+	+
Chitinase	nd	nt	nt	nt	nt	nt
β-1,3 glucanase	nd	nt	nt	nt	nt	nt
Cellulase	poor	nt	nt	nt	nt	nt
Peptides	++	nt	nt	nt	nt	+[v]

nd not detected
nt not tested

All letters after figures indicate no significant difference between means of treatments ($P = 0.05$).

(i) Colony morphology on TZCA medium
(ii) root colonisation measured as $\log_{10}$ CFU per mg fresh root weight.
(iii) siderophore production measured as mean distance from the edge of the colony to the limit of iron chelation in the medium in mm after 48 hours incubation.
(iv) Inhibition measured by mean radial hyphal growth of *Ggt* in mm after 4 days incubation. Hyphal growth in control treatment with no bacteria = 12.95 mm.
(v) Peptide production detected for mutant TM1120 but differences observed when compared to *Pc*2140 on TLC plates

APPENDIX VI: PUBLICATIONS AND PRESENTATIONS

Ross, I.L. and Ryder, M.H. (1994) Hydrogen cyanide production by a biocontrol strain of *Pseudomonas corrugata*: Evidence that cyanide antagonises the take-all fungus *in vitro*. In: (Ryder, M.H., Stephens, P.M. and Bowen, G.D. eds.) Improving Plant Productivity With Rhizosphere Bacteria. Proceedings of the Third International Workshop on Plant Growth-Promoting Rhizobacteria. pp131-133. CSIRO, Adelaide.

Ross. I.L. (1994) Interactions of Microbial Siderophores in Soil. (Abstract) Symposium presentation at the Annual Scientific Meeting for the Australian Society for Microbiology. Melbourne, September, 1994.

Ross, I.L. and Ryder, M.H. (1995) Suppression of take-all of wheat by *Pseudomonas corrugata* strain 2140 by the production of water-soluble antibiotics. (Abstract) Poster presentation at the 10th Biennial Conference for the Australasian Plant Pathology Society. Christchurch, N.Z. August, 1995.

Ross, I.L. and Ryder, M.H. (1996) Complementation of a Tn*5*-induced mutant of *P. corrugata* strain 2140 restores biocontrol activity against the take-all fungus. (Abstract) Poster presentation for the Molecular Plant-Microbe Interactions 8th. International Congress. Knoxville, TN. July, 1996.

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