



**ECOLOGY AND BIOLOGICAL CONTROL OF
AGROBACTERIUM VITIS, THE GRAPEVINE
CROWN GALL PATHOGEN**

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Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
(Faculty of Agriculture and Natural Resources)

June, 1994

Awarded 1995

TABLE OF CONTENTS

ACKNOWLEDGMENTS	1
PREFACE	2
ABBREVIATIONS	3
LIST OF FIGURES	4
LIST OF TABLES	7
SUMMARY	9

Chapter One

INTRODUCTION AND AIMS

1.1 GENERAL INTRODUCTION	12
1.2 PROJECT AIMS	15

Chapter Two

REVIEW OF LITERATURE

2.1 NOMENCLATURE OF <i>AGROBACTERIUM</i>	16
2.1.1 The genus <i>Agrobacterium</i>	16
2.1.2 Pathogenicity of <i>Agrobacterium</i>	17
2.1.3 Stable taxonomy	17
2.2 ECOLOGY OF <i>AGROBACTERIUM VITIS</i>	18
2.2.1 Host specificity	18
2.2.2 Ecological specialisation	19
2.2.3 Host defence mechanisms	20
2.2.4 Aerial galls on grapevine	21
2.2.5 Detection of <i>A. vitis</i>	22
2.2.6 Eradication of <i>A. vitis</i>	24
2.3 TUMOUR FORMATION BY <i>AGROBACTERIUM</i>	25
2.3.1 Bacterial attachment to plant cells	26
2.3.2 Plasmid-borne virulence	27
2.3.3 Initiation of the disease process	29

2.3.4	Inter-kingdom DNA transfer	30
2.3.5	Production and utilisation of opines.....	31
2.3.6	Specific loci determine host range	32
2.4	BIOLOGICAL CONTROL OF CROWN GALL.....	34
2.4.1	Biological control of stone fruit crown gall.....	34
2.4.2	Mode of action of K84	34
2.4.3	Agrocin 84 production and sensitivity	35
2.4.4	Other potential biocontrol agents.....	37
2.4.5	Biological control of grapevine crown gall.....	37
2.5	CONCLUSIONS.....	40

Chapter Three

MATERIALS AND METHODS

3.1	BACTERIAL STRAINS	42
3.1.1	Culture and storage of bacterial strains.....	42
3.1.1.1	Culture of bacterial strains	42
3.1.1.2	Preparation of washed bacteria.....	42
3.1.1.3	Storage of bacterial strains.....	42
3.2.1	Identification of <i>A. vitis</i> using a monoclonal antibody.....	45
3.2.1.1	Colony identification using a dot immunoassay	45
3.2.2	Screening for polygalacturonase activity.....	45
3.2.2.1	Polygalacturonase plate assay	45
3.2.3	Screening for antagonism.....	46
3.2.3.1	Stonier's medium.....	46
3.2.3.2	Sensitive strain overlay assay.....	46
3.3	BACTERIAL GENETIC MANIPULATIONS	47
3.3.1	Transfer of a <i>pehA</i> clone to <i>Agrobacterium</i> strains	47
3.3.1.1	Bacterial matings.....	47
3.3.1.2	Recipients of a <i>pehA</i> clone	47
3.3.1.3	Plasmid visualisation using gel electrophoresis	50
3.3.2	Transposon-tagging of an <i>A. vitis</i> pathogen with Tn501	50
3.3.2.1	Transposon mutagenesis with Tn501	50
3.4	EXPERIMENTS WITH BACTERIA	53
3.4.1	Growth characteristics of <i>Agrobacterium</i> strains.....	53
3.4.1.1	Growth rate in TY broth.....	53
3.4.1.2	Death rate in nutrient-free liquid media	53

3.4.1.3	Viability of bacteria on selective media.....	54
3.4.2	Sensitivity of bacteria to plant anti-microbial compounds.....	54
3.4.2.1	Preparation of bacteria	54
3.4.2.2	Inoculation of phosphate buffer containing anti- microbial compounds.....	54
3.4.3	Fitness of genetically modified bacteria.....	55
3.4.3.1	Preparation of bacteria	55
3.4.3.2	Serial transfer of dual cultures	55
3.4.4	Dual culture of a pathogen with antagonistic strains in broth.....	56
3.4.4.1	Preparation of bacteria	56
3.4.4.2	Co-inoculation of dual cultures	56
3.5	GRAPEVINE MATERIAL.....	57
3.5.1	Storage and treatment of grapevine material.....	57
3.5.1.1	Storage of grapevine material.....	57
3.5.1.2	Thermotherapy of grapevine material	57
3.6	EXPERIMENTS INVOLVING GRAPEVINE MATERIAL.....	57
3.6.1	Grapevine cane-segment bioassay.....	57
3.6.1.1	Preparation of cane segments.....	57
3.6.1.2	Preparation of bacteria	58
3.6.1.3	Inoculating bacteria to cane segments.....	58
3.6.1.4	Extraction of bacteria from cane segments	58
3.6.1.5	Statistical analysis	60
3.6.2	Inoculation of artificially wounded grapevines.....	60
3.6.2.1	Artificial wounding of grapevine stems.....	60
3.6.2.2	Inoculation of bacteria to wounds	60
3.6.3	Glasshouse testing of antagonistic strains on grapevines.....	60
3.6.3.1	Experimental conditions and design.....	60
3.6.3.2	Application of antagonistic strains.....	61
3.6.3.3	Application of mercury resistant pathogen.....	61
3.6.3.4	Sampling pathogen numbers in the soil.....	62
3.6.3.5	Sampling pathogen numbers in the vascular system	62

Chapter Four

A BIOASSAY TO INVESTIGATE POPULATION DYNAMICS OF BACTERIA IN THE VASCULAR SYSTEM OF GRAPEVINES

4.1	INTRODUCTION	64
4.2	EXPERIMENTS	65
4.2.1	Population dynamics of <i>A. vitis</i> and <i>A. rhizogenes</i> in a grapevine cane-segment bioassay	65
4.2.1.1	Recovery of bacteria on selective media.....	66
4.2.1.2	Bacterial recovery from a grapevine cane-segment bioassay over time.....	66
4.2.1.3	Bacterial growth rates	68
4.2.1.4	Bacterial death rates	68
4.2.2	Endogenous <i>A. vitis</i> in the grapevine cane-segment bioassay	71
4.2.2.1	Effect of thermotherapy of grapevine cuttings on the recovery of bacteria from the bioassay	71
4.2.2.2	Influence of competition by <i>A. vitis</i> on the recovery of <i>A. rhizogenes</i> from the bioassay	73
4.2.2.3	Effect of <i>A. vitis</i> -free grapevine segments on the recovery of bacteria from the bioassay	76
4.2.3	Influence of segment type in the grapevine cane-segment bioassay	79
4.2.3.1	Effect of nodal and internodal grapevine segments on recovery of bacteria from the bioassay	79
4.3	DISCUSSION.....	79

Chapter Five

ADAPTATIONS OF *AGROBACTERIUM VITIS* FOR SURVIVAL IN THE GRAPEVINE VASCULAR SYSTEM

5.1	INTRODUCTION	87
5.2	EXPERIMENTS	89
5.2.1	Sensitivity of agrobacteria to toxic plant compounds.....	89
5.2.1.1	Incubation of three <i>Agrobacterium</i> strains with	

	phenolic, quinnic and phytoalexin compounds that are associated with plant defence.....	89
5.2.1.2	Screening agrobacteria for sensitivity to p-benzoquinone.....	90
5.2.2	Moderating quinone toxicity with ascorbic acid in the grapevine cane-segment bioassay	95
5.2.2.1	Effect of ascorbic acid on bacterial survival in the bioassay	95
5.2.3	Effect of polygalacturonase activity on bacterial recovery from the grapevine cane-segment bioassay.....	97
5.2.3.1	Construction of transconjugant strains	97
5.2.3.2	Fitness of transconjugant strains	101
5.2.3.3	Recovery of bacteria on selective media with antibiotics.....	101
5.2.3.4	Effect of modified Peh activity on recovery of <i>Agrobacterium</i> strains from the grapevine cane-segment bioassay	103
5.3	DISCUSSION.....	106

Chapter Six

CHARACTERISATION OF BACTERIOCIN PRODUCING STRAINS INHIBITORY TO *AGROBACTERIUM VITIS*

6.1	INTRODUCTION.....	113
6.2	EXPERIMENTS.....	114
6.2.1	Growth and inhibitory properties of antagonistic strains.....	114
6.2.1.1	Inhibition of <i>A. vitis</i> strains on solid media	114
6.2.1.2	Growth rate of antagonistic strains	116
6.2.1.3	Death rate of antagonistic strains	116
6.2.1.4	Screening antagonistic strains for polygalacturonase activity and tolerance to benzoquinone.....	121
6.2.2	Population dynamics of a transposon-tagged <i>A. vitis</i> pathogen in the presence of antagonistic strains	121
6.2.2.1	Recovery of transposon-tagged <i>A. vitis</i> K1345 on media containing mercury.....	124
6.2.2.2	Fitness of the transposon-tagged <i>A. vitis</i> strain K1345	124

6.2.2.3	Pathogen displacement by antagonistic strains in TY broth.....	127
6.2.2.4	Pathogen displacement by antagonistic strains in Stonier's broth.....	129
6.2.2.5	Pathogen displacement by antagonistic strains in the grapevine cane-segment bioassay	129
6.2.3	Bacterial antagonism in artificial wound sites on grapevine stems	131
6.2.3.1	Preventing gall formation by <i>A. vitis</i> K377 at wound sites with antagonistic strains.....	131
6.3	DISCUSSION.....	137

Chapter Seven

GLASSHOUSE TESTING OF THERMOTHERAPY AND BIOLOGICAL CONTROL AGENTS AS A CONTROL STRATEGY FOR GRAPEVINE CROWN GALL

7.1	INTRODUCTION	145
7.2	EXPERIMENTS	146
7.2.1	Impact of thermotherapy on gall formation by endogenous <i>A. vitis</i>	146
7.2.1.1	Gall formation following artificial wounding of thermotherapy treated cuttings.....	147
7.2.2	Protection of thermotherapy treated cuttings from crown gall using antagonistic bacteria	149
7.2.2.1	Experimental design	149
7.2.2.2	Incidence of gall formation on grapevines treated with antagonistic strains	150
7.2.2.3	Pathogen numbers in the soil around grapevines treated with antagonistic strains	150
7.2.2.4	Pathogen numbers in the vascular fluid of grapevines treated with antagonistic strains.....	153
7.2.2.5	Re-analysis of results to include data from grapevines that were dipped into a pathogen suspension before planting.....	153
7.3	DISCUSSION.....	160

Chapter Eight

GENERAL CONCLUSIONS.....	166
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APPENDICES

APPENDIX A: Culture media.....	171
APPENDIX B: Antibiotics and other anti-microbial compounds	175
APPENDIX C: Miscellaneous buffers.....	176
APPENDIX D: Publications from this thesis.	177
APPENDIX E. Data from figures and tables.	178

BIBLIOGRAPHY	209
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ACKNOWLEDGMENTS

I sincerely thank my supervisors Professor Allen Kerr, Dr Peter Murphy and Dr Maarten Ryder for their support and guidance during my candidature. In particular, for Allen's ongoing interest in my project after his retirement and for Peter and Maartens' willingness to take on a project not integrated with their existing research.

Support and assistance from people at the Waite Institute was offered without hesitation. I would specially like to thank Kevin Sanderson and Nick McClure for their suggestions, advice and friendship. I am also indebted to Lynne Giles for statistical analyses, Jenny Groom for photography and Terry Feckner for unfailing laboratory support. Thanks also to Professor Tom Burr of Cornell University for a number of bacterial strains.

I have been very fortunate to have made so many good friends at the Waite Institute. For their warmth and humour, I cannot thank them enough. Especially Andrew Geering, Kathy Ophel-Keller, Susan Chambers, David Miller, Margaret Wexler, Gary Rosewarne, Suzanne Charwat, Madhu, Nicki Featherstone, Scott Donner, Natalie Cavanagh, Heather Fraser, Julie Nicol, Kim Tynan, Steve Thomas, Chris Saint and Brett Ingham.

The continual support of my family during my studies has been invaluable, in particular, I thank my father for his enthusiasm and encouragement throughout.

Finally, and most importantly, I thank Jamie Brady for her friendship. When times were difficult, her patience and support made it possible to forge on.

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.

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ABBREVIATIONS

1A	Brisbane & Kerr medium for <i>A. tumefaciens</i> (Appendix A)
2E	Brisbane & Kerr medium for <i>A. rhizogenes</i> (Appendix A)
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
LB	Luria broth medium (Appendix A)
LHR	Limited host range
MSALTS	Minimal salts buffer (Appendix C)
Peh	Polygalacturonase
Ri	Root inducing
RO	Reverse osmosis
RS	Roy & Sasser medium for <i>A. vitis</i> (Appendix A)
SDDW	Sterile double distilled water
SED	Standard error difference
T-DNA	Transferred DNA
Ti	Tumour inducing
TY	Tryptone yeast medium (Appendix A)
WHR	Wide host range
WSD	Wholly significant difference

LIST OF FIGURES

1. Crown gall of grapevine (*Vitis vinifera*).
2. Gall formation at the graft union of a grafted grapevine (*Vitis vinifera*).
3. Complementation of an *A. vitis* Peh⁻ mutant (CG50) with a *pehA* clone derived from *A. vitis* K377.
4. Map of pNMM2.
5. Conjugal transfer of Tn501 to *A. vitis* K377 using the suicide vector pNMM2.
6. Apparatus used to inoculate and extract vascular fluid from segments of grapevine canes.
7. Sampling sites for pathogen colonisation of grapevines after treatment with antagonistic strains.
8. Effect of the duration of incubation on recovery of *A. vitis* K377 and *A. rhizogenes* K27 from a grapevine cane-segment bioassay.
9. Growth of *A. vitis* K377 and *A. rhizogenes* K27 when incubated in TY broth at 28°C.
10. Survival of *A. vitis* K377 and *A. rhizogenes* K27 when incubated in MSALTS (Appendix C) solution at 25°C.
11. Effect of thermotherapy of grapevine cuttings on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay.
12. Effect of type of grapevine segment on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay.
13. Polygalacturonase activity of *A. vitis* strains CG49 (a), CG50 (b) and K1336 (c) on agarose plates containing polygalacturonate and stained with ruthenium red.

14. Plasmids in donor (*E.coli* K1153), parent (*A. vitis* CG50) and transconjugant (*A. vitis* K1336) strains after separation by gel electrophoresis.
15. Fitness co-efficients of the genetically modified *Agrobacterium* strains *A. vitis* K1336, *A. vitis* CG50, *A. tumefaciens* K1329, *A. tumefaciens* K1341 and *A. rhizogenes* K1312 when competing against respective wild-type parents *A. vitis* CG49, *A. vitis* CG49, *A. tumefaciens* C58, *A. tumefaciens* HLB2 and *A. rhizogenes* K27.
16. Inhibition of *A. vitis* K377 by *A. tumefaciens* HLB2 on Stonier's medium.
17. Growth of the bacterial strains *A. tumefaciens* HLB2, *P. fluorescens* K315, *A. vitis* E26, *A. vitis* K1069 and *A. vitis* F2/5 when incubated in TY broth at 28°C.
18. Survival of the bacterial strains *A. tumefaciens* HLB2, *P. fluorescens* K315, *A. vitis* E26, *A. vitis* K1069 and *A. vitis* F2/5 when incubated in MSALTS solution (Appendix C) at 25°C.
19. Fitness co-efficients of the transposon-tagged (Tn501) *A. vitis* K1345 when competing against its wild-type parent *A. vitis* K377.
20. Pathogen displacement in TY broth when *A. vitis* K1345 was co-inoculated with antagonistic strains *A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 and *A. tumefaciens* K1341 and incubated for 48 hours.
21. Pathogen displacement in Stonier's broth when *A. vitis* K1345 was co-inoculated with antagonistic strains *A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 and *A. tumefaciens* K1341 and incubated for 48 hours.
22. Tumour formation at artificial wound sites co-inoculated with *A. tumefaciens* HLB2 and *A. vitis* K377.

23. Effect on tumour formation at artificial wound sites on grapevine stems when sites are pre-inoculated with an antagonistic strain (*A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 or *A. tumefaciens* K1341) 24 hours before inoculation with an equal volume of a suspension of *A. vitis* K1345.
24. Effect on tumour formation at artificial wound sites on grapevine stems when sites are co-inoculated with an antagonistic strain (*A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 or *A. tumefaciens* K1341) and an equal volume of a suspension of *A. vitis* K1345.
25. Effect of different biocontrol strains on the percentage of gall formation.
26. Effect of different biocontrol strains on numbers of the mercury resistant pathogens (*A. vitis* K1345) in the soil.
27. Effect of different biocontrol strains on numbers of mercury resistant pathogens (*A. vitis* K1345) in xylem fluid.

LIST OF TABLES

1. Some bacterial strains documented as antagonistic toward crown gall pathogens.
2. Origin and source of bacterial strains used in this study.
3. Important plasmids and their carrier strains used in this study.
4. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 on undefined media and selective media.
5. Effect of thermotherapy of grapevine material on the level of endogenous *A. vitis* recovered from the grapevine cane-segment bioassay.
6. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 from a grapevine cane-segment bioassay when the strains were inoculated either separately or in combination with each other.
7. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 from a grapevine cane-segment bioassay when the grapevine canes (Mission/Sultana seedling) used had no endogenous *A. vitis*.
8. Viability of three *Agrobacterium* strains when incubated with phytoalexin, quinone and phenolic compounds for 24 hours.
9. Viability of 27 *Agrobacterium* strains when incubated in a solution of 10 µg/ml benzoquinone for 24 hours at 25°C.
10. Effect of ascorbic acid added to the incubation buffer on recovery of strains *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay.
11. Polygalacturonase activity of transconjugant and wild-type *Agrobacterium* strains.
12. Bacterial recovery on selective media with antibiotics.

13. Recovery of *Agrobacterium* strains with and without polygalacturonase activity from the grapevine cane-segment bioassay.
14. Bacterial strains used for inhibition studies in chapter six and chapter seven.
15. Survey of *A. vitis* isolates for sensitivity to antagonistic strains.
16. Characteristics of antagonistic strains.
17. Recovery of bacterial strains on TY and RS media amended with different concentrations of mercuric chloride.
18. Effect of antagonistic strains on the recovery of Tn501-tagged *A. vitis* K1345 from the grapevine cane-segment system bioassay.
19. Performance of antagonistic strains in pathogen displacement studies and in the artificial wound assay.
20. Incidence and size of gall formation at artificial wound sites on grapevines that have been previously treated with thermotherapy.
21. Effect of different pathogen application methods on performance of biocontrol strains in restricting numbers of mercury resistant pathogens in grapevine xylem fluid.
22. Performance of antagonistic strains when data from grapevines dipped into a pathogen suspension is included with data from grapevines planted into pathogen infested soil.

SUMMARY

Effective biological control of a microbial pathogen, such as *Agrobacterium vitis*, requires an understanding of the biology and ecology of the pathogen. The first part of this study investigated whether *A. vitis* strains have any special adaptations for colonising the vascular system of grapevines. The second part investigated a number of antagonistic strains isolated from different ecological backgrounds. Results from screening assays for potential biocontrol strains were compared with the results from a glasshouse pot trial.

To examine the systemic nature of *A. vitis*, a bioassay that used dormant grapevine cane segments was devised. After a fourteen day incubation the bioassay indicated at least a ten-fold difference in survival between *A. vitis* strain K377 and *A. rhizogenes* strain K27, a rhizosphere inhabiting species. The decline in numbers of strain K27, which was more rapid than the natural rate of cell death, suggested that a plant defence mechanism was involved.

A major component of the host-defence response in xylem vessels of *Vitis vinifera* is immobilisation of microbes by a pectin-based gel. Bacterial polygalacturonases, also known as pectinases, can break down plant pectins. Consequently, the hypothesis that polygalacturonase (Peh) production by *A. vitis* may affect vascular colonisation was proposed. When compared using the grapevine cane-segment bioassay an *A. vitis* Peh⁻ mutant was recovered at ten-fold lower levels than an isogenic Peh⁺ strain. Expression of the cloned *pehA* gene in other species of *Agrobacterium* gave variable results, with improved recovery for one strain, *A. tumefaciens* K1329, but no differences for other strains. These results suggest that the *A. vitis* *peh* gene plays some role in bacterial survival in grapevines, as determined by the bioassay, although other mechanisms must also be involved.

Another component of the plant defence system is the production of anti-microbial compounds in response to elicitors liberated by microorganisms. Phytoalexins, phenolics and quinones are often associated with microbial infection of plants and these compounds can be toxic to bacteria. The tolerance of *Agrobacterium* strains was tested to the grapevine phytoalexin resveratrol, to the phenolic compounds chlorogenic acid and catechol and to the quinone benzoquinone. All compounds except benzoquinone were equally non-toxic to the bacteria tested. *A. vitis* strains and *A. tumefaciens* strains isolated from the grapevine vascular system proved to be more tolerant to benzoquinone than other *Agrobacterium* strains, indicating that tolerance to benzoquinone may be an advantage for systemic colonisation.

In the second part of this study, a number of agrocin-producing non-pathogenic bacteria were tested for biocontrol of grapevine crown gall. As part of this control strategy, the effectiveness of thermotherapy upon dormant grapevine cuttings in eliminating endogenous *A. vitis* was examined. Dormant cuttings that had been treated using the recommended thermotherapy of 50°C for 30 minutes and planted into steam treated soil in pots were grown for a number of months and then artificially injured by drilling holes into the stem. Gall formation only occurred in wounds on grapevines with the longest period (41 months) between thermotherapy and artificial wounding. The thermotherapy treatment used in this experiment may not completely eradicate *A. vitis* and, subsequently, numbers could regenerate to levels capable of initiating gall formation. However, it cannot be ruled out that the grapevines may have become recontaminated from an external source of infection.

Bacterial attachment to binding sites exposed by wounding is an essential step in the tumour formation process. Competition for such binding sites by non-pathogenic bacteria can reduce gall formation by pathogenic bacteria and may be a good screening process for potential biocontrol strains. Co-inoculation of artificial wound sites with non-pathogenic bacterial strains, including four bacteriocin producing strains, and a sensitive pathogenic strain resulted in reduced gall formation compared with inoculation with the pathogenic strain alone. *A. vitis* strain F2/5, an agrocin-producing strain and the only antagonistic strain with some tolerance to benzoquinone, resulted in complete inhibition of gall formation.

The ability of the same bacteriocin-producing non-pathogens to protect *A. vitis*-free potted vines from crown gall and re-infestation from soil-borne *A. vitis* was assessed. An *A. vitis* pathogen was tagged with a transposon conferring mercury resistance, enabling it to be easily distinguished from other bacteria. All strains resulted in significantly less gall formation than the control treatment, even *A. vitis* K1069 which was inhibitory to the pathogen when tested *in vitro*. Two agrocin-producing *A. vitis* strains, F2/5 and E26, isolated in South Africa and China respectively, completely prevented gall formation even when the grapevines had been dipped into a suspension of the pathogen before planting. Of the strains tested, only strain F2/5 resulted in lower densities of pathogen in both the rhizosphere and vascular tissue. Strain F2/5 has promise as a biocontrol agent of grapevine crown gall and warrants further investigation in field trials.

CHAPTER ONE

INTRODUCTION AND AIMS

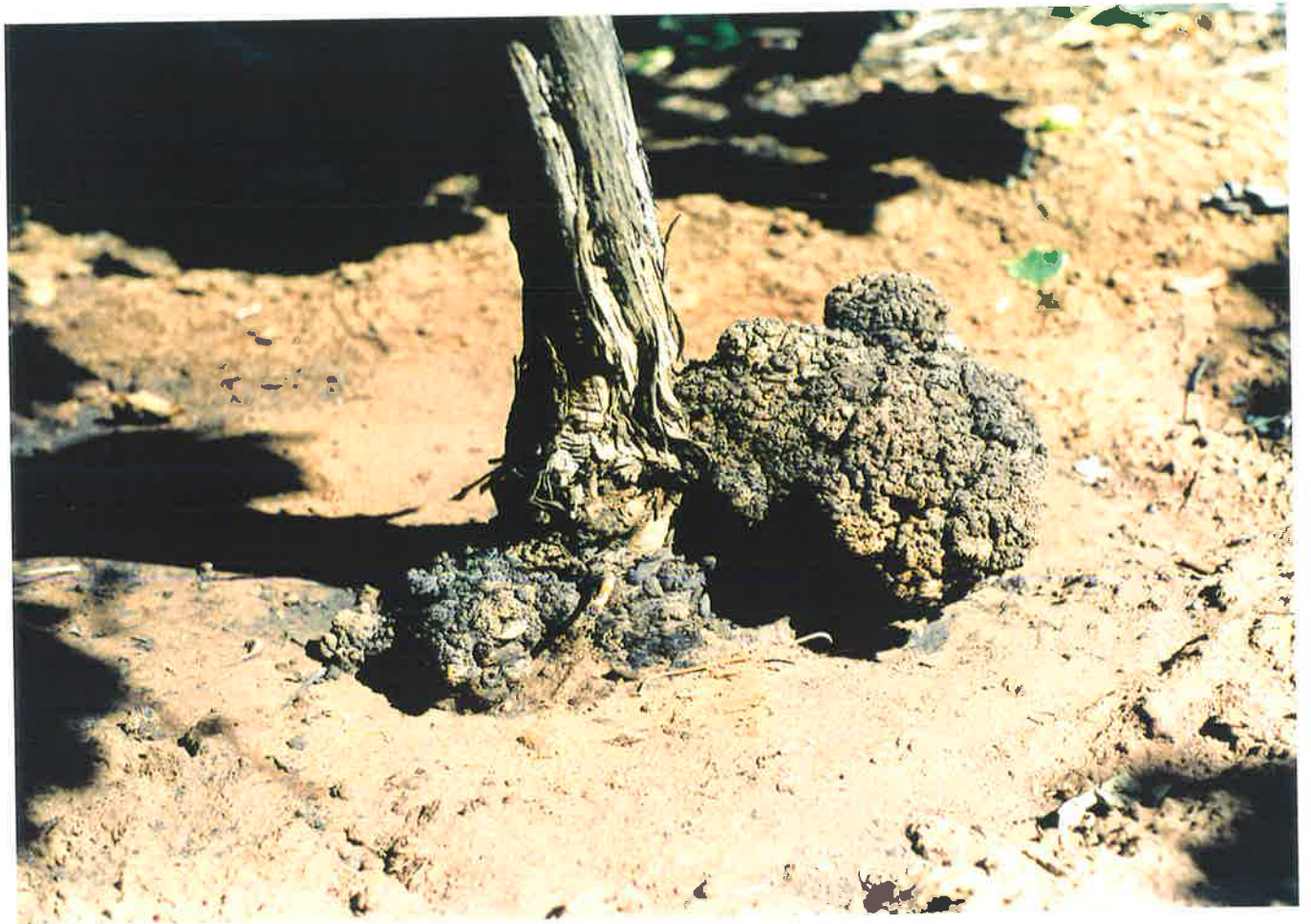
1.1 GENERAL INTRODUCTION

Detection of the grapevine crown gall pathogen, *Agrobacterium vitis* Ophel & Kerr 1990, in plant material is difficult as it can survive systemically (Lehoczky, 1968). The endophytic nature of *A. vitis* means it can be unknowingly distributed in apparently healthy propagation material (Lehoczky, 1971; Katz & Burr, 1983) and consequently, it is widespread throughout viticultural regions of the world (Panagopoulos & Psallidas, 1973; Loubser, 1978; Burr & Hurwitz, 1981; Sánchez-Serrano *et al.*, 1985; Ophel *et al.*, 1988; Jäger *et al.*, 1990).

Although the pathogen is widespread, the damage it imparts is often underestimated as even mild gall formation can reduce the productivity and longevity of grapevines (Fig. 1). Gall formation is commonly associated with cold-sensitive cultivars in frost-prone vineyards (Burr, 1978) and can manifest itself as aerial galls as well as crown galls. Even in arid areas not prone to frost, crown gall can reduce vigour and yield of grapevines; although in arid areas, galls are normally associated with graft injury as opposed to frost related injury which is more prevalent in cold climate regions (Schroth *et al.*, 1988).

Curative treatments for grapevine crown gall have proven ineffectual. The topical application of bacteriocidal chemicals to galls, a common practice of

Figure 1. Crown gall of grapevine (*Vitis vinifera*). The grapevine was a Calmeria scion grafted onto a Ramsey rootstock in a vineyard at Mildura, Australia.



viticulturists in some regions (Kan-Rice, 1988), has little effect as the presence of the inciting bacteria is unnecessary to sustain the gall (Braun & White, 1942). It is also unlikely that such an application would have much impact on systemic populations of *A. vitis*, although there is no evidence to support this.

Burr & Katz (1984) found that grapevine cuttings were a major source of infection and that a control strategy should include the use of planting and grafting material free of *A. vitis*. Several approaches were developed with the aim of eliminating *A. vitis* from grapevine propagating material, including; selection and growth of green-wood tips, *in vitro* shoot tip culture and thermotherapy of infested dormant cuttings. Each treatment has advantages and disadvantages and these will be discussed in the literature review. Importantly, these techniques can be used to produce grapevines free of the crown gall pathogen.

A. vitis can also be detected in soil near vines, although rarely is it found in soil beyond the grapevine rhizosphere (Burr & Katz, 1983; 1984). This indicates that soil from a diseased vineyard site may be a potential source of pathogenic *A. vitis*. Recent evidence that *A. vitis*-free vines can become recontaminated within sixteen months of being planted into infected vineyard soil was presented by Goodman *et al.* (1993). Considering this, an appropriate strategy to maintain 'clean' grapevines would be to plant them into non-vineyard soil. However, many vigneron wish to replant sites that have been previously used for grapevines and may therefore be contaminated. Hence, a mechanism is needed to prevent reinfestation and subsequent disease.

Biological control may offer a way of protecting *A. vitis*-free grapevines from disease when they are planted into infested soil. Effective control of crown gall of stone fruit crops is possible when they are treated with the biocontrol agent strain K84 but strain K84 is ineffective against *A. vitis* isolates (Kerr & Panagopoulos, 1977). Although there are no reports of successful biological control of grapevine crown gall in the field, there have been several bacterial strains isolated which are inhibitory towards the *A. vitis* isolates tested (Staphorst *et al.*, 1985; Webster *et al.*, 1986; Chen & Xiang, 1986; Liang *et al.*, 1990). Further work is necessary to determine if any of these strains will protect healthy grapevines from soil-borne infection.

1.2 PROJECT AIMS

The aims of this project were:

1. To examine various strains of bacteria for their potential as biological control agents of grapevine crown gall and, in particular, to see whether *A. vitis*-free propagating material could be protected by the bacteria when planted into soil infested with *A. vitis*.
2. To investigate the ecological niche of *A. vitis* and determine what phenotypic traits are required for bacteria to be competitive under these conditions.
3. To study the mechanisms involved in successful biological control by comparing results from *in vitro* and *in planta* assays with those from biocontrol experiments on potted grapevines.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 NOMENCLATURE OF *AGROBACTERIUM*

2.1.1 The genus *Agrobacterium*

The organism responsible for inciting crown gall was discovered by Smith & Townsend (1907) and named *Bacterium tumefaciens*. Conn (1942) subsequently placed *B. tumefaciens* in the genus *Agrobacterium*, a member of the family *Rhizobiaceae*.

The classification of *Agrobacterium* and *Rhizobium* has generated some confusion. Fast-growing *Rhizobium* species form a close relationship with *Agrobacterium* when examined using biochemical and DNA hybridisation techniques. Proposals that *Agrobacterium* should be combined into *Rhizobium* with a new genus, *Phytomyxa*, for the slow-growing rhizobia were suggested (Graham, 1964; Heberlein *et al.*, 1967). This classification was adopted by some (Moffett & Colwell, 1968; White, 1972) but has not continued, with *Agrobacterium* remaining a separate genus.

The genus *Agrobacterium* was classified into species from information based on pathogenicity. The taxonomy of *Agrobacterium* has been reviewed by Kersters & De Ley (1984). Allen & Holding (1974) proposed that *A. tumefaciens* contains strains causing crown gall, *A. rhizogenes* contains strains causing hairy root, *A. rubi* contains strains causing cane gall on *Rubus*

spp. and *A. radiobacter* contains non-pathogens.

2.1.2 Pathogenicity of *Agrobacterium*

Nomenclature based on pathogenicity proposed by Allen & Holding (1974) was shown to be unsuitable, as *A. tumefaciens* could not be distinguished from *A. radiobacter* using biochemical and DNA hybridisation tests (Graham, 1964; De Ley *et al.*, 1966; Heberlein *et al.*, 1967). The genes for pathogenicity were shown to be carried on a plasmid that can be transferred from pathogenic to non-pathogenic strains (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). Consequently, *A. radiobacter* could be transformed into *A. tumefaciens* by way of plasmid transfer, a situation that does not lead to clear classification.

2.1.3 Stable taxonomy

Effective taxonomy needs to be based upon stable chromosomal characteristics and not upon transient attributes, even if they are agronomically important. Taxonomic studies using biochemical tests found isolates clustered into two major groups that did not correspond to pathogenicity (Keane *et al.*, 1970; White, 1972). The taxonomic position was clear; however, suitable nomenclature could not be agreed upon. It was proposed that *A. radiobacter*, *A. tumefaciens*, *A. rhizogenes* and *A. rubi* be included into a single species, *A. radiobacter*, which was divided into two distinct biotypes (Keane *et al.*, 1970; Kerr *et al.*, 1978). Biotype 1 contained *A. radiobacter* and *A. tumefaciens* strains and biotype 2 contained *A. rhizogenes* strains, with *A. rubi* strains being merged with the biotype 2 cluster. A varietal epithet was used to indicate pathogenicity. Studies by Kersters *et al.* (1973) confirmed clustering of isolates into biotype 1 and

biotype 2 categories, but found *A. rubi* isolates formed their own distinct cluster. Holmes & Roberts (1981) preferred separate species for *A. tumefaciens* and *A. rhizogenes*, disagreeing with Kerr *et al.* (1978) in preferring the retention of *A. rubi* and the removal of *A. radiobacter* as species. They proposed *A. tumefaciens*, *A. rhizogenes* and *A. rubi* with 'state' epithets (saprophytic, tumorigenic and rhizogenic) to indicate pathogenicity.

A third biotype was suggested by Kerr & Panagopoulos (1977) for grapevine isolates which could not be allocated to either biotype 1 or biotype 2. Ophel & Kerr (1990) found biotype 3 isolates were distinct in phenotype and DNA homology from other *Agrobacterium* species and suggested *A. vitis* to indicate the specificity of these strains for grapevines. In recent studies using fatty acid analysis and carbon utilisation to determine relatedness it was confirmed that *Agrobacterium* forms four distinct clusters and proposed that biotypes be elevated to species (Bouzar *et al.*, 1993).

In this manuscript *Agrobacterium* strains will be referred to as *A. tumefaciens* (previously biotype 1), *A. rhizogenes* (previously biotype 2), *A. vitis* (previously biotype 3) and *A. rubi* regardless of the presence or type of plasmid harboured.

2.2 ECOLOGY OF AGROBACTERIUM VITIS

2.2.1 Host specificity

Reports from around the world confirm that *A. vitis* exhibits strong host specificity towards grapevines (Loubser, 1978; Perry & Kado, 1982; Ma *et al.*, 1987; Ophel & Kerr, 1987; Bien *et al.*, 1990; Haas *et al.*, 1991), although

several reports of systemic *A. tumefaciens*, possibly *A. vitis*, isolated from chrysanthemum have also been recorded (Miller, 1975; Bazzi & Rosciglione, 1982; Jones & Raju, 1988). Grapevine sap, roots and galls are the sites from which *A. vitis* is predominantly isolated (Burr & Katz, 1983; Perry & Kado, 1981). *A. tumefaciens* has been isolated from these same sites (Burr & Katz, 1984; Burr *et al.*, 1987b) albeit at much lower levels than *A. vitis* and the *A. tumefaciens* isolates are generally non-tumorigenic (Bien *et al.*, 1990) with a few exceptions (Sánchez-Serrano *et al.*, 1985; Thies *et al.*, 1991). Consequently, *A. tumefaciens* is considered to play only a minor role in gall formation on grapevine.

2.2.2 Ecological specialisation

A. vitis has marked host specificity for *Vitis vinifera* and it is speculated that ecological adaptation of *A. vitis* has contributed to this. The process by which *A. vitis* moves between the soil and the grapevine vascular system is an important aspect of this specialisation. *A. vitis* has been isolated from necrotic lesions on grapevine roots (Burr *et al.*, 1987b) and subsequently shown to be responsible for this grapevine-specific root decay (Burr *et al.*, 1987a). It was suggested that *A. vitis* may penetrate the root vascular system through these lesions, and thus have a mechanism through which it can migrate from the soil into the grapevine vascular system. Ophel *et al.* (1990b) found that polygalacturonase activity in combination with endoglucanase activity were required for grapevine-specific root decay because *A. tumefaciens* transconjugants with either functional polygalacturonase or endoglucanase clones were alone unable to induce root decay. Evidence from an *A. vitis* polygalacturonase-minus mutant suggested that polygalacturonase activity was also involved in multiplication of bacteria at wound sites and attachment to plant roots (Brisset *et al.*, 1991). The mutants

were also unable to induce root decay and were diminished in tumorigenicity, both of which are possibly important virulence factors (Rodriguez-Palenzuela *et al.*, 1991). On a different aspect of ecological specialisation, Szegedi (1985) proposed that tartrate utilisation by *A. vitis* may play a role in bacterial survival, as tartrate is present at significant concentrations in the xylem of *V. vinifera*. These findings suggest that *A. vitis* has a degree of specialisation for the ecological niche provided by grapevines.

2.2.3 Host defence mechanisms

As *A. vitis* forms a close association with grapevines, interactions between bacteria and host defence mechanisms are inevitable. An important aspect of plant defence is production of anti-microbial compounds in response to microbial elicitors. One type of inducible response is phytoalexin production (Cruickshank & Perrin, 1960). In grapevines, phytoalexin-like compounds called stilbenes, including resveratrol, ϵ -viniferin and pterostilbene, are induced by fungal and bacterial pathogens (Langcake & Pryce, 1977; Langcake *et al.*, 1979). Similarly, phenolic compounds are mobilised by plant cells in response to injury or to invading organisms (Farkas & Király, 1962). Polyphenol oxidases then play an important role in rapidly oxidising released phenolic compounds to highly reactive quinones (Mayer, 1987). Quinones bind unspecifically to proteins reducing or extinguishing their biological activity (Leatham *et al.*, 1980; Zaprometov, 1992) and are toxic to most bacteria (Geiger, 1946). However, the degree to which phytoalexins and phenolic compounds contribute to defence against micro-organisms in the grapevine vascular system is not known.

Along with anti-microbial biochemical defence mechanisms, there are also mechanisms that prevent the spread of systemic bacteria. The most well

studied localisation mechanism is vascular occlusion (Beckman & Zaroogian, 1967). There are three main occlusion processes: tylosis formation involving hypertrophy of xylem parenchyma, where xylem parenchyma cells expand until the lumen of adjacent vessels becomes blocked and prevents passage of xylem contents; gummatation along the inner surface of vessels that traps debris, including fungal spores, hyphae and bacteria; and gel formation, a three-dimensional net-like pectin matrix within the vessel lumen (Beckman, 1964). In *Vitis vinifera* grapevines infected with Pierce's Disease bacteria, the main mechanism of vascular occlusion is production of pectin gel with very little tyloses or gums (Fry & Milholland, 1990).

2.2.4 Aerial galls on grapevine

The cause of aerial gall formation, or secondary tumours, on plants has been of interest to researchers for a considerable time. Smith *et al.* (1912) observed that secondary tumours on tomato were formed from tumour strands originating from primary tumours. It was suggested that stem elongation carried the secondary tumour and tumour strand up from the primary tumour (Riker, 1923). However, in subsequent studies by Stapp (1936) no such strands could be found, this led him to propose that secondary tumours may be formed by bacteria that spread in xylem vessels. Wilson & Magie (1964) found evidence supporting Stapp's theory while investigating *Pseudomonas savastanoi* in olive and oleander.

In grapevine, a possible explanation for aerial gall formation was first proposed by Lehoczky (1968), who isolated tumorigenic *A. tumefaciens* from the sap of bleeding canes of diseased grapevines. Such aerial tumours tend to be closely correlated with frost injury. Seasons with severe frosts in Ontario led to a predominance of cane and trunk galls according to an early

report by Chamberlain (1962). This report was verified by Burr & Hurwitz (1981) in New York State vineyards. However, in arid areas with lower frost risk than in cold climate areas Schroth *et al.* (1988) observed aerial galls were found less frequently. Therefore, gall formation tends to be a function of the presence of systemic pathogenic bacteria coinciding with plant cell injury, such as that arising from severe frosts or grafting wounds (Fig. 2).

2.2.5 Detection of *A. vitis*

Isolation of systemic bacteria is not straightforward, as the bacteria must first be extracted from the woody tissue. Early studies by Lehoczy (1968) relied on sampling sap exuding from cut canes but this technique could only be successfully used during Spring when there was adequate root pressure to force sap through the canes. A water displacement method was developed that forced water through cane sections while collecting the exudate (Tarbah & Goodman, 1986; Tarbah & Goodman, 1987). This technique had the benefit of being able to be used on dormant grapevine material, was laboratory based and was more sensitive than sampling exuding sap from canes. A modification of the water displacement method allowed water to be drawn through the segments under vacuum (Bazzi *et al.*, 1987) which simplified the process and made it by far the most effective technique. Another approach was to sample by drilling holes into wood and collecting filings (Jäger, personal communication); however, this technique can be time consuming and the precision of the results is yet to be confirmed.

Figure 2. Gall formation at the graft union of a grafted grapevine (*Vitis vinifera*). The grapevine was a Sultana scion grafted onto a Ramsey rootstock in a vineyard at Menindee, Australia. The arrow (◄) indicates the gall.



Selective media have been developed by several researchers in an attempt to streamline isolation and identification of *A. vitis*. Brisbane & Kerr (1983) developed a useful medium, 3DG; but widespread acceptance has been limited because certain *A. tumefaciens* strains readily grow on it and *A. vitis* strains isolated from chrysanthemum cannot. Another selective medium, RS, developed by Roy & Sasser (1983) has been more widely accepted but also has limitations: *A. vitis* colony morphology can be variable; numerous other endophytic bacteria are able to grow on it (Bishop *et al.*, 1989); and the recovery rate can be as low as thirty percent (Goodman *et al.*, 1987).

These selective media aid in semi-selective recovery of *A. vitis* but are not verification of identity. A monoclonal antibody developed by Bishop *et al.*, (1989) proved rapid and reliable for diagnosis of *A. vitis* strains from diverse origins (Bazzi *et al.*, 1988). An alternative possibility has been reported by Jäger *et al.* (1989) who successfully used gas chromatography of whole cell fatty acids to identify *A. vitis* isolates, but this technique requires expensive equipment that may not be readily available to most laboratories.

2.2.6 Eradication of *A. vitis*

Rapid and accurate detection of *A. vitis* greatly facilitated the development of techniques to eradicate the pathogen from grapevine propagation material. Tarbah & Goodman (1986) suggested greenwood tip cuttings may be good propagating material after they found *A. vitis* numbers in apical sections of hardwood cuttings were less than in basal sections. However, *in situ* detection of large numbers of *A. vitis* in the primary xylem vessels of green Spring shoots by Stefani & Bazzi (1989) indicates that such phytosanitary techniques should be used with caution. Burr *et al.* (1987c) obtained *A.*

vitis-free vines by *in vitro* culture of the shoot tips from early season green shoots. This method has proven reliable but it is time consuming and consequently can be costly.

A rapid and cost effective method of producing *A. vitis*-free propagating material is disinfestation of contaminated dormant cuttings using thermotherapy. Burr *et al.* (1989) found thermotherapy of dormant cuttings at fifty degrees Celsius for thirty minutes reduced *A. vitis* populations to below the detectable limit. Thirty minutes of thermotherapy had no detrimental effect on vine vitality in experiments conducted by Bazzi *et al.* (1991) and Wample *et al.* (1991) added to this with the finding that the greatest tolerance to thermotherapy is shown by cuttings taken from grapevines during mid-winter dormancy. Ophel *et al.* (1988) demonstrated reduced crown gall incidence in similarly heat treated cuttings in a field nursery, but found the pathogen was not eliminated. This simple thermotherapy technique has been readily adopted by industry and government organisations in Australia where complete eradication of *A. vitis* has not been necessary for effective control. However, in cold regions where the risk of frost injury is higher thermotherapy may not be the most appropriate technique to use.

2.3 TUMOUR FORMATION BY AGROBACTERIUM

Tumour formation involves a complicated interaction between bacterium and plant cells. A closer understanding of the disease process should provide knowledge that helps improve biological control of the crown gall disease. Much of the research into tumour formation has concentrated on the mechanisms of oncogenic *A. tumefaciens* strains as this species is widely distributed and well characterised. These mechanisms are equally applicable

to *A. vitis* strains, although subtle differences in certain *A. vitis* oncogenes can influence tumour formation on some hosts but this is discussed in section 2.3.6 on host range determination.

2.3.1 Bacterial attachment to plant cells

The attachment of agrobacteria to plant cells is an integral step in the process of tumour formation. In experiments with *A. tumefaciens* on Pinto bean leaves, Lippincott & Lippincott (1969) showed tumour formation could be inhibited by prior or co-inoculation with non-tumorigenic strains. It was proposed that specific attachment sites must be present in the wound for the infection process to proceed and that certain bacteria have cell surface properties enabling them to compete for and bind to those sites. Tumour formation was also inhibited when a solution containing components of plant cell walls was added to a suspension of pathogenic bacteria and then applied to a wound site (Lippincott *et al.*, 1977). Consequently, it was suggested that the active component contained binding sites with which the pathogens interacted before they could reach binding sites exposed in the wounded plant cells. However, when the ability of bacteria to attach was lost due to mutation, tumour formation was not inhibited by inoculation of these mutants before virulent strains (Douglas *et al.*, 1982). These chromosomal mutants were mapped to two loci, *chvA* and *chvB* (Douglas *et al.*, 1985). *chvA* and *chvB* are structurally and functionally related to *ndvA* and *ndvB* of *Rhizobium*, and are also involved in site specific binding (Dylan *et al.*, 1986; Cangelosi *et al.*, 1987), indicating that bacterial attachment plays a key role in plant-microbe interactions.

Along with *chvA* and *chvB* related binding, fibril formation is involved in attachment of bacteria to plant cells. Using scanning electron microscopy

Matthysse *et al.* (1981) observed fibril formation following attachment of *Agrobacterium* to plant cells. The fibrils secured bacteria to the cell surface and entrapped other bacteria resulting in large clusters next to the plant cell wall. When chromosomal mutants were generated with altered polysaccharide composition, they were shown to have poor plant cell attachment and attenuated virulence on a range of hosts (Thomashow *et al.*, 1987). The mutations were mapped to a locus, *pscA*, distinct from *chvA* and *chvB*, although with a similar phenotype. Cellulose fibrils have some role in attachment but may not be essential, as Douglas *et al.* (1982) demonstrated that a *chvA* mutant which had produced fibrils was still unable to attach.

2.3.2 Plasmid-borne virulence

A majority of the loci involved in the transformation process have been localised not to the chromosome, but to a plasmid. Kerr (1969) presented evidence for transfer of virulence from a plant pathogenic *Agrobacterium* strain to a saprophytic strain. This transfer of virulence from oncogenic to non-oncogenic strains was due to the transfer of a large tumour inducing (Ti) or root inducing (Ri) plasmid (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975; White & Nester, 1980). Hooykaas *et al.* (1977) demonstrated that the transfer of an *A. tumefaciens* Ti plasmid to a *R. trifolii* (now called *R. leguminosarum* biovar *trifolii*) strain resulted in a tumour inducing *Rhizobium* strain. Mutagenesis studies on *A. tumefaciens* confirmed that most genes for virulence were located on the Ti plasmid in a section known as the virulence (*vir*) region, although some were chromosomally located (Garfinkel & Nester, 1980). Complementation analysis of Tn5 Ti plasmid mutants by Klee *et al.* (1982) determined several essential loci. The virulence region of pTiA6 was shown to consist of six distinct loci; *virA*, *virB*, *virC*, *virD*, *virE* and *virG*. Mutations in *virA*, *virB*, *virD* or *virG* eliminated the

transformation process completely and mutations in *virC* or *virE* affected the efficiency of transformation and the host range (Stachel & Nester, 1986). Two further loci were identified, *virF* and *virH*, that were specific to octopine-type Ti plasmids and influenced host range as well (Hooykaas *et al.*, 1984; Stachel & Nester, 1986).

The *vir* loci belong to a single regulon the transcriptional activation of which is positively controlled by the expression of *virA* and *virG* gene products (Stachel & Zambryski, 1986). It was proposed that plant signal molecules may be recognised by the *virA* gene product which subsequently activates the *virG* protein and enables it to switch on the remaining *vir* loci (Winans *et al.*, 1986; Leroux *et al.*, 1987). Yanofsky *et al.* (1986) determined that the *virD* locus encodes an endonuclease that breaks the DNA at the border of the transferred DNA (T-DNA). The function of the *virD* locus has been likened to bacterial mobilisation (*mob*) genes. Evidence by Buchanan-Wollaston *et al.* (1987) that the mobilisation functions of a wide host range plasmid, RSF1010, could mediate the transfer of plasmids from *Agrobacterium* into a plant cell, as well as between bacteria strengthened the analogy between bacterial conjugation and *Agrobacterium* mediated DNA transfer to plant cells. The *virC* locus, in combination with a conserved DNA sequence designated overdrive, enhances the cleavage process of *virD* (Toro *et al.*, 1988) but how this is achieved is yet to be determined. The *virE* locus has been implicated in protection and transport of T-DNA by Gietl *et al.* (1987) who isolated a sequence-non specific single-stranded DNA binding protein encoded by the *virE* locus. The functions of the *virB* loci have not been clearly elucidated but protein analysis indicates most are membrane-associated proteins and may function as a channel across the bacterial membrane for the T-DNA to pass through (Shirasu *et al.*, 1990). The *vir* region of *Agrobacterium* Ti plasmids has been extensively reviewed in recent

years (Binns & Thomashow, 1988; Zambryski, 1992).

2.3.3 Initiation of the disease process

The *vir* region of the Ti plasmid is activated by various molecules of plant origin. Stachel *et al.* (1986a; 1986b) purified molecules involved in induction of the *vir* regulon from exudates of wounded plant cells. The purified molecules were characterised as acetosyringone and alpha-hydroxy acetosyringone Stachel *et al.* (1985b). These compounds although abundant in wounded plant tissue are present at relatively low levels in healthy tissue. Besides these plant phenolics, it was shown that various plant sugars induce the *vir* regulon (Ankenbauer & Nester, 1990). Furthermore, a chromosomal virulence gene, *chvE*, that produces a galactose and glucose binding protein was characterised by Huang *et al.* (1990) and mutations in *chvE* significantly reduced induction of the *vir* region.

Certain plant compounds also influence bacterial activity by stimulating chemotaxis, a process that may increase the chance of virulent bacteria encountering a susceptible wounded plant cell. The chemotactic response may be affected by the presence of the Ti plasmid; however, the extent and the method of modulation are unknown. Ashby *et al.* (1987) observed chemotaxis only in strains with Ti plasmids. Parke *et al.* (1987) demonstrated that several aromatic compounds that served as *vir* inducers were also chemo-attractants in either the presence or absence of the Ti plasmid. In contrast, Hawes *et al.* (1988) found that strains both with and without a Ti plasmid expressed attraction to pea root tips and root cap cells; indicating that chemotaxis is presumably a chromosomal trait. The Ti plasmid loci may add specificity in some cases. This subject is further covered by Winans (1992), who reviewed recent work on molecular signals between

agrobacteria and plants.

2.3.4 Inter-kingdom DNA transfer

Following induction of the *vir* genes on the Ti plasmid, a discrete segment of Ti plasmid is integrated into the plant nuclear genome. Braun & White (1942) speculated that susceptible plant cells must be permanently altered prior to tumorigenesis but it was Chilton *et al.* (1977; 1978) who were the first to provide evidence of the stable incorporation of a section of plasmid DNA into a plant cell. The position of the transferred DNA (T-DNA) in the transformed plant cell was further localised by Willmitzer *et al.* (1980), who did not find T-DNA in mitochondria or chloroplasts (plant cell organelles of bacterial origin) but only in the nucleus.

The T-DNA segment was subsequently characterised. Studies to define the T-DNA by Yadav *et al.* (1982) discovered the region was flanked by specific sequences consisting of 25 base pair imperfect direct repeats. Expanding on this, Wang *et al.* (1984) found the right T-DNA border region was essential for tumorigenicity as mutants with this region deleted were unable to transform plants. Several loci are located within the T-DNA border regions; three are important for oncogenicity and the others are involved in opine synthesis (discussed in the following section). The oncogenes code for enzymes that synthesise plant hormones and include the *ipt* loci involved in cytokinin synthesis (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchman *et al.*, 1985) and *iaaM* and *iaaH* necessary for production of auxin (Schroder *et al.*, 1984; Thomashow *et al.*, 1984). These plant hormones of bacterial origin create an imbalance in the plant cell hormone levels, leading to cell proliferation and result in either the tumorous (Ti) or hairy root (Ri) phenotype.

2.3.5 Production and utilisation of opines

The unusual class of amino acid derivatives produced by transformed crown gall and hairy root cells are known collectively as “opines” (Tempé *et al.*, 1977). This subject was reviewed by Tempé & Goldman (1982) and Winans (1992). The genes responsible for the catabolism and synthesis of opines are distinct from each other and occur on the same oncogenic plasmids (Watson *et al.*, 1975; Bomhoff *et al.*, 1976; Kerr & Roberts, 1976; Montoya *et al.*, 1977). The catabolic genes are expressed in *Agrobacterium* strains harbouring oncogenic plasmids and Petit & Tempé (1978) hypothesised that the synthetic genes were expressed in the transformed plant cells. Such production of specific compounds by transformed plant cells, and their utilisation by related bacteria, confer a nutritional and ecological advantage over other rhizosphere inhabitants. This unique system was described by Petit *et al.* (1978a) as the opine concept; “...substances synthesized by the cells of the host-plant in response to a stimulus from the pathogen. Their presence creates favorable environmental conditions for the pathogen and contribute to its dissemination”. However, subsequent studies have shown that opine utilisation is not limited to pathogenic agrobacteria. Various non-pathogenic agrobacteria can utilise opines, indicating that in some cases genes for opine utilisation are not located on the Ti plasmid (Lippincott *et al.*, 1973; Montoya *et al.*, 1978). There are also reports of opine catabolising pseudomonads (Köhn & Beiderbeck, 1982; Brisbane & Kerr, 1983). Ten percent of the bacteria isolated from natural savanna by Bouzar & Moore (1987) were opine utilisers and eighty-three percent of these opine-utilising isolates were neither agrobacteria nor fluorescent pseudomonads. These results indicate that the source and role of opines beyond the crown gall environment needs to be further investigated.

Opines may thus not provide such an exclusive source of nutrition as first thought; however, they have been implicated in another ecological advantage. Some opines can induce conjugal transfer of plasmids between agrobacteria, subsequently enhancing dissemination and survival of these pathogenic plasmids (Petit *et al.*, 1978b).

2.3.6 Specific loci determine host range

Host range refers to the variety of host plants in which agrobacteria can induce tumour formation following artificial inoculation. Most *Agrobacterium* isolates have a large host range but it can vary significantly between species and even strains (De Cleene & De Ley, 1976; Anderson & Moore, 1979). The host range of *Agrobacterium* isolates is determined largely by genetic factors on the Ti plasmid (Loper & Kado, 1979), although chromosomal loci, such as *ros*, *chvD* and *chvE*, can have some affect (Close *et al.*, 1987; Winans *et al.*, 1988; Huang *et al.*, 1990). *A. vitis* Ti plasmids have been classified into two main categories, wide host range (WHR) and limited host range (LHR) plasmids (Thomashow *et al.*, 1980; Knauf *et al.*, 1982); with the LHR *A. vitis* types being the subject of much investigation. The discovery by Knauf *et al.* (1984) that the Ti plasmid pTiAg162 from a LHR strain consisted of two T-DNA regions, T_A-DNA and T_B-DNA, when all characterised WHR Ti plasmids have only one T-DNA region indicates that distinct structural differences exist between the plasmid types.

The narrow host range of certain *A. vitis* strains can be attributed to aberrations in either the LHR T-DNA compared with WHR T-DNA or in the corresponding *vir* regions (Yanofsky *et al.*, 1985a). Evidence for genes from the T-DNA affecting host range came from Hoekema *et al.* (1984) when they

complemented a LHR strain with the T-DNA from a WHR strain and extended the host range. The mechanism by which the WHR T-DNA extended the host range of the LHR strains was complementation of an ineffective *ipt* locus with an active one (Buchholz & Thomashow, 1984; Hoekema *et al.*, 1984; Yanofsky *et al.*, 1985b). The *ipt* locus, and other cytokinin loci (Inze *et al.*, 1984), are essential for tumour formation on most plants but not on grapevines. In fact, infection of grapevines by *Agrobacterium* strains with a functional *ipt* locus leads to tissue necrosis. However, the *iaa* loci that are required for auxin synthesis are essential for tumour formation on grapevines (Bonnard *et al.*, 1989; Huss *et al.*, 1990). So from an evolutionary standpoint it seems likely that the *ipt* locus has become inactive in the Ti plasmids of many of the *A. vitis* strains that are closely associated with grapevines.

Host range in *Agrobacterium* is determined not only by genes in the T-DNA and bacterial chromosome but by genes in the *vir* region as well. Although WHR T-DNA extends the host range of LHR Ti plasmids, Yanofsky *et al.* (1985a) found that *virA* and *virG* from the *vir* region of a WHR Ti plasmid were necessary to completely restore the host range. The DNA and proteins of LHR *virA* showed little homology to that of WHR *virA* (Leroux *et al.*, 1987; Ma *et al.*, 1987; Turk *et al.*, 1993) and Leroux *et al.* (1987) speculated that as the proteins showed greatest divergence at their binding regions the LHR *virA* may recognise different, possibly grapevine specific, molecules. Other *vir* genes with some involvement in host range determination include *virF* (Hooykaas *et al.*, 1984; Jarchow *et al.*, 1991), *virC* (Hille *et al.*, 1984; Hooykaas *et al.*, 1984; Yanofsky & Nester, 1986) and *virE* (Hirooka & Kado, 1986). However, the exact nature of how these *vir* loci interact with tumour formation on different hosts is yet to be revealed. Zambryski (1992) has reviewed research on Ti plasmid-determined host range.

2.4 BIOLOGICAL CONTROL OF CROWN GALL

2.4.1 Biological control of stone fruit crown gall

Crown gall of stone fruit is caused by pathogenic strains of *A. rhizogenes* and *A. tumefaciens*. Kerr (1992) has recently reviewed research on crown gall inciting bacteria and their biological control. A non-pathogenic *A. rhizogenes* strain was isolated from a peach gall by New & Kerr (1972) that was inhibitory towards many of the pathogenic *Agrobacterium* isolates tested. The inhibitory isolate, strain K84, was found to be effective as a biocontrol agent when plant roots were dipped into a suspension of the bacteria (Kerr, 1972). A number of laboratories obtained strain K84 for testing and found it to be an effective biocontrol agent for crown gall on stone fruit and rose crops (Kerr & Panagopoulos, 1977; Dhanvantari, 1983; Farkas & Haas, 1985; López *et al.*, 1987).

2.4.2 Mode of action of K84

The efficiency with which K84 protected susceptible crops led to much interest and research into the mechanisms of control. Kerr & Htay (1974) produced evidence that one mechanism of control by K84 was antibiotic production. They termed the antibiotic bacteriocin 84 but it was later changed to agrocin 84 (Engler *et al.*, 1975). Non-oncogenic strains were not sensitive to agrocin 84 and it was demonstrated that agrocin 84 sensitivity was determined by the Ti plasmid (Engler *et al.*, 1975). Kerr & Tate (1984) have reviewed the mechanisms of biocontrol by strain K84.

In biochemical studies by Roberts *et al.* (1977), agrocin 84 was isolated and

characterised as a nucleotide analogue, a new type of bacteriocin. Further studies on agrocin 84 by Tate *et al.* (1979) pinpointed the substituent that confers specificity of agrocin 84 to pathogenic strains harbouring nopaline type Ti plasmids. The selectivity of agrocin 84 was due to selective uptake of the agrocin (Murphy & Roberts, 1979), by a permease gene usually involved with the uptake of an opine called agrocinopine A (Ellis & Murphy, 1981). The agrocinopine A permease transports agrocin 84 into the cell where it prevents DNA replication and consequently stops growth (Das *et al.*, 1978).

2.4.3 Agrocin 84 production and sensitivity

With much of the biochemical nature of K84 elucidated, interest turned to the genetic mechanisms concerned and to determine if agrocin 84 production was the only factor involved in biocontrol. Ellis & Kerr (1978) found agrocin 84 production was coded for by a small plasmid, later established as pAgK84 (Slota & Farrand, 1982). pAgK84 could be conjugally transferred between bacteria (Farrand *et al.*, 1985). Transfer of pAgK84 to non-agrocin producing strains resulted in transconjugants that produced agrocin 84 and were effective biocontrol agents. However, it was noted that several of the agrocin 84 producing transconjugants did not offer effective control and that perhaps agrocin 84 production, although vital for inhibition, was not the only factor in successful biocontrol by K84 (Ellis & Kerr, 1978; Ellis *et al.*, 1979). Later evidence supported Ellis & Kerr's hypothesis: Cooksey & Moore (1982) observed that a K84 agrocin minus mutant strain prevented tumour formation when co-inoculated with an agrocin sensitive pathogen, although it has recently been reported that strain K84 produced another agrocin, agrocin 434 (Donner *et al.*, 1993), that is effective against most *A. rhizogenes* strains and may account inhibition when agrocin 84 is inactivated; and Shim *et al.* (1987) found that agrocin 84 producing *A. tumefaciens* strains were

less effective biocontrol agents than agrocin 84 producing *A. rhizogenes* strains.

With evidence that pAgK84 could be transferred among agrobacteria and thus confer the ability to produce and to exhibit resistance to agrocin 84, it seemed there may be a possibility that pathogens could acquire immunity to agrocin 84 in nature. Panagopoulos *et al.* (1979) isolated agrocin 84 producing pathogens from the gall of a plant that had been treated with a mixture of K84 and pathogenic strains. To prevent the transfer of pAgK84 occurring on a wide scale and consequently diminishing the usefulness of K84 as a biocontrol agent, the transfer region of the plasmid needed to be rendered inoperative. Jones *et al.* (1988) constructed a deletion mutant of K84 that was unable to transfer conjugatively between bacteria. This genetically engineered bacterium, strain K1026, proved as efficient as K84 in preventing crown gall in open air pot trials (Jones & Kerr, 1989; Jones *et al.*, 1991). K1026 was registered as “Nogall®” and became the first commercially released genetically engineered organism (Kerr, 1989).

Another threat against the effectiveness of biocontrol strains K84 and K1026 that would also lead to agrocin 84 immune pathogens was the acquisition of a Ti plasmid by the biocontrol strain, K84. This situation is unlikely though as both strains harbour another plasmid, pAtK84b, that has the same incompatibility group as nopaline type Ti plasmids (Clare *et al.*, 1990). Plasmids of the same incompatibility group are unable to reside within a bacterial cell at the same time.

2.4.4 Other potential biocontrol agents

Success of K84, and later K1026, inspired a number of research groups to search for more biocontrol agents of crown gall (Table 1). Cooksey & Moore (1980) isolated *Penicillium* and *Bacillus* strains that reduced the incidence of crown gall but not as effectively as K84. Henderson *et al.* (1983) isolated D286, a non-tumorigenic strain, which produced an agrocin with a broader host range than agrocin 84. Agrocin 286 was active against strains carrying nopaline, octopine and agropine Ti plasmids but it was not tested for biocontrol. Another agrocin from strain K84 was detected in a derivative strain, K434, which lacked pAgK84 (Donner *et al.*, 1993). This new agrocin, agrocin 434, inhibited all *A. rhizogenes* strains tested including non-pathogens, so it is thought sensitivity may be located on the chromosome. However, no *A. tumefaciens*, *A. vitis* or *A. rubi* strains were sensitive to agrocin 434. No new biocontrol agents are yet able to match the performance of strains K84 and K1026 for control of stone fruit and rose crown gall; it would seem effective biological control is more elusive than at first thought.

2.4.5 Biological control of grapevine crown gall

Early studies screening for biocontrol agents of grapevine crown gall were unsuccessful (Kerr & Panagopoulos, 1977; Panagopoulos *et al.*, 1978) and the prospect of effective biocontrol was considered remote. The efficient biocontrol agent of crown gall on stone fruit and roses, K84, was ineffective against *A. vitis* strains (Kerr & Panagopoulos, 1977). However, Dhanvantari (1983) detected *in vitro* sensitivity of *A. vitis* strains to K84 on amended Stonier's medium and prior inoculation of K84 suspensions led to some suppression of gall development on grapevines.

Table 1. Some bacterial strains documented as antagonistic toward crown gall pathogens.

Antagonistic strain	Host on which crown gall is prevented	Source
<i>A. rhizogenes</i> K84	Stone fruit	New & Kerr, 1972
<i>A. rhizogenes</i> D286	Stone fruit	Hendson <i>et al.</i> , 1983
<i>A. rhizogenes</i> K1026	Stone fruit	Jones <i>et al.</i> , 1988
<i>A. rhizogenes</i> K434	Stone fruit	Donner <i>et al.</i> , 1993
<i>A. vitis</i> F2	Grapevine	Staphorst <i>et al.</i> , 1985
<i>A. rhizogenes</i> J86	Grapevine	Webster <i>et al.</i> , 1986
<i>A. tumefaciens</i> HLB2	Grapevine	Chen & Xiang, 1986
<i>A. vitis</i> E26	Grapevine	Liang <i>et al.</i> , 1990

More recently, a number of promising agrocin-producing strains have been isolated. In South Africa, Staphorst *et al.* (1985) isolated agrocin producing non-pathogenic *A. vitis* strains including A4, F2, L1, I2, B5 and H6. Also in South Africa, Webster *et al.* (1986) isolated J86 a tumorigenic *A. rhizogenes* strain producing an agrocin inhibitory to *A. vitis* strains. The Ti plasmid of strain J86 was later cured thus enabling the strain to be used as a potential biocontrol agent (Webster *et al.*, 1986). In China, non-pathogenic *A. tumefaciens* strain HLB2 was isolated from a hop gall and was inhibitory to *A. vitis* strains (Chen & Xiang, 1986). In the same country an *A. vitis* non-pathogen, strain E26, was isolated from grapevine and was found to produce an agrocin that inhibited other *A. vitis* strains (Liang *et al.*, 1990).

The biocontrol potential of these agrocin-producing strains has not been fully investigated and there is evidence that only limited control of grapevine crown gall may be possible. Burr & Reid (1994) found *A. vitis* F2/5 (originally F2; Staphorst *et al.*, 1985) effectively prevented gall formation on artificially inoculated grapevines by certain *A. vitis* strains but not by all of those tested. Another antagonist, HLB2, was found to prevent gall formation on grape shoots when co-inoculated at a 1:1 ratio (Chen & Xiang, 1986). The potential of HLB2 as a biocontrol was further investigated by Pu & Goodman (1993b) who found tumour formation on grapevine explants was only reduced with HLB2 inoculated before the pathogen or at a higher concentration. Application of HLB2 to healthy grapevines planted into *Agrobacterium*-infested vineyard soil reduced the incidence of tumour formation to 22%, less effective than the fumigant Vorlex (16%). Furthermore, the grapevines were still reinfested by pathogenic *A. vitis* regardless of treatment (Pu & Goodman, 1993a). Yet another antagonist, *A. vitis* E26, was shown to inhibit gall formation on young grapevine shoots

and sunflower seedlings and to colonise wound sites effectively but biocontrol potential was not investigated (Liang *et al.*, 1990).

The capacity of these potential biocontrol strains to prevent reinfestation by soil-borne *A. vitis* has not been addressed, except in the case of strain HLB2 which was unsuccessful (Pu & Goodman, 1993a). Consequently, further characterisation and comparison of these potential biocontrol strains seems warranted.

2.5 CONCLUSIONS

Agrobacterium isolates cluster into four groups when characterised biochemically and these distinct groups have been made into four species; *A. tumefaciens*, *A. rhizogenes*, *A. vitis* and *A. rubi*. Each species is host specific with the exception of *A. tumefaciens* which is found on a broad range of hosts. *A. vitis* is closely associated with grapevines. That *A. vitis* causes a host-specific root decay and colonises grapevines systemically suggests a degree of host specialisation. Tumour formation by *A. vitis* is also host specific as most pathogenic isolates have a LHR Ti plasmid rather than a WHR plasmid seen in other *Agrobacterium* species. This close association of *A. vitis* with grapevines may have implications in biocontrol. Effective biocontrol of *A. vitis* will likely require a biocontrol agent to colonise grapevines as efficiently as *A. vitis* pathogens.

Research on the mechanisms of biocontrol by K84, used to control crown gall of stone fruit, suggested that agrocin production was an important factor in reducing tumour formation but other elements were also involved. Bacterial attachment is a pivotal step in the tumour formation process. Inhibition of attachment through competition for attachment sites has been

documented and may be a facet of successful biocontrol.

To achieve successful biocontrol of grapevine crown gall a more thorough understanding of the processes involved in preventing gall formation is necessary. The relative importance of bacteriocin production, competition for binding sites and ecological competence of biocontrol agents in controlling gall formation by *A. vitis* needs to be established. Finally, from a more practical perspective, the efficacy of biocontrol agents in protecting healthy grapevines from soil-borne infection is a fundamental cultural consideration.

CHAPTER THREE

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

3.1.1 Culture and storage of bacterial strains

3.1.1.1 *Culture of bacterial strains*

Non-selective growth of *Agrobacterium* and *Pseudomonas* strains was on TY agar (Appendix A. Culture media) or in TY broth (reciprocating shaker 50 r.p.m.) at 28°C for 48 hours. *E. coli* was grown on LB agar (Appendix A. Culture media) or in LB broth (reciprocating shaker 200 r.p.m.) at 37°C for 24 hours. For details on the origin and source of all the bacterial strains used in this thesis see Table 2.

3.1.1.2 *Preparation of washed bacteria*

A 48 hour TY broth culture was aseptically centrifuged at 4,000 r.p.m. for 15 minutes and the pellet resuspended in an equal volume of sterile MSALTS (Appendix C. Miscellaneous buffers).

3.1.1.3 *Storage of bacterial strains*

For long term storage of bacteria, cultures were lyophilised and kept at 4°C. For short to medium term storage, cultures were mixed with 15% glycerol and kept at -80°C.

Table 2. Origin and source of bacterial strains used in this study.

Bacterial strain	Pathogenic	Plant host	Source (origin)
<i>A. vitis</i> Ag57	yes	grapevine	Panagopoulos, Greece
<i>A. vitis</i> CG49	yes	grapevine	Burr, USA
<i>A. vitis</i> CG50	yes	grapevine	Burr, USA
<i>A. vitis</i> K306	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K374	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K375	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K376	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K377	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K1070	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K1072	yes	grapevine	Kerr, Australia
<i>A. vitis</i> K1336	yes	grapevine	This study
<i>A. vitis</i> K1345	yes	grapevine	This study
<i>A. vitis</i> CG673	no	grapevine	Burr, USA
<i>A. vitis</i> CG675	no	grapevine	Burr, USA
<i>A. vitis</i> CG676	no	grapevine	Burr, USA
<i>A. vitis</i> CG678	no	grapevine	Burr, USA
<i>A. vitis</i> CG680	no	grapevine	Burr, USA
<i>A. vitis</i> E26	no	grapevine	Liang, China
<i>A. vitis</i> F2/5	no	grapevine	Staphorst, South Africa
<i>A. vitis</i> K1069	no	grapevine	Kerr, Australia

Table 2. Continued

Bacterial strain	Pathogenic	Plant host	Source (origin)
<i>A. rhizogenes</i> ICPBTR101	yes	unknown	De Ley, unknown
<i>A. rhizogenes</i> K27	yes	Peach	Kerr, Australia
<i>A. rhizogenes</i> K1312	yes	Peach	This study
<i>A. rhizogenes</i> K118	yes	Peach	Kerr, Australia
<i>A. tumefaciens</i> K122	yes	peach	Kerr, Australia
<i>A. tumefaciens</i> C58	yes	cherry	Dickey, USA
<i>A. tumefaciens</i> K1329	yes	cherry	This study
<i>A. tumefaciens</i> K42	no	unknown	Lockhead, Canada
<i>A. tumefaciens</i> HBL2	no	hops	Chen, China
<i>A. tumefaciens</i> K1341	no	hops	This study
<i>A. tumefaciens</i> K1073	no	grapevine	Kerr, Australia
<i>A. tumefaciens</i> K1074	no	grapevine	Kerr, Australia
<i>A. tumefaciens</i> K1075	no	grapevine	Kerr, Australia
<i>E. coli</i> HB101::pRK2013	-	-	Figurski, USA
<i>E. coli</i> HB101::pVK102	-	-	Ditta, USA
<i>E. coli</i> HB101::pVK102::P9-P3	-	-	Ophel, Australia
<i>E. coli</i> HB101::pNMM2	-	-	McClure, Australia
<i>P. fluorescens</i> K315	no	grapevine	Kerr, Australia

3.2 CHARACTERISATION OF BACTERIA

3.2.1 Identification of *A. vitis* using a monoclonal antibody

3.2.1.1 Colony identification using a dot immunoassay

A. vitis colony morphology can be variable on RS medium (Appendix A. Culture media). So identification of *A. vitis* colonies grown on RS medium was verified using an *A. vitis*-specific monoclonal antibody developed by Bishop *et al.* (1989). Individual colonies representative of *A. vitis* morphology were aseptically transferred from RS plates to 50 µl of sterile double-distilled water (SDDW) and tested in a dot immunoassay for binding to the *A. vitis* specific monoclonal antibody. This assay has been previously used (Ophel *et al.*, 1988) and is a modification of that described by Leach *et al.* (1987).

3.2.2 Screening for polygalacturonase activity

3.2.2.1 Polygalacturonase plate assay

A modification of the Ried and Collmer (1985) method was used to screen for polygalacturonase (Peh) activity in *Agrobacterium* and *E. coli* strains. Media containing polygalacturonic acid (Appendix A. Culture media) was spotted with strains that had been grown for 48 hours on TY agar. After incubation for 24 hours at 28°C the bacteria were removed from the plate by rinsing with SDDW, then the plate was stained with 0.05% w/v ruthenium red (Sigma Chemical Co.) for 30 minutes at room temperature. Clear spots on the plates due to breakdown of the polygalacturonic acid substrate indicate Peh activity.

3.2.3 Screening for antagonism

3.2.3.1 *Stonier's medium*

Stonier (1960) used a defined medium to test for bacteriocin production. This medium was amended by adding the vitamins biotin, calcium pantothenate and nicotinic acid. The amount of phosphate in the media was increased to improve buffering capacity and thus protect from pH inactivation any bacteriocins that may be produced. For a description of the modified Stonier's medium see Appendix A.

3.2.3.2 *Sensitive strain overlay assay*

Strains to be tested for bacteriocin production were grown on TY agar for 48 hours at 28°C. Then a loopful of each strain was spot inoculated onto a plate of amended Stonier's medium and incubated at 28°C for 48 hours. The culture was then removed with a sterile swab and for ten minutes the petri-dish was inverted over a filter paper soaked in chloroform to kill any remaining antagonistic bacteria. Following chloroform treatment, the plate was aired for 20 minutes in a laminar flow cabinet. Five-hundred microlitres of washed cell suspension (Materials & Methods, section 3.1.1.2) of a strain to be tested for sensitivity to the antagonist strain was added to 10 ml of soft buffered agar (Appendix A. Culture media). Prior to adding the bacteria, the soft buffered agar was cooled to 40°C in a water bath. This suspension was poured over the Stonier's agar plate that the antagonistic strain had been grown on. Once the soft buffered agar overlay had set, the plates were incubated for at least 48 hours at 28°C and then checked for zones of inhibition in the growth of overlay strains.

3.3 BACTERIAL GENETIC MANIPULATIONS

3.3.1 Transfer of a *pehA* clone to *Agrobacterium* strains

3.3.1.1 *Bacterial matings*

Transfer of plasmids from *E. coli* donors to *Agrobacterium* recipients was achieved through triparental matings using the mobilisation (helper) plasmid, pRK2013 (Ditta *et al.*, 1980). See Table 3 for a list of plasmids used in this thesis. Donor, recipient and helper strains were mixed in a patch on a TY agar plate. A loopful of each strain was also placed individually on the plate as a control. The cultures were incubated for 24 hours at 28°C before each patch was suspended in 500 µl MSALTS. Serial dilutions of the suspensions were spread onto selective media. The transconjugants were selected on Bergersen's medium (Appendix A. Culture media) containing antibiotics (Appendix B. Antibiotics). As *E. coli* is unable to grow on Bergersen's medium, only *Agrobacterium* colonies appear after the mating. Therefore, Bergersen's medium with antibiotics selects for agrobacteria that have received a plasmid encoding the relevant antibiotic resistance.

3.3.1.2 *Recipients of a *pehA* clone*

Using the triparental mating technique, a *pehA* clone in pVK102 (Ophel *et al.*, 1990b) was mobilised into a number of *Agrobacterium* strains, including; *A. vitis* CG50, *A. tumefaciens* C58, *A. tumefaciens* HLB2 and *A. rhizogenes* K27. See Figure 3. for a diagram illustrating the complementation of Peh activity in *A. vitis* CG50 with a *pehA* clone derived from *A. vitis* strain K377.

Table 3. Important plasmids and their carrier strains used in this study.

Plasmid	<i>E. coli</i> host	Resistance ¹	Source
pRK2013	HB101	Km	Figurski & Helinski, 1979
pVK102	HB101	Tc, Km	Ditta <i>et al.</i> , 1980
pVK102::P9P3 ²	HB101	Tc	K. Ophel
pNMM2 ³	HB101	Hg	N. McClure
pNJ5000	HB101	Tc	Grinsted <i>et al.</i> , 1982

¹ The full names and details of these anti-microbial compounds are given in Appendix B (Antibiotics and other anti-microbial agents).

² See Materials & Methods, section 3.3.1.2 for more details on pVK102::P9P3.

³ See Materials & Methods, section 3.3.2.1 for more details on pNMM2.

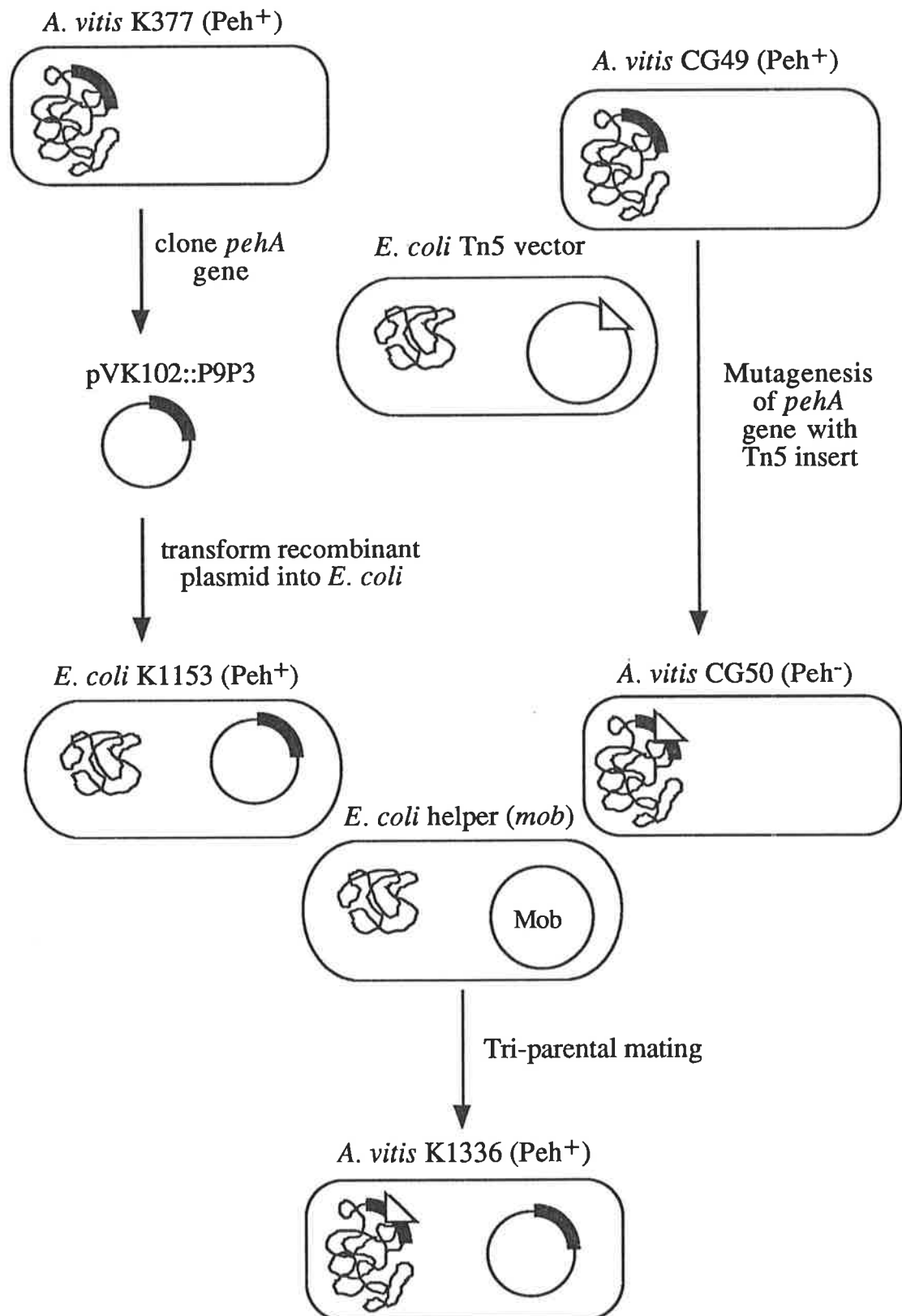


Figure 3.

Complementation of an *A. vitis* *Peh*⁻ mutant (CG50) with a *pehA* clone derived from *A. vitis* K377.

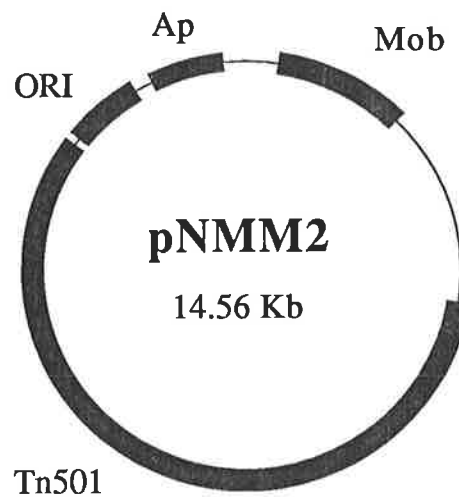
3.3.1.3 *Plasmid visualisation using gel electrophoresis*

The presence of a transferred plasmid was verified using gel electrophoresis. To prevent plasmid shearing, bacterial cells were lysed *in situ* according to the method of Eckhardt (1978). Plasmid DNA was visualised using the DNA stain ethidium bromide in conjunction with ultraviolet light. This procedure was used to ensure that plasmids had been successfully transferred to antibiotic resistant recipients and that antibiotic resistance was not due to a spontaneous mutation.

3.3.2 **Transposon-tagging of an *A. vitis* pathogen with Tn501**

3.3.2.1 *Transposon mutagenesis with Tn501*

A transposon conferring mercury resistance, known as Tn501, was introduced into target bacteria using the suicide vector pNMM2. Plasmid pNMM2 is a derivative of plasmid pVS982 (Grinsted *et al.*, 1982) into which the Mob region of plasmid pRP4 has been cloned (N. McClure, personal communication) (Fig. 4). This suicide vector system is similar to that developed for Tn5 by Simon (1984). The plasmid pNMM2 has a pBR322 based replication origin that only functions in *E. coli* and close relatives. The presence of the pRP4 Mob region allows transfer to a wide range of gram negative recipients, if essential transfer functions are provided *in trans* (Simon *et al.*, 1983). Once pNMM2 enters a non-enteric recipient it is unable to replicate and mercury resistance can only be acquired by transposition of Tn501 into the recipient genome. In this donor system, transfer functions were provided in the donor *E. coli* strain by the plasmid pNJ5000 which is cloned from pRP4 (Grinsted *et al.*, 1982). The transposon Tn501 was introduced into *A. vitis* strain K377 by conjugal transfer of the suicide vector pNMM2 (Fig. 5). Transposition of Tn501 into the bacterial genome was



ORI - encodes an origin of replication (ColE1); originates from pVS982

Ap - encodes ampicillin resistance; originates from pVS982

Mob - encodes mobilisation functions; originates from pRP4

Tn501 - encodes the Tn501 transposon with mercury resistance; originates from pVS982

Figure 4. Map of pNMM2.

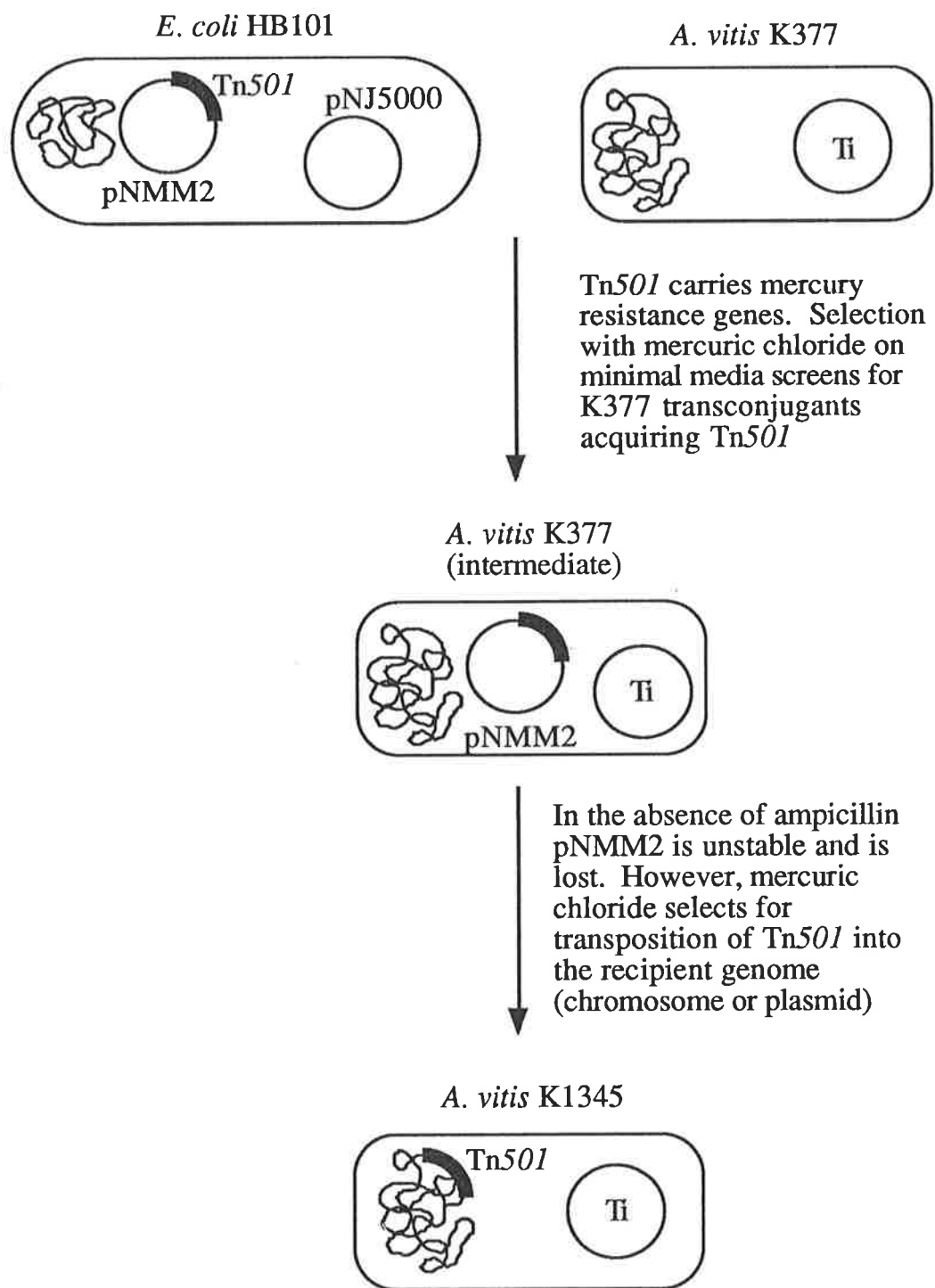


Figure 5. Conjugal transfer of Tn501 to *A. vitis* K377 using the suicide vector pNMM2.

selected for on RS media containing 1 mg/ml of mercuric chloride (Appendix B. Antibiotics).

3.4 EXPERIMENTS WITH BACTERIA

3.4.1 Growth characteristics of *Agrobacterium* strains

3.4.1.1 Growth rate in TY broth

All growth rate experiments were carried out in TY broth. A loopful of culture was placed into 5 ml of TY in a screw cap McCartney bottle and incubated for 48 hours. From this, a volume of culture was added to 50 ml of TY in a 200 ml medical flat bottle to bring the initial cell density to approximately 10^5 cells/ml. A sample from each replicate was taken, diluted in MSALTS and spread onto TY agar plates to verify the initial cell density. The inoculated medical flat bottles were placed on a reciprocating shaker (50 r.p.m) and incubated at 28°C. Sampling was initiated after 10 hours, once the cultures had entered log phase. Samples were taken from the culture vessels every two or three hours, depending on the growth rate, for 12 hours. The samples were diluted and spread onto TY agar plates in 100 µl aliquots. Colonies were counted after a further 48 hour incubation at 28°C and Colony Forming Units (CFU) were plotted against incubation time for each strain. Lines were fitted to the plotted data, the slope of which represents the growth rate.

3.4.1.2 Death rate in nutrient-free liquid media

A loopful of culture was placed into 100 ml of TY in a 200 ml medical flat bottle and shaken in a 28°C incubator for 48 hours. Bacterial suspensions were placed into FalconTM centrifuge tubes in 10 ml aliquots and spun at 4,000 r.p.m. for 15 minutes, supernatants were discarded and equal volumes

of sterile MSALTS aliquoted into the tubes. This procedure was repeated three times for all replicates of each strain then samples were taken from each, diluted and plated onto TY agar plates as an indication of the initial concentration of inoculum. The tubes were placed in a 25°C incubator and sampled weekly. The number of CFU's was plotted against sample time for each strain. Curves were fitted to the plotted data, the slope of which represents the death rate.

3.4.1.3 Viability of bacteria on selective media

Serial dilutions were made from washed cell suspensions (Materials & Methods, section 3.1.1.2) of *Agrobacterium* strains and plated onto TY media and selective media. The recovery rate was calculated by dividing the number of CFU's on selective media by the number of CFU's on TY media and multiplying by one hundred. In experiments where bacterial strains are recovered on selective media, the recovery rates of those strains on selective media are taken into account.

3.4.2 Sensitivity of bacteria to plant anti-microbial compounds

3.4.2.1 Preparation of bacteria

The number of viable bacterial cells in a washed cell suspension (Materials & Methods, section 3.1.1.2) was recorded by plating dilutions onto TY agar plates. An aliquot of the washed cell suspension was added to phosphate buffer containing anti-microbial compounds (see below).

3.4.2.2 Inoculation of phosphate buffer containing anti-microbial compounds

Phosphate buffer (Appendix C. Miscellaneous buffers) was added to McCartney bottles in 10 ml aliquots and sterilised in an autoclave. Anti-

microbial compounds (0.02g) were dissolved in 0.5 ml ethanol and added to 4.5 ml of Tris buffer (Appendix C. Miscellaneous buffers). The anti-microbial solutions (0.4% w/v) were filter sterilised and 50 µl added to each 10 ml aliquot of phosphate buffer. The final concentration of anti-microbial compound was 0.002% w/v. The Tris buffer containing ethanol but with no anti-microbial compound was also added to phosphate buffer and used as a control treatment. One hundred microlitres of washed cell suspension was added to each 10 ml aliquot of phosphate buffer with anti-microbial agent and incubated on a reciprocating shaker (50 r.p.m) at 25°C for 24 hours. Following incubation the suspensions were diluted and plated onto TY agar plates.

3.4.3 Fitness of genetically modified bacteria

3.4.3.1 Preparation of bacteria

Genetically modified strains were inoculated into TY broth containing antibiotics, while their wild-type parent strains were inoculated into TY broth and incubated for 48 hours. The bacterial suspensions were washed (Materials & Methods, section 3.1.1.2) and diluted (1:100). Then 50 µl from each wild-type strain 1:100 dilution and 50 µl from each corresponding modified strain 1:100 dilution were added to McCartney bottles containing 5 ml TY broth.

3.4.3.2 Serial transfer of dual cultures

The co-inoculated TY broth was incubated for 48 hours and then diluted (1:100) by aliquoting 100 µl to 10 ml MSALTS. Fifty microlitres of the diluted dual culture was subsequently transferred to McCartney bottles containing 5 ml of fresh TY broth. Serial dilutions of the dual cultures were plated onto TY agar and TY agar amended with antibiotics. This transfer

procedure was repeated every 48 hours at least five times. The ratio of resistant to sensitive colony numbers was plotted against the number of transfers. The gradient of this line represents the fitness coefficient of the genetically modified strain in comparison with the wild-type strain (Stewart & Levin, 1973).

3.4.4 Dual culture of a pathogen with antagonistic strains in broth

3.4.4.1 Preparation of bacteria

A transposon-tagged *A. vitis* pathogen, strain K1345 (Materials & Methods, section 3.3.2.1), was tested for sensitivity to antagonistic strains (Table 14). All strains were cultured for 48 hours in TY broth; 3 mg/ml of mercuric chloride was used in the K1345 culture to maintain selection for the mercury resistance marker genes. The cell suspensions were then washed (Materials & Methods, section 3.1.1.2).

3.4.4.2 Co-inoculation of dual cultures

Suspensions of pathogenic and antagonistic strains were added in 50 µl aliquots to McCartney bottles containing 5 ml of either TY or Stonier's broth to give a one to one ratio of pathogen to antagonist cells. Strain K1345 was co-inoculated with strain K377, its wild-type progenitor, as a control. The co-inoculated cultures were incubated for 48 hours then a range of serial dilutions were plated onto TY agar containing 3 mg/ml mercuric chloride. The mercury resistant colonies were counted after 48 hours of incubation.

3.5 GRAPEVINE MATERIAL

3.5.1 Storage and treatment of grapevine material

3.5.1.1 Storage of grapevine material

Canes were removed from dormant French Colombard grapevines and immersed in a Chinosol[®]W (Hoechst) solution (5g/L) for two hours to reduce the risk of decay during storage. The treated cuttings were wrapped in newspaper, sealed in plastic bags and then placed into cold storage at 4°C until needed. Upon removal from cold storage, cuttings were soaked in reverse osmosis (RO) water for 72 hours to remove Chinosol residues.

3.5.1.2 Thermotherapy of grapevine material

To reduce systemic populations of *A. vitis*, dormant grapevine cuttings were placed in a hot water bath (50°C) for 30 minutes as described by Burr *et al.* (1989). The temperature was carefully maintained within one degree of the specified temperature. Upon removal from the hot water bath the treated cuttings were immersed in cool RO water to reduce the temperature of the cuttings and thus lessen the possibility of bud damage.

3.6 EXPERIMENTS INVOLVING GRAPEVINE MATERIAL

3.6.1 Grapevine cane-segment bioassay

3.6.1.1 Preparation of cane segments

Dormant French Colombard cuttings were cut into segments approximately 5 cm long, each segment containing a single node. These nodal segments were

sorted into groups of ten with an average weight of 5.5 g. Ten replicates per treatment were used for all bioassay experiments.

3.6.1.2 *Preparation of bacteria*

The bacterial strains to be tested in the bioassay were grown for 48 hours in 5 ml TY. One hundred microlitres of each suspension was transferred to 50 ml TY and grown for a further 48 hours. Washed cell suspensions (Materials & Methods, section 3.1.1.2) were added to the dormant cane segments in 500 µl aliquots. A titre of diluted suspension was plated onto TY agar to record the inoculum levels of each strain added to the grapevine segments.

3.6.1.3 *Inoculating bacteria to cane segments*

The bacterial suspension was drawn into the nodal sections under vacuum using the apparatus detailed in Figure 6. The procedure was a modification of the method used by Bazzi *et al.* (1987) to sample the vascular fluid of grapevine cuttings. The inoculated cane segments were placed into separate plastic bags for each treatment and incubated for 14 days at 25°C.

3.6.1.4 *Extraction of bacteria from cane segments*

After incubation, the inoculated cane segments were blotted dry and sprayed with a 70% ethanol solution to surface sterilise them. The browned ends were aseptically removed in equivalent portions, then the segments were placed onto a vacuum apparatus (Fig. 6). The vascular system of each cane segment was flushed with 500 µl MSALTS and the washings collected in separate test tubes. The vascular washings were diluted and spread onto agar plates selective for agrobacteria. All grapevine cane-segment bioassay experiments were repeated at least once.

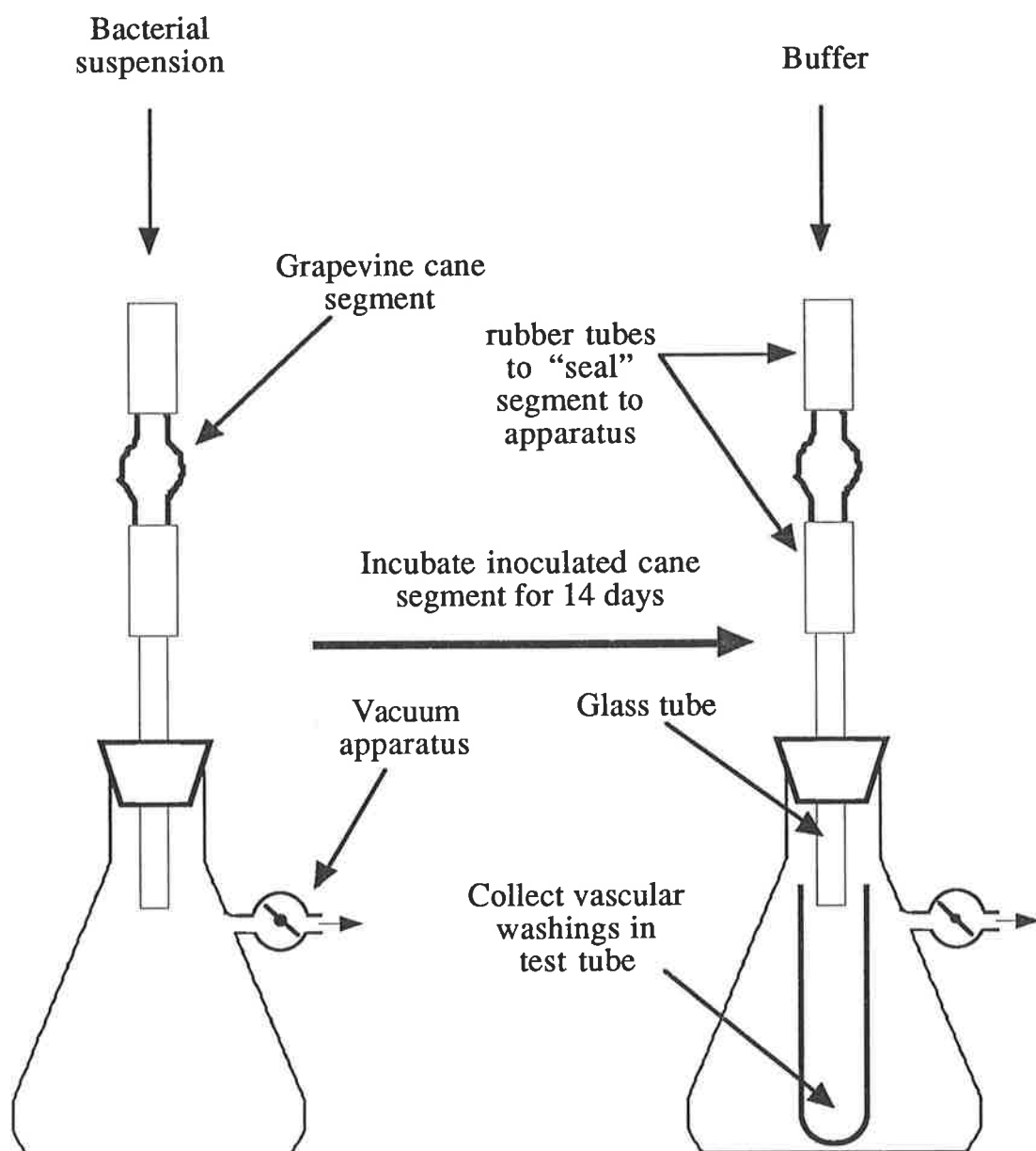


Figure 6. Apparatus used to inoculate and extract vascular fluid from segments of grapevine canes.

3.6.1.5 Statistical analysis

An analysis of variance was performed on the data using the Genstat statistical package (Rothamsted Experimental Station, 1977) and if the variation permitted ($p < 0.05$), the residual degrees of freedom and the residual mean square were used to calculate Tukey's Wholly Significant Difference (WSD) statistic from tables with a 5% significance level (Tukey, 1953; Volk, 1969). Tukey's statistic was used for multiple comparisons of means, with means differing by more than the calculated statistic considered significantly different.

3.6.2 Inoculation of artificially wounded grapevines

3.6.2.1 Artificial wounding of grapevine stems

Grapevines grown in pots in a glasshouse were wounded as described by Burr & Reid (1994). Inoculation sites were sprayed with a 100% (w/v) ethanol solution before the holes were bored with a 4 mm diameter drill. The hole depth was approximately 5 mm to where the drill bit reached the cortex. The volume of the hole was large enough to contain 80 μ l of fluid.

3.6.2.2 Inoculation of bacteria to wounds

Eighty microlitres of washed cell suspension (Materials & Methods, section 3.1.1.2) was aliquoted to each wound site, then the wound was wrapped in parafilm to prevent drying out.

3.6.3 Glasshouse testing of antagonistic strains on grapevines

3.6.3.1 Experimental conditions and design

This experiment was carried out in a containment glasshouse under C1

regulations. The room was maintained between 15°C and 25°C with the relative humidity ranging from 50% to 75%. The grapevines were grown in steam treated soil in 7.5 cm (diameter) x 12 cm (height) pots. The plants were watered every second day with tap water and fertilised with Fish Liquid Fertiliser™ (Harrison's) every three weeks. The pots were placed on saucers to prevent cross-contamination.

3.6.3.2 *Application of antagonistic strains*

Two methods of applying antagonistic strains were used. A pre-root application (pre-inoculation) where the basal ends of heat-treated cuttings were dipped in a bacterial suspension (cell density 10^9 cells/ml) for 30 seconds then planted in sand to encourage root development. Four weeks later, upon removal from sand, a pathogen treatment was applied. The second method was a post-root application (co-inoculation) where heat-treated cuttings were removed from sand after roots had developed and the roots dipped in a bacterial suspension (cell density 10^9 cells/ml) for 30 seconds. After allowing 10 minutes for the roots to dry, a pathogen treatment was applied and the grapevine was planted in soil.

3.6.3.3 *Application of mercury resistant pathogen*

The transposon-tagged, mercury resistant pathogen was also inoculated in two ways. The root dip treatment involved taking rooted grapevines that had received the biocontrol application treatment and dipping the roots into a pathogen suspension (cell density 10^9 cells/ml) for 10 seconds; the treated grapevines were then planted into soil. This treatment was a positive control treatment to ensure maximum penetration of the pathogen. The second pathogen application treatment involved adding the pathogenic bacterial suspension to soil (cell density 4×10^8 cells/cm³ soil), then planting the biocontrol inoculum treated grapevines.

3.6.3.4 Sampling pathogen numbers in the soil

Soil samples (one gram/grapevine) taken from the base of grapevine cuttings (Fig. 7) were suspended in 5 ml of MSALTS solution and shaken for 60 minutes. Serial dilutions were prepared then plated onto selective media and incubated for 6 days at 28°C.

3.6.3.5 Sampling pathogen numbers in the vascular system

Each grapevine was dissected into three nodal segments (Fig. 7). The length and position of each segment were recorded. The vascular system of each segment was then flushed with 500 µl of MSALTS and the vascular fluid collected (Materials & Methods, section 3.6.1.4). Serial dilutions were plated onto selective media and incubated for 6 days at 28°C.

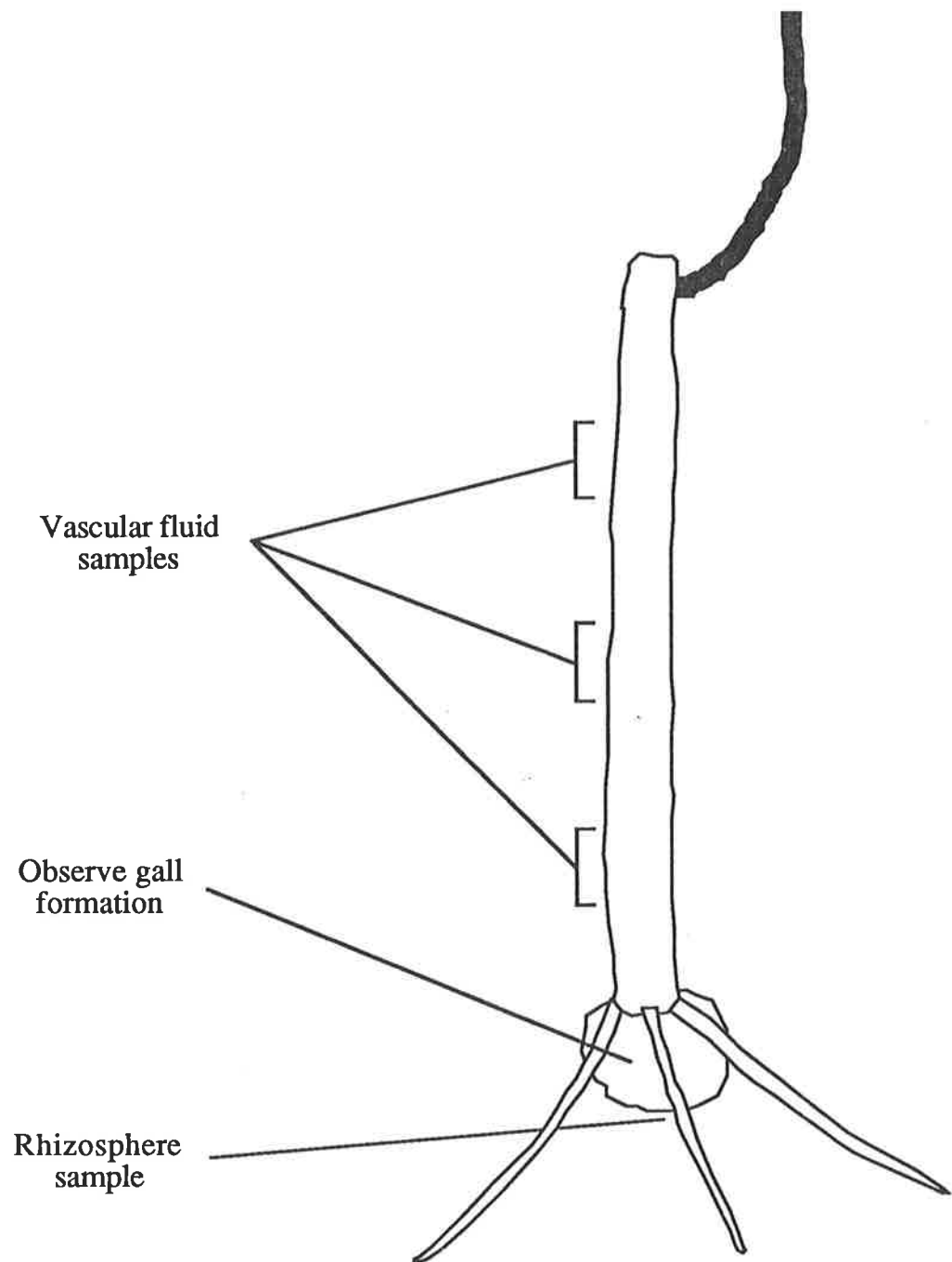


Figure 7. Sampling sites for pathogen colonisation of grapevines after treatment with antagonistic strains.

CHAPTER FOUR

A BIOASSAY TO INVESTIGATE POPULATION DYNAMICS OF BACTERIA IN THE VASCULAR SYSTEM OF GRAPEVINES

4.1 INTRODUCTION

Numerous reports have suggested that *A. vitis* can spread systemically through the vascular system of grapevines (Lehoczky, 1971; Burr & Katz, 1983; Burr & Katz, 1984). Tarbah & Goodman (1987) confirmed the systemic spread of an antibiotic resistant mutant by reisolating it thirty centimetres above the point of inoculation. Further research suggested that *A. vitis* was confined to xylem vessels in the grapevine vascular system (Burr *et al.*, 1987c; Tarbah & Goodman, 1988a; Stefani & Bazzi, 1989). However, Jäger *et al.* (1990) could not detect *A. vitis* in the bleeding sap but observed the rind tissue to be the main source of *A. vitis* in German vineyards. While the reports do not completely agree on the location of *A. vitis* in the grapevine, the systemic nature of colonisation by the bacteria is not in doubt.

The vascular system is a specialised ecological niche and a demanding environment for microbial colonisation. Entry into xylem vessels from the soil or plant surface is difficult as the vascular system is protected by several layers of tissue, but wounds spanning the plant surface to the xylem vessels can allow rapid access for microbes (Goodman, 1986). Once inside xylem vessels, microbes must withstand plant defence mechanisms that are activated in response to cell damage and the presence of micro-organisms (Beckman,

1989). In addition, xylem vessels have limited carbon resources, although they are relatively high in nitrogenous compounds (Roubelakis-Angelakis & Kliwer, 1979). Glutamine, malic acid, citric acid and tartaric acid are the main carbon containing compounds in grapevine xylem fluid (Andersen & Brodbeck, 1991), so utilisation of such compounds by a bacterium may offer a competitive advantage in the vascular system.

A comparison between two *Agrobacterium* species from different ecological niches may give some insight into the different requirements for effective colonisation of those niches. *A. vitis* cells are often isolated from the vascular system of grapevines, whereas *A. rhizogenes* cells have never been reported to colonise plants systemically but are normally isolated from the rhizosphere of plants. Comparison of *A. vitis* with *A. rhizogenes* should reveal different ecological adaptations, as mechanisms required for survival in the rhizosphere are likely to be different from those necessary for survival in a plant's vascular system. An examination of rhizosphere colonisation of peach roots by *A. rhizogenes* and *A. vitis* strains indicated much stronger root colonisation by the *A. rhizogenes* strain (K. Ophel, thesis). Such a comparison of colonisation in grapevine vascular tissue has not previously been attempted.

4.2 EXPERIMENTS

4.2.1 Population dynamics of *A. vitis* and *A. rhizogenes* in a grapevine cane-segment bioassay

In this experiment, a bioassay was designed to study the ecology of *A. vitis* K377 and *A. rhizogenes* K27 in the grapevine vascular system. The population dynamics of the two strains were compared in a grapevine cane-

segment bioassay. To ensure that any differences in survival between the two strains in the bioassay were not due to detectable differences in bacterial growth characteristics, *in vitro* growth rates and death rates were calculated and taken into account.

4.2.1.1 *Recovery of bacteria on selective media*

To study bacterial populations in non-sterile environments, such as the grapevine vascular system, it is necessary to discern between cells of interest and other cells. Selective media 2E (Brisbane & Kerr, 1983) and RS (Roy & Sasser, 1983) have been developed that allow good growth of a selected species of *Agrobacterium* while limiting the growth of other species but these media do not recover cells as efficiently as nutrient-rich, undefined media. The viability of *A. vitis* K377 on RS selective media (Appendix A. Culture media) and of *A. rhizogenes* K27 on 2E selective media (Appendix A. Culture media) was determined (Materials & Methods, section 3.4.1.3) and compared with the viability of respective strains on undefined TY media (Appendix A. Culture media). The recovery rate was calculated by dividing the number of colony forming units (CFU) on selective media by the number of CFU's on TY media and multiplying by one hundred. The recovery rate of *A. vitis* strain K377 on RS media was 50.26% compared with 96.79% for *A. rhizogenes* strain K27 on 2E media (Table 4). The recovery rates of K377 and K27 were taken into account in subsequent experiments involving recovery of these bacteria on selective media.

4.2.1.2 *Bacterial recovery from a grapevine cane-segment bioassay over time*

Bacterial survival in the grapevine vascular system was studied *in vitro* by inoculating grapevine cane segments with bacteria and observing the

Table 4. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 on undefined media and selective media

Bacterial strain	Selective media	% recovery ¹
<i>A. vitis</i> K377	RS ²	52.26
<i>A. rhizogenes</i> K27	2E ³	96.79

¹ Calculated by dividing the number of CFU's on selective media by the number of CFU's on undefined media (TY media developed by Beringer, 1974) and multiplying by one hundred.

² Media selective for *A. vitis*, developed by Roy & Sasser (1983). See Appendix A.

³ Media selective for *A. rhizogenes*, developed by Brisbane & Kerr (1983). See Appendix A.

number of cells recovered from those segments after an incubation period. Suspensions of *A. vitis* K377 and *A. rhizogenes* K27 were inoculated to grapevine cane-segments and incubated for 0, 2, 4, 7, 10 and 14 days (Materials & Methods, sections 3.6.1.1-3). The vascular fluid was extracted from the incubated cane-segments and plated onto selective media (Materials & Methods, section 3.6.1.4). An analysis of variance indicated that *A. rhizogenes* K27 cells were recovered at significantly ($p < 0.001$) lower levels than *A. vitis* K377 from day 7 onwards (Fig. 8).

4.2.1.3 *Bacterial growth rates*

While there were differences in the number of *A. vitis* K377 and *A. rhizogenes* K27 cells recovered from grapevine cane-segments, it is possible that those differences could be attributed to differences in the growth rates or death rates of the respective bacteria. Consequently, growth rates of *A. vitis* K377 and *A. rhizogenes* K27 in TY broth (Appendix A. Culture media) were compared (Materials & Methods, section 3.4.1.1). Six replicates of each strain were sampled. As sampling was confined to the log phase of the growth curve, the closest approximations of the data were linear plots. An analysis of variance of the slope of the curves indicated that the growth rate of *A. vitis* K377, 0.180 log CFU/ml/hour, was significantly ($p < 0.01$) higher than the growth rate of *A. rhizogenes* K27, 0.137 log CFU/ml/hour, when grown in TY broth (Fig. 9).

4.2.1.4 *Bacterial death rates*

The rate at which *A. vitis* K377 and *A. rhizogenes* K27 cells died, termed 'death rate', during incubation in a nutrient-free buffer solution known as MSALTS (Appendix C. Miscellaneous buffers) was determined (Materials & Methods, section 3.4.1.2). This was done to establish that the observations from the grapevine cane-segment bioassay were not a function of natural

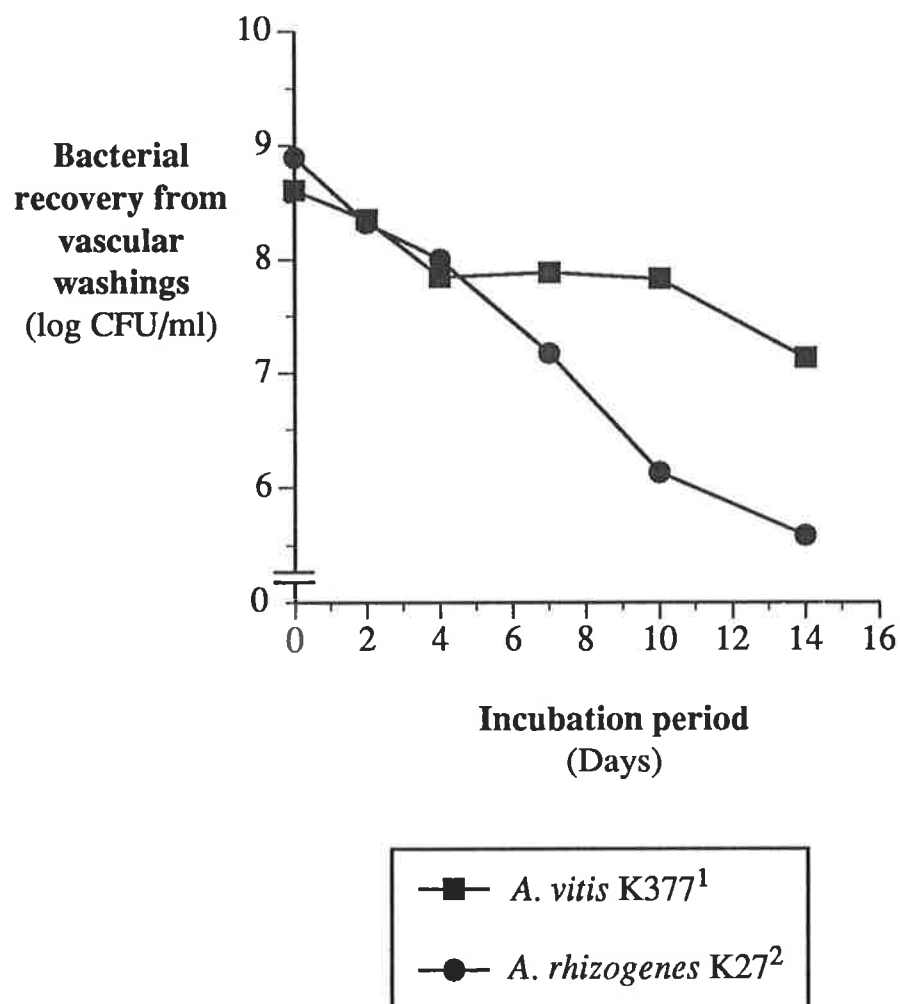


Figure 8.

Effect of the duration of incubation on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 cells from a grapevine cane-segment bioassay.

¹⁻² The number of K377 and K27 cells added to the grapevine cane segments was 9.19 and 9.23 (log CFU/ml) respectively. The recovery of K27 cells from the grapevine cane-segment bioassay was significantly ($p < 0.001$) lower than the recovery of K377 cells after day 7.

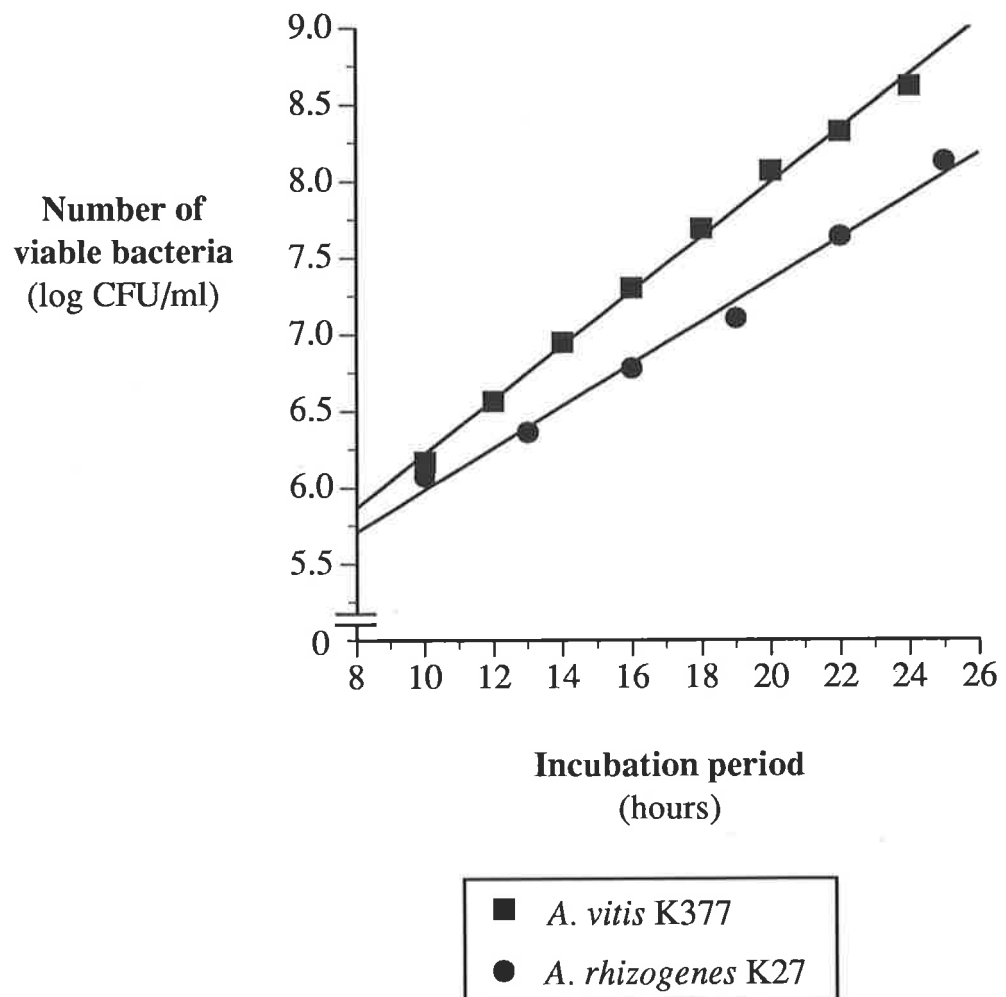


Figure 9. Growth of *A. vitis* K377 and *A. rhizogenes* K27 when incubated in TY broth at 28°C. The slope of the fitted line indicates growth rate. The slopes of the lines were significantly ($p < 0.01$) different.

bacterial death rates. Ten replicates per strain were used. The rate of decrease in viable cell count when incubated in MSALTS was calculated by fitting curves to the data and comparing the slopes of these curves. An analysis of variance performed on the slopes of the fitted curves (Fig. 10) indicated that *A. vitis* K377 had a significantly ($p < 0.01$) higher death rate, 0.947 log CFU/ml/day, than *A. rhizogenes* K27, 0.931 log CFU/ml/day. This suggests that the lower number of *A. rhizogenes* K27 cells recovered in the grapevine cane-segment bioassay, in comparison to *A. vitis* K377 cells, is not a reflection of differences in the natural death rates of the two strains.

4.2.2 Endogenous *A. vitis* in the grapevine cane-segment bioassay

Endogenous *A. vitis* were detected in the grapevine material used in the grapevine cane-segment bioassay, so thermotherapy was used to eradicate these bacteria. The effects of thermotherapy on grapevine cuttings and the presence of endogenous *A. vitis* on the survival and recovery of introduced bacteria from the bioassay were examined.

4.2.2.1 Effect of thermotherapy of grapevine cuttings on the recovery of bacteria from the bioassay

To determine the impact thermotherapy of grapevine cuttings has on the subsequent survival of introduced bacteria in those cuttings, both heat treated and untreated cuttings were prepared. The grapevine cuttings were prepared for use in the cane segment bioassay (Materials & Methods, section 3.6.1.1) but half were immersed in a water bath at 50°C for 30 minutes to eradicate endogenous *A. vitis* cells (Materials & Methods, section 3.5.1.2). Both the treated and untreated cane segments were used in the grapevine cane-segment bioassay (Materials & Methods, sections 3.6.1.2-4) and

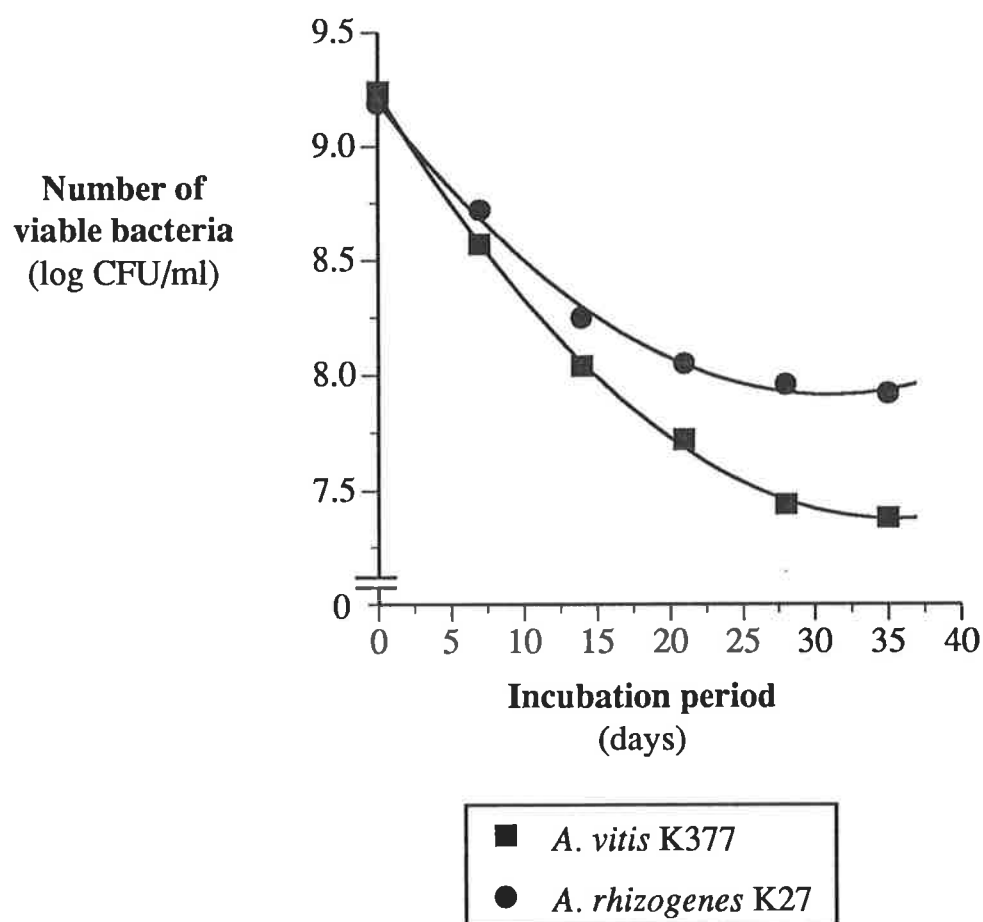


Figure 10. Survival of *A. vitis* K377 and *A. rhizogenes* K27 when incubated in MSALTS (Appendix C) solution at 25°C. The slope of the fitted curve indicates death rate. The slope of the curve for K377 was significantly ($p < 0.01$) lower than that of K27.

inoculated either with a suspension of *A. vitis* K377 or *A. rhizogenes* K27, or left uninoculated. Detection of *A. vitis* cells in uninoculated, untreated grapevine cane segments (Table 5) suggested endogenous *A. vitis* populations were present in the grapevine cuttings used. An analysis of variance indicated significant ($p < 0.001$) differences between means. To rank the means Tukey's statistic was calculated (0.271) and means differing by more than this value were considered significantly different (Tukey's WSD; Materials & Methods, section 3.6.1.5). The recovery of *A. vitis* K377 cells from grapevine segments treated with heat was unaffected, whereas the recovery of *A. rhizogenes* K27 cells was significantly higher in the heat-treated segments than in the untreated segments but still significantly lower than *A. vitis* K377 numbers (Fig. 11).

4.2.2.2 *Influence of competition by A. vitis on the recovery of A. rhizogenes from the bioassay*

As *A. rhizogenes* K27 cells were recovered at higher levels from heat treated cane segments than from untreated cane segments and the treated cane segments were free of endogenous *A. vitis*, it is possible that competition from endogenous *A. vitis* was resulting in lowered K27 numbers in the untreated cane segments. To determine the effect of competition by *A. vitis* on *A. rhizogenes* K27, an equivalent number of *A. vitis* K377 cells were inoculated either alone or in combination with *A. rhizogenes* K27 to heat treated grapevine cane segments (Materials & Methods, sections 3.5.1.2, 3.6.1.1-4). Similarly, strain K27 was inoculated alone or in combination with strain K377. An analysis of variance of the data indicated significant ($p < 0.001$) differences between means. Tukey's statistic was calculated (0.252) to rank the means (Tukey's WSD; Materials & Methods, section 3.6.1.5). The recovery of *A. rhizogenes* K27 cells from the grapevine cane-

Table 5. Effect of thermotherapy of grapevine material on the level of endogenous *A. vitis* recovered from the grapevine cane-segment bioassay.

Thermotherapy ¹	Endogenous <i>A. vitis</i> ² (log CFU/ml)
no	5.67
yes	0

- ¹ Grapevine cuttings were heat treated (50°C for 30 minutes) before use in the grapevine cane-segment bioassay.
- ² The number of *A. vitis* cells recovered on RS medium (Roy & Sasser, 1983) from vascular washings of uninoculated cane segments in the grapevine cane-segment bioassay.

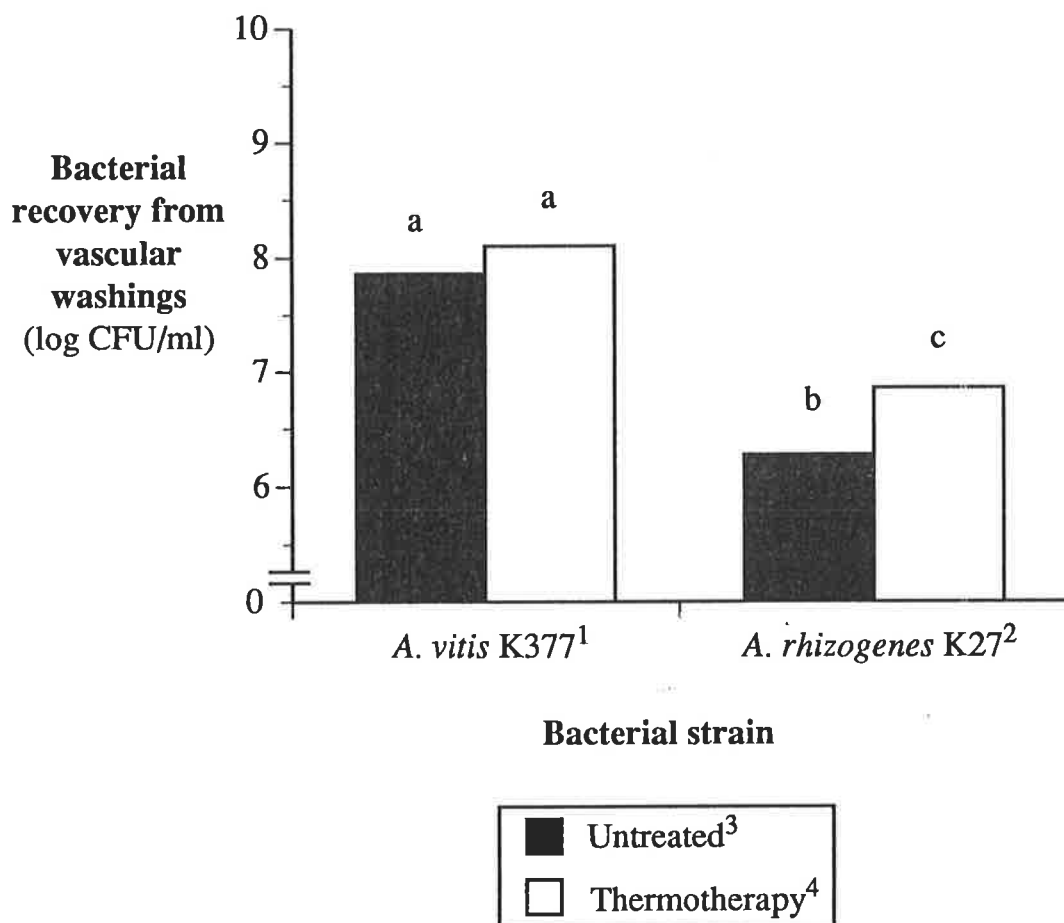


Figure 11. Effect of thermotherapy of grapevine cuttings on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay. ¹⁻² The number of K377 and K27 cells inoculated to the grapevine segments were 9.10 and 9.23 (log CFU/ml) respectively. ³ Untreated refers to cuttings that have not been heat treated. ⁴ Thermotherapy refers to cuttings that have been heat treated at 50°C for 30 minutes. Different letters (a-c) indicate means that differ by more than Tukey's statistic (0.271) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

segment bioassay was significantly lower when co-inoculated with *A. vitis* K377 cells than when inoculated singly. Numbers of *A. vitis* K377 cells were not significantly different in the two treatments (Table 6).

4.2.2.3 *Effect of A. vitis-free grapevine segments on the recovery of bacteria from the bioassay*

To determine whether endogenous *A. vitis* cells reduce the recovery of bacterial strains inoculated to the grapevine cane-segment bioassay, the endogenous *A. vitis* cells must be removed to enable a comparison of bacterial recovery both in their presence and in their absence. Previous experiments (this study) have used thermotherapy to reduce endogenous *A. vitis* cells. However, grapevine material with no detectable endogenous *A. vitis* was used to eliminate the possibility that heat treatment of grapevine cuttings may be having an effect other than removing bacteria. Cuttings were taken from a grapevine that had been grown from seed (parents; Mission x Sultana) and were prepared in the same fashion as French Colombard cuttings (Materials & Methods, section 3.6.1.1) then used in the grapevine cane-segment bioassay (Materials & Methods, sections 3.6.1.2-4). An analysis of variance on the data indicated a significant ($p < 0.001$) difference, so Tukey's statistic was used (0.458) to rank the means (Tukey's WSD; Materials & Methods, section 3.6.1.5). In these segments, no endogenous *A. vitis* could be detected in uninoculated cane segments after incubation and *A. rhizogenes* K27 cells were recovered at significantly lower levels than that of *A. vitis* K377 cells (Table 7).

Table 6. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 from a grapevine cane-segment bioassay when the strains were inoculated either separately or in combination with each other.

Strain	Bacterial recovery from vascular washings (log CFU/ml)	
	Control ¹	Co-inoculated ²
<i>A. vitis</i> K377 ³	7.63 ^a	7.75 ^a
<i>A. rhizogenes</i> K27 ⁴	6.71 ^b	6.37 ^c

¹ *A. vitis* K377 and *A. rhizogenes* K27 inoculated separately into grapevine cane segments.

² *A. vitis* K377 and *A. rhizogenes* K27 co-inoculated into grapevine cane segments.

³ Number of K377 cells inoculated to grapevine cane segments was 8.89 (log CFU/ml).

⁴ Number of K27 cells inoculated to grapevine cane segments was 8.90 (log CFU/ml).

^{a-c} Different letters indicate means that differ by more than Tukey's statistic (0.252) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

Table 7. Recovery of *A. vitis* K377 and *A. rhizogenes* K27 from a grapevine cane-segment bioassay when the grapevine canes used (Mission/Sultana seedling) had no endogenous *A. vitis*.

Inoculation treatment	Bacterial recovery from vascular washings (log CFU/ml)
Uninoculated ¹	0
<i>A. vitis</i> K377 ²	6.54 ^a
<i>A. rhizogenes</i> K27 ³	5.47 ^b

¹ In this treatment, vascular washings of uninoculated segments were plated onto RS medium (Roy & Sasser, 1983) to select for *A. vitis* cells.

² Number of K377 cells inoculated to grapevine cane segments was 8.88 (log CFU/ml).

³ Number of K27 cells inoculated to grapevine cane segments was 8.62 (log CFU/ml).

^{a-b} Different letters indicate means that differ by more than Tukey's statistic (0.458) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

4.2.3 Influence of segment type in the grapevine cane-segment bioassay

Nodal and internodal regions of a grapevine cane may have different physiological activities. This experiment investigated whether bacterial recovery from the bioassay was different in segments from the nodal region and the internodal region.

4.2.3.1 Effect of nodal and internodal grapevine segments on recovery of bacteria from the bioassay

Grapevine canes were cut into two types of segment; those containing nodal sections and those containing internodal sections. Both types were similar in length and weight. The two types of cane segment were used in the grapevine cane-segment bioassay (Materials & Methods, sections 3.6.1.1-4) and inoculated with either *A. vitis* K377 or *A. rhizogenes* K27. An analysis of variance indicated the means were significantly ($p < 0.001$) different. To rank the means Tukey's statistic was calculated (0.278) (Tukey's WSD; Materials & Methods, section 3.6.1.5). Strain K377 was recovered at similar levels in both types of grapevine segment while strain K27 was recovered at significantly lower levels in the nodal segments compared with the internodal segments (Fig. 12).

4.3 DISCUSSION

A bioassay to examine the ecology of *A. vitis* in the grapevine vascular system was developed using cane segments from dormant grapevines. Bacteria were inoculated into the cane segments by vacuum infiltration of the vascular system, the segments were incubated, then vascular washings

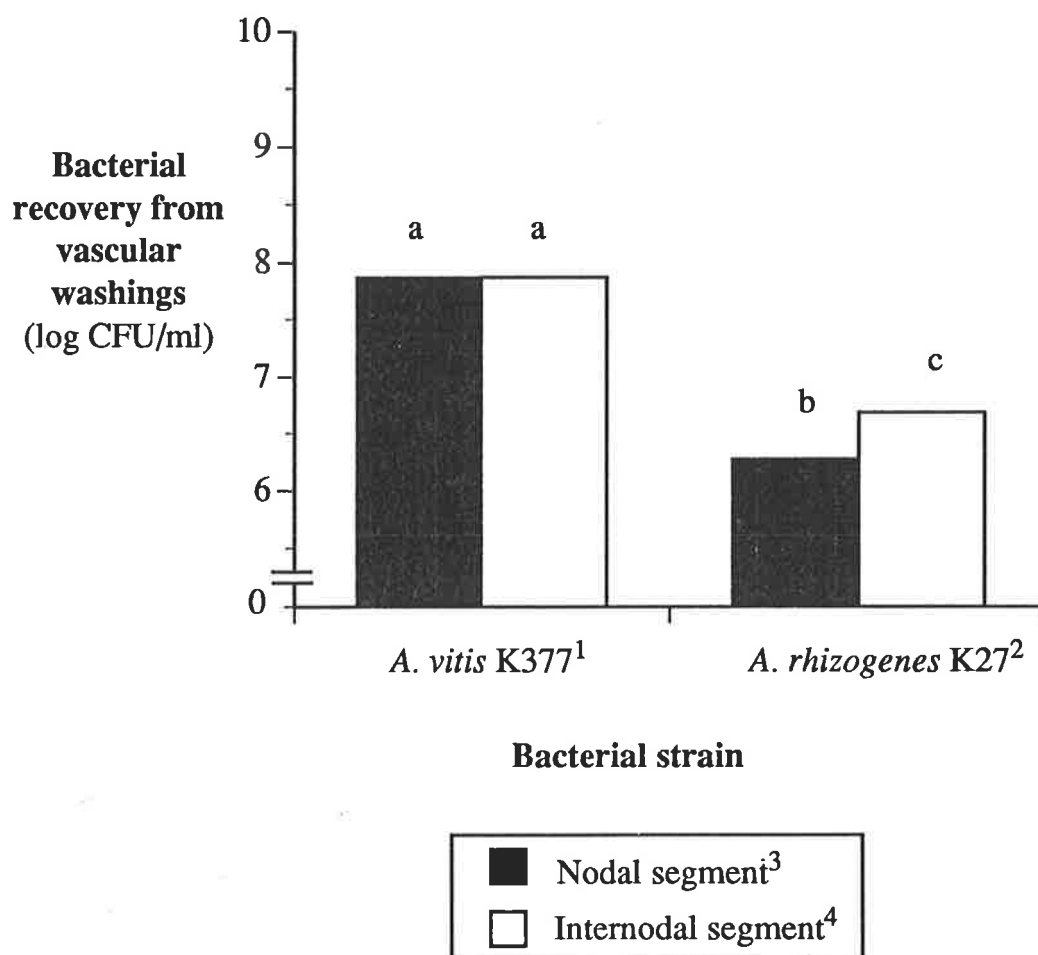


Figure 12. Effect of type of grapevine segment on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay. ¹⁻² The number of K377 and K27 cells inoculated to the grapevine segments were 9.10 and 9.23 (log CFU/ml) respectively. ³ Nodal segment refers to grapevine cane-segments including a nodal region. ⁴ Internodal segment refers to grapevine cane-segments with no nodal region. Different letters (a-c) indicate means that differ by more than Tukey's statistic (0.278) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

were extracted from the segments under a vacuum (Bazzi *et al.*, 1987) and plated onto selective media to enumerate viable bacteria. Two *Agrobacterium* species from different ecological backgrounds were examined. *A. vitis* K377 was isolated from grapevine vascular fluid and *A. rhizogenes* K27 was isolated from the rhizosphere of a peach tree. In the grapevine cane-segment bioassay, numbers of *A. rhizogenes* K27 cells declined ten-fold more than *A. vitis* K377 cells. Since strain K27 cells had a significantly lower death rate in nutrient-free media than strain K377, the differences between the recovery of cells of K27 and K377 from the grapevine cane-segment bioassay could not be attributed to different rates of natural cell death. However, the growth rate of strain K377 in rich, liquid media was higher than strain K27. It is possible that the difference in growth rates of the two strains may account for the different rates of recovery from the bioassay but as the nutrient supply in grapevine xylem fluid is low and bacterial numbers were high, the opportunity for sustained bacterial growth would be limited. In fact, bacterial numbers of both strains decreased during the bioassay, suggesting that potential growth rates of bacteria were not being fulfilled. Consequently, results from the grapevine cane-segment bioassay were considered to be a real indication of plant-bacterial interactions rather than of bacterial growth characteristics.

Endogenous *A. vitis* were present in the grapevine segments used in the bioassay but at levels ten-fold lower than the lowest numbers of artificially inoculated bacteria recovered from the bioassay. Nonetheless, the endogenous *A. vitis* cells may have affected the results obtained. Consequently, thermotherapy of the grapevine cuttings, as described by Burr *et al.* (1989), was used to reduce the endogenous *A. vitis* population. The effect thermotherapy of grapevine material had on the subsequent recovery of inoculated bacteria from the bioassay was then examined. Unexpectedly,

it was found that thermotherapy of grapevine segments had an influence on the recovery of *A. rhizogenes* K27 from those segments; bacterial numbers were significantly higher in the heat treated grapevine segments than in the untreated segments but still significantly lower than *A. vitis* K377 numbers. While thermotherapy of grapevine segments had no significant effect on the recovery of *A. vitis* K377 cells from the bioassay. It is possible that the thermotherapy was having some effect on processes that influence bacterial survival and recovery from the grapevine vascular system, possibly by reducing competition from endogenous *A. vitis* and other endogenous bacteria, or by interfering with plant defence mechanisms. For instance, Wample *et al.* (1991) demonstrated that, under certain conditions, vine vitality can be adversely affected by thermotherapy. When cuttings were treated at temperatures over 54°C, they found variation in the vitality of canes between different cultivars and the time of year at which thermotherapy was performed. It is likely that cell metabolic processes concerned with plant defence as well as vine vitality could be affected by thermotherapy in sensitive cultivars.

The impact of competition from endogenous *A. vitis* on survival and recovery of *A. rhizogenes* in the bioassay was investigated by comparing cell recovery when two strains, *A. vitis* K377 and *A. rhizogenes* K27, were co-inoculated and when they were inoculated singly. Strain K27 numbers were slightly, yet significantly, reduced when incubated with equal numbers of strain K377 indicating that competition can have an effect when *A. vitis* numbers are very high and equivalent to *A. rhizogenes* numbers.

Grapevine material with no detectable *A. vitis* was used in the bioassay to eliminate the possibility of competition by endogenous *A. vitis* without the use of thermotherapy. No *A. vitis*-free French Colombard grapevines could

be obtained, so cuttings were used from a *V. vinifera* (Mission x Sultana) grapevine that had been grown from seed. Cell numbers of *A. rhizogenes* K27 were significantly lower than those of *A. vitis* K377 after incubation in the segments, although both strains were at lower levels than in French Colombard cane segments. The lower recovery of strain K27 compared with strain K377 in the segments with no endogenous *A. vitis* suggests that it is a factor(s) other than competition contributing to differences in recovery of the two strains from the grapevine cane-segment bioassay.

One possible explanation for the difference in numbers of *A. vitis* K377 and *A. rhizogenes* K27 recovered from the grapevine cane-segment bioassay is utilisation of specific nutrients by *A. vitis*. Analysis of xylem exudate of grapevines by Andersen & Brodbeck (1991) indicated that the fluid would provide little nourishment for microorganisms in the way of utilisable carbon compounds. The most abundant organic compounds were glutamine (2.3 mM), malic acid (2.1 mM), citric acid (1 mM) and tartaric acid (0.7 mM). As carbon is limited, it is possible that catabolism of one or more of these compounds by a bacterial strain may improve the survival of that strain in the vascular system of grapevines. Tartrate utilisation by *A. vitis* has been documented by Szegedi (1985), who proposed that it may contribute to bacterial survival in the grapevine.

Specific nutrient resources produced by a plant can influence microbial competitiveness (Tepfer *et al.*, 1988). In an experiment with opine-producing transgenic plants Farrand *et al.* (1994) studied competition between opine-catabolising bacteria and near-isogenic bacteria unable to utilise opines. While rhizosphere carbon was found not to be a limiting factor, the utilisation of a specific carbon compound, an opine, still offered the opine catabolising strain a competitive advantage. On the phylloplane where carbon was a

limiting resource, the advantage of the opine catabolising strain was even greater.

Whether nutrient utilisation by bacteria was a factor in decreasing numbers of *A. rhizogenes* K27 relative to numbers of *A. vitis* K377 in the grapevine cane-segment bioassay could not be easily assessed by direct means. However, when the effect of incubating bacteria in broth with no nutrients after 14 days is compared with the change in bacterial numbers in the bioassay after 14 days, the decrease in *A. rhizogenes* K27 numbers is 500-fold greater (8-fold for *A. vitis* K377) in the bioassay than in nutrient-free medium. This suggests that although nutrient availability could have been having some effect, other factors besides nutrient availability were more important. The rapid and large decrease in *A. rhizogenes* K27 numbers after day 7 in the bioassay (Fig. 8) indicates that a process directly affecting the survival and recovery of strain K27 may be activated at this time.

The large decrease in the number of *A. rhizogenes* K27 cells compared with *A. vitis* K377 cells recovered from the bioassay may be the result of a plant defence response. There are two likely explanations for the differences between the strains. Either plant defences are activated in the presence of both strains and strain K377 is more tolerant to them than strain K27, or the plant defences are activated by strain K27 but not strain K377. Evasion of plant defences by plant pathogens has been previously documented by Kearney *et al.* (1988). They found that in bacterial spot disease of pepper the bacterial pathogen, *Xanthomonas campestris*, could evolve to overcome plant resistance by evading host recognition. A mutation in the bacterial gene, the product of which induces the pepper's defence mechanisms, enabled these bacteria to overcome plant resistance. The hypothesis that *A. vitis* K377 may be evading a plant defence response that *A. rhizogenes* K27

was activating was tested in the co-inoculation bioassay experiment. That strain K377 numbers were equally high in the singly and co-inoculated treatments suggests that strain K27 is not eliciting a host-response that *A. vitis* K377 is sensitive to and would normally evade. It seems more likely that plant defences are being activated by both strains and that *A. vitis* is more tolerant to them than is *A. rhizogenes*.

Plant defence mechanisms can be more active in some plant tissue than in others. The number and complexity of xylem vessels is greater in nodal regions of grapevine canes than in internodal regions (Stefani & Bazzi, 1989) and presumably, the tissue surrounding the vessels is more physiologically and biochemically active. Therefore, if host-associated phenomena are affecting survival and recovery of *A. rhizogenes* K27 from the bioassay then the effect should be most prominent in the nodal regions. Recovery of *A. vitis* K377 cells from the bioassay was similar in both nodal and internodal segments which is curious as Stefani & Bazzi (1989) visualised, using immunofluorescent staining, *in situ* *A. vitis* cells were localised mainly in the xylem vessels of the nodal regions of grapevines. Perhaps the length of incubation in the bioassay is insufficient for this effect to be detected. Numbers of *A. rhizogenes* K27 were recovered at significantly lower levels from the nodal segments than the internodal segments. This strengthens the argument that the survival of strain K27 is being restricted to a greater extent than strain K377 by plant defence factors, although it cannot be dismissed that such factors may be enhanced in the bioassay. Nonetheless, the grapevine cane-segment bioassay developed in this chapter clearly detects differences in the survival and recovery of *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine vascular system.

The hypothesis that *A. vitis* K377 may be better adapted to host-defence mechanisms than *A. rhizogenes* K27 in the grapevine vascular system is put forward and will be examined in the following chapter.

CHAPTER FIVE

ADAPTATIONS OF *AGROBACTERIUM VITIS* FOR SURVIVAL IN THE GRAPEVINE VASCULAR SYSTEM

5.1 INTRODUCTION

Development and testing of a grapevine cane-segment bioassay in Chapter Four highlighted the reduced survival and recovery of *A. rhizogenes* K27 cells in comparison to *A. vitis* K377 cells. Differences in survival were probably due to host-related phenomena and *A. vitis* K377 was apparently better adapted than *A. rhizogenes* K27 to endure such conditions. This chapter seeks to investigate what defences plants have to restrict vascular colonising bacteria and to determine what mechanisms bacteria use to improve survival under such conditions.

It is a well-documented phenomenon that plants produce anti-microbial compounds in response to bacterial invasion. Bacterial enzymes, or their products, and secondary metabolites are detected by plant cells which then implement a defence response (Hammerschmidt *et al.*, 1982; Darvill & Albersheim, 1984). Anti-microbial agents such as phytoalexins, phenolics and quinone compounds are major components of a plant defence response (Cruickshank & Perrin, 1960; Farkas & Király, 1962). Working in conjunction with the anti-microbial biochemical responses are a series of physical fortifications that can include; lignification of cell walls (Vance *et al.*, 1980), callose deposition (Allison & Shalla, 1974) or vascular occlusion

(Beckman, 1964), if the microbe is systemic.

Research on a bacterium, *Xylella fastidiosa* (Davis *et al.*, 1978), that can colonise grapevines systemically has indicated that resistance to the bacterium is directly related to the ability of the plant to prevent bacterial movement along the vascular system. Mollenhauer & Hopkins (1976) found that grapevines tolerant to Pierce's Disease, such as *V. rotundifolia*, produced large quantities of gums and tyloses in response to infection with *Xylella fastidiosa*, whereas susceptible grapevines, such as *V. vinifera*, produced only small amounts of gums and tyloses. It was later suggested by Hopkins (1985) that virulence was related to the multiplication of *Xylella fastidiosa* and may be governed by ability to move both through and between vessels. Limiting movement of bacteria effectively reduces nutrients available for bacterial growth and enhances biochemical defence mechanisms.

French Colombard, a *V. vinifera* cultivar susceptible to Pierce's Disease, was shown to produce large amounts of pectin gel with minimal tyloses or gums in response to infection with Pierce's Disease bacteria (Fry & Milholland, 1990). It was suggested that pectin was produced as a mechanism of resistance to the vascular infection. However, the role of pectin gels in constraining colonisation of *A. vitis* in the vascular system of *V. vinifera* cultivars is yet to be determined.

In this chapter the possible adaptations of *A. vitis* to survival in the vascular system of grapevines were examined. Adaptations that may enhance survival include overcoming or evading host defence responses and improving mobility through the xylem vessels. Consequently, sensitivity of agrobacteria to the phytoalexin resveratrol and to a number of phenolic compounds was tested and the grapevine cane-segment bioassay was used

to investigate whether bacterial polygalacturonases, enzymes that degrade pectin, affect survival of agrobacteria in the grapevine vascular system.

5.2 EXPERIMENTS

5.2.1 Sensitivity of agrobacteria to toxic plant compounds

Part of the host defence-response is the production of compounds, such as phytoalexins, quinones and phenolics, that are inhibitory to micro-organisms. The aim of this experiment was to expose three *Agrobacterium* strains; *A. vitis* K377, *A. tumefaciens* C58 and *A. rhizogenes* K27, to various plant defence-associated compounds and compare the tolerance of the bacteria to these compounds. The compounds used in the following experiments were chosen as general representatives of toxic plant compounds.

5.2.1.1 Incubation of three Agrobacterium strains with phenolic, quinnic and phytoalexin compounds that are associated with plant defence

The sensitivity of *A. vitis* K377, *A. tumefaciens* C58 and *A. rhizogenes* K27 was tested (Materials & Methods, sections 3.4.2.1-2) to the following: the phenolic compounds catechol (10 µg/ml) and chlorogenic acid (10 µg/ml); resveratrol (10 µg/ml) a known grapevine phytoalexin; the quinnic compound benzoquinone (1 µg/ml, 10 µg/ml, 20 µg/ml and 100 µg/ml); and a combination of benzoquinone (10 µg/ml) and the antioxidant ascorbic acid (10 µg/ml). Bacteria were also inoculated to a control medium containing buffer but no inhibitory compounds. To assess the effect of inhibitory compounds on bacterial strains, the number of viable bacteria was determined after incubation of the bacteria with the inhibitory compound. Five replicates per treatment were used and viable colonies were enumerated on

TY media. An analysis of variance indicated that there were significant ($p < 0.001$) differences between the inhibitory effects of the various compounds (Table 8). Only benzoquinone proved inhibitory to the bacterial strains after a 24 hour incubation, with increasing inhibition as the concentration was increased. *A. vitis* K377 exhibited more tolerance of benzoquinone than strains *A. tumefaciens* C58 and *A. rhizogenes* K27. The other compounds tested had no effect when added at 10 µg/ml on the viability of the bacterial strains. For all strains ascorbic acid neutralised the toxicity of p-benzoquinone when added to the buffer at an equal concentration to p-benzoquinone.

5.2.1.2 Screening agrobacteria for sensitivity to p-benzoquinone

Twenty-seven *Agrobacterium* strains (Table 9) were tested for tolerance to 10 µg/ml p-benzoquinone during a 24 hour incubation (Materials & Methods, sections 3.4.2.1-2). Bacteria were inoculated to a control medium containing the buffer used in the benzoquinone treatment but with no inhibitory compound. Four replicates per treatment were used. The viable bacteria were counted and the replicates averaged for each strain. An analysis of variance indicated that there were significant ($p < 0.001$) differences between the tolerance of different strains to benzoquinone. The number of *A. vitis* strains (13 out of 17) that exhibited a degree of tolerance was slightly higher than that of *A. tumefaciens* strains (4 out of 7) but it should be noted that three of the four *A. tumefaciens* strains with some tolerance were isolated from grapevine xylem fluid. Furthermore, the three *A. tumefaciens* strains from grapevine were amongst the most tolerant strains from all species tested. One of three *A. rhizogenes* strains showed a degree of tolerance to benzoquinone. When the strains were grouped into species and an analysis of variance was performed, there were no significant differences between the species.

Table 8. Viability of three *Agrobacterium* strains when incubated with phytoalexin, quinone and phenolic compounds for 24 hours.

Compound	Concentration ($\mu\text{g/ml}$)	Number of viable bacteria ¹		
		<i>A. vitis</i> K377 ² (log CFU/ml)	<i>A. tumefaciens</i> C58 ² (log CFU/ml)	<i>A. rhizogenes</i> K27 ² (log CFU/ml)
Control ³	0	6.94	7.35	7.31
Catechol	10	7.15	7.51	7.20
Chlorogenic acid	10	7.23	7.40	7.21
Resveratrol	10	7.24	7.36	7.22
Benzoquinone	1	5.79	7.18	7.35
Benzoquinone	10	2.25	0.32	0.46
Benzoquinone	20	2.13	0	0.46
Benzoquinone	100	0	0	0
Benzoquinone and Ascorbic acid	10	6.82	7.49	7.44

Table 8. Continued.

- ¹ Number of bacteria able to form colonies on TY media (Appendix A) following a 24 hour incubation in buffer containing potentially anti-microbial compounds.
- ² There were significant ($p < 0.001$) differences between the strains.
- ³ Same buffer solution as used in other treatments to suspend anti-microbial compounds.

Table 9. Viability of 27 *Agrobacterium* strains when incubated in a solution of 10 µg/ml benzoquinone for 24 hours at 25°C.

Species	Strain ¹	Viable bacteria ²	
		Control ³ (log CFU/ml)	Benzoquinone (log CFU/ml)
<i>A. vitis</i>	Ag57	6.93	1.78
<i>A. vitis</i>	CG49	6.83	2.35
<i>A. vitis</i>	E26	7.39	0
<i>A. vitis</i>	F2/5	6.77	0.94
<i>A. vitis</i>	K306	6.98	0.58
<i>A. vitis</i>	K374	6.90	0
<i>A. vitis</i>	K375	6.94	1.50
<i>A. vitis</i>	K376	6.83	1.08
<i>A. vitis</i>	K377	6.92	1.00
<i>A. vitis</i>	K1069	7.03	0
<i>A. vitis</i>	K1070	6.74	1.36
<i>A. vitis</i>	K1072	6.86	1.65
<i>A. vitis</i>	#673	6.92	0.37
<i>A. vitis</i>	#675	7.05	0
<i>A. vitis</i>	#676	6.85	0.25
<i>A. vitis</i>	#678	6.83	1.12
<i>A. vitis</i>	#680	6.99	2.74

Table 9. Continued.

Species	Strain ¹	Viable bacteria ²	
		Control ³ (log CFU/ml)	Benzoquinone (log CFU/ml)
<i>A. tumefaciens</i>	C58	6.94	0.90
<i>A. tumefaciens</i>	HLB2	7.10	0
<i>A. tumefaciens</i>	K42	6.97	0
<i>A. tumefaciens</i>	K122	6.97	0
<i>A. tumefaciens</i> ⁴	K1073	7.12	2.94
<i>A. tumefaciens</i> ⁴	K1074	7.27	1.89
<i>A. tumefaciens</i> ⁴	K1075	7.44	3.13
<i>A. rhizogenes</i>	ICPBTR101	6.93	0
<i>A. rhizogenes</i>	K27	6.95	0
<i>A. rhizogenes</i>	K118	6.88	1.59

¹ There were significant ($p < 0.001$) differences between the strains.

² Number of bacteria able to form colonies on TY media (Appendix A) following a 24 hour incubation in buffer or buffer containing benzoquinone.

³ Same buffer solution as used to suspend benzoquinone but without the anti-microbial compound.

⁴ These *A. tumefaciens* strains were isolated from grapevine sap.

5.2.2 Moderating quinone toxicity with ascorbic acid in the grapevine cane-segment bioassay

Phenolic compounds can be oxidised to quinone compounds by peroxidases and polyphenol oxidases. If production of these oxidases is part of the grapevine defence response then quinone-based compounds might accumulate following bacterial inoculation of grapevines. It was demonstrated previously that ascorbic acid tempered the antibacterial effect of benzoquinone, most likely by reducing benzoquinone to its corresponding phenolic compound. Whether ascorbic acid would improve bacterial survival in the grapevine cane-segment bioassay by moderating any possible accumulation of toxic quinones was examined.

5.2.2.1 Effect of ascorbic acid on bacterial survival in the bioassay

A. vitis K377 and *A. rhizogenes* K27 cells were inoculated to grapevine cane segments as described (Materials & Methods, sections 3.6.1.1-4). Three types of incubation buffer were used to suspend the bacteria; MSALTS buffer (Appendix C. Miscellaneous buffers), MSALTS buffer amended with 0.01% ascorbic acid and MSALTS buffer amended with 0.1% ascorbic acid. After incubation in the grapevine cane-segment bioassay, the recovery of bacteria from the vascular fluid was enumerated on selective media (Appendix A. Culture media). An analysis of variance indicated significant ($p < 0.001$) differences between the means. Tukey's statistic was calculated (0.282) and was used to determine differences between means (Tukey's WSD; Materials & Methods, section 3.6.1.5). Numbers of strain K377 were significantly higher than those of strain K27 (Table 10) in each treatment. However, there were no significant differences between numbers of strain K377 recovered from cane segments inoculated with the different levels of ascorbic acid.

Table 10. Effect of ascorbic acid added to the incubation buffer on recovery of strains *A. vitis* K377 and *A. rhizogenes* K27 from the grapevine cane-segment bioassay.

Strain	Bacterial recovery		
	(log CFU/ml)		
	Control ¹	0.01% ascorbic acid ²	0.1% ascorbic acid ²
<i>A. vitis</i> K377 ³	7.52 ^a	7.52 ^a	7.40 ^a
<i>A. rhizogenes</i> K27 ⁴	6.15 ^b	6.45 ^b	6.44 ^b

¹ Inoculation buffer consists of MSALTS (Appendix C).

² Inoculation buffer consists of MSALTS amended with ascorbic acid.

³ Number of K377 cells inoculated to grapevine cane segments was 9.15 (log CFU/ml).

⁴ Number of K27 cells inoculated to grapevine cane segments was 8.89 (log CFU/ml).

^{a-b} Different letters indicate means that differ by more than Tukey's statistic (0.282) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

Similarly, ascorbic acid amended buffer had no effect on recovery of strain K27.

5.2.3 Effect of polygalacturonase activity on bacterial recovery from the grapevine cane-segment bioassay

In the vascular system of some *V. vinifera* cultivars, pectin gels have been cited as part of the defence response to systemic bacteria (Fry & Milholland, 1990). This experiment was designed to determine whether polygalacturonase (Peh) activity enhances bacterial survival and recovery from the grapevine cane-segment bioassay. Agrobacteria were genetically modified to remove or add polygalacturonase activity and then tested in the bioassay.

5.2.3.1 Construction of transconjugant strains

The *pehA* gene, coding for Peh activity, was cloned from *A. vitis* strain K377 into a vector, pVK102 (Knauf & Nester, 1982), and replicated in *E. coli* (Ophel & Kerr, unpublished data). This clone, P9-P3, was conjugally transferred (Materials & Methods, sections 3.3.1.1-2) to *A. vitis* Peh⁻ mutant CG50, *A. tumefaciens* C58, *A. tumefaciens* HLB2 and to *A. rhizogenes* K27 in separate triparental matings (Table 11). The transconjugant strains were tested for polygalacturonase production (Fig. 13) (Materials & Methods, section 3.2.2.1) and plasmids were visualised using agarose gel electrophoresis (Materials & Methods, section 3.3.1.3). Figure 14 shows the plasmids in transconjugant strain *A. vitis* K1336 after separation by gel electrophoresis.

Table 11. Polygalacturonase activity of transconjugant and wild-type *Agrobacterium* strains.

Species	Original designation	Transconjugant designation	Plasmid or transposon	Resistance ¹	Phenotype ²
<i>A. vitis</i>	CG49	-	-	-	Peh ⁺
<i>A. vitis</i>	CG49	CG50	Tn5	-	Peh ⁻
<i>A. vitis</i>	CG50	K1336	Tn5, pVK102::P9-P3	Km, Tc	Peh ⁺
<i>A. tumefaciens</i>	C58	-	-	-	Peh ⁻
<i>A. tumefaciens</i>	C58	K1329	pVK102::P9-P3	Tc	Peh ⁺
<i>A. tumefaciens</i>	HLB2	-	-	-	Peh ⁻
<i>A. tumefaciens</i>	HLB2	K1341	pVK102::P9-P3	Tc	Peh ⁺
<i>A. rhizogenes</i>	K27	-	-	-	Peh ⁻
<i>A. rhizogenes</i>	K27	K1312	pVK102::P9-P3	Tc	Peh ⁺

¹ The full names and details of these anti-microbial compounds are given in Appendix B (Antibiotics and other anti-microbial agents).

² Peh⁺ represents polygalacturonase activity as determined by the method of Ried and Collmer (1985), Peh⁻ represents no detectable polygalacturonase activity.

Figure 13. Polygalacturonase activity of *A. vitis* strains CG49 (a), CG50 (b) and K1336 (c) on agarose plates containing polygalacturonate and stained with ruthenium red.

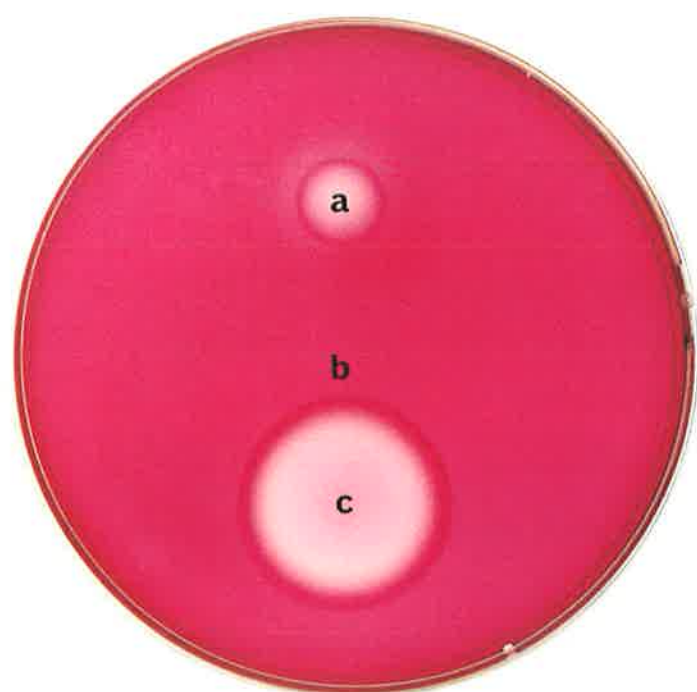
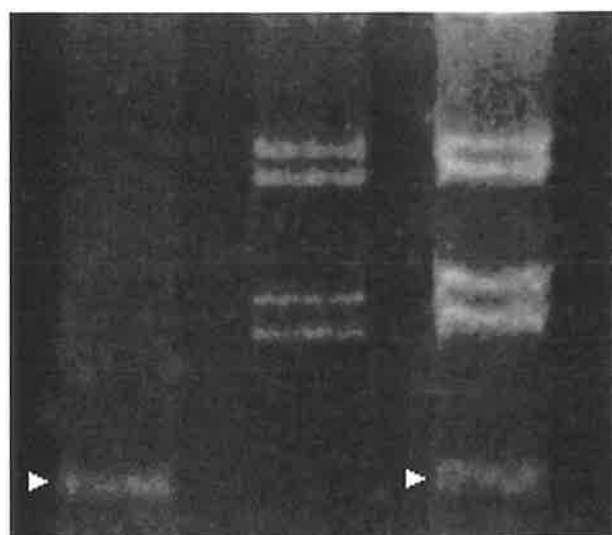


Figure 14. Plasmids in donor (*E.coli* K1153), parent (*A. vitis* CG50) and transconjugant (*A. vitis* K1336) strains after separation by gel electrophoresis. The arrows (◄) indicate the plasmid pVK102::P9P3 that was transferred from *E. coli* K1153 into *A. vitis* K1336.

K1153 CG50 K1336



5.2.3.3 Recovery of bacteria on selective media with antibiotics

-101-

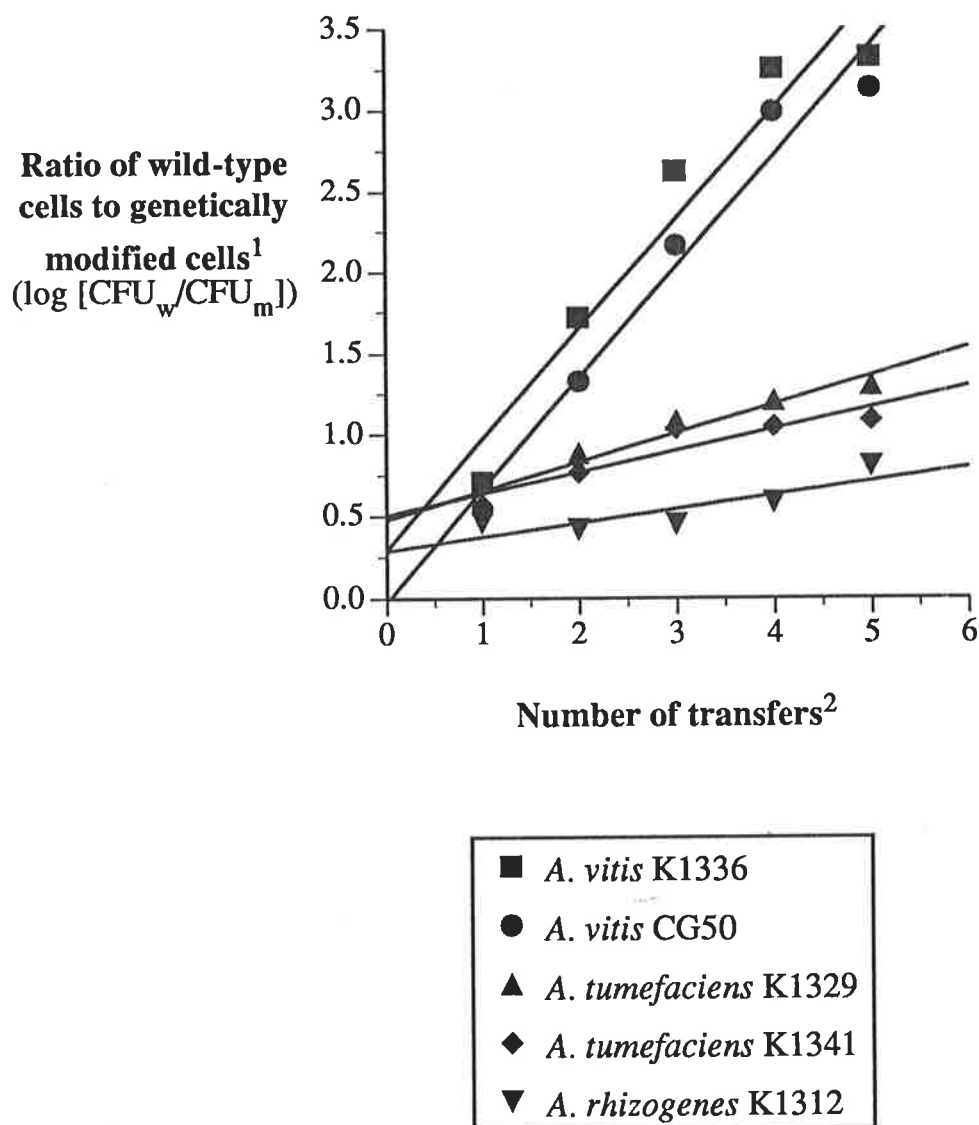


Figure 15.

Fitness co-efficients of the genetically modified *Agrobacterium* strains *A. vitis* K1336, *A. vitis* CG50, *A. tumefaciens* K1329, *A. tumefaciens* K1341 and *A. rhizogenes* K1312 when competing against respective wild-type parents *A. vitis* CG49, *A. vitis* CG49, *A. tumefaciens* C58, *A. tumefaciens* HLB2 and *A. rhizogenes* K27. ¹ The rate at which the log of the ratio of wild-type cells (CFU_w) to genetically modified cells (CFU_m) increases is the fitness co-efficient. ² Genetically modified and wild-type parent cells were co-inoculated to TY broth and incubated for 48 hours, the dual culture was then transferred to fresh TY broth. The slope of each line was significantly greater than zero (more than twice the standard error).

five replicates per treatment. Percentage recovery was calculated by comparing the recovery of strains on TY media with their recovery on selective media (amended with antibiotics for transconjugants). In all cases, the percentage recovery of the transconjugant strains on selective media with antibiotics was lower than that of the wild-type strains on selective media with no antibiotics (Table 12).

5.2.3.4 *Effect of modified Peh activity on recovery of Agrobacterium strains from the grapevine cane-segment bioassay*

Recovery of *A. vitis*, *A. tumefaciens* and *A. rhizogenes* strains from the bioassay (Materials & Methods, sections 3.6.1.1-3.6.1.4) was compared with recovery of isogenic counterparts containing clones conveying Peh activity. The bacteria were recovered on the selective media (with antibiotics in the case of transconjugant strains) described in the previous section (Table 12). Various Peh⁻ and Peh⁺ strains were compared (Table 13). An analysis of variance was performed and there were significant ($p < 0.001$) differences between means. The means were ranked using Tukey's statistic (0.484) (Tukey's WSD; Materials & Methods, section 3.6.1.5). The Peh⁻ *A. vitis* strain (CG50) complemented with the *pehA* clone (strain K1336) was recovered at higher levels than the Peh⁻ *A. vitis* strain. Similarly, higher numbers of a Peh⁺ *A. tumefaciens* strain (K1329) were recovered than the Peh⁻ *A. tumefaciens* strain (C58). However, expression of the Peh⁺ phenotype by strains *A. rhizogenes* K27 and *A. tumefaciens* HLB2 had no effect on their recovery from the bioassay.

Table 12. Bacterial recovery on selective media with antibiotics.

Bacterial strain	Phenotype ¹	Selective media	Antibiotic ²	% recovery ³
<i>A. vitis</i> CG49	Peh ⁺	RS ⁴	-	30.80
<i>A. vitis</i> CG50	Peh ⁻	RS	Km	25.88
<i>A. vitis</i> K1336	Peh ⁺	RS	Tc, Km	22.93
<i>A. rhizogenes</i> K27	Peh ⁻	2E ⁵	-	96.79
<i>A. rhizogenes</i> K1312	Peh ⁺	2E	Tc	92.35
<i>A. tumefaciens</i> C58	Peh ⁻	1A ⁶	-	90.51
<i>A. tumefaciens</i> K1329	Peh ⁺	1A	Tc	84.26

¹ Peh⁺ represents polygalacturonase activity as determined by the method of Ried and Collmer (1985), Peh⁻ represents no detectable polygalacturonase activity.

² The full names and details of these antibiotics are given in Appendix B (Antibiotics and other anti-microbial agents).

³ Calculated by dividing number of CFU's on selective media by the number of CFU's on TY media (Beringer, 1974) and multiplying by one hundred.

⁴ Media selective for *A. vitis*, developed by Roy & Sasser (1983).

⁵ Media selective for *A. rhizogenes*, developed by Brisbane & Kerr (1983).

⁶ Media selective for *A. tumefaciens*, developed by Brisbane & Kerr (1983).

Table 13. Recovery of *Agrobacterium* strains with and without polygalacturonase activity from the grapevine cane-segment bioassay.

Strain	Polygalacturonase activity ¹	No. of bacteria (log CFU/ml)	
		Inoculated ²	Recovered ³
<i>A. vitis</i> CG49	+	9.46	8.08 ^a
<i>A. vitis</i> CG50	-	9.38	6.37 ^b
<i>A. vitis</i> K1336	+	9.26	7.79 ^a
<i>A. tumefaciens</i> C58	-	9.45	5.44 ^a
<i>A. tumefaciens</i> K1329	+	9.25	6.09 ^b
<i>A. tumefaciens</i> HLB2	-	9.32	6.46 ^a
<i>A. tumefaciens</i> K1341	+	9.31	6.12 ^a
<i>A. rhizogenes</i> K27	-	9.20	6.75 ^a
<i>A. rhizogenes</i> K1312	+	9.08	6.67 ^a

¹ Polygalacturonase activity refers to clear zones in polypectate agar plates as described by Ried and Collmer (1985).

² The number of viable cells inoculated to grapevine cane segments, as determined by CFU's on TY media (Appendix A).

³ The number of viable cells recovered from vascular washings from the grapevine cane-segment bioassay, as determined by CFU's on selective media with adjustment for percentage recovery of different media (Chapter 5, section 5.2.3.3).

^{a-b} Different letters indicate means that differ by more than Tukey's statistic (0.484) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

5.3 DISCUSSION

The host-specific nature of *A. vitis* has prompted several researchers to investigate mechanisms that facilitate the colonisation of grapevines by *A. vitis*. Brisset *et al.* (1991) presented evidence that polygalacturonase production by *A. vitis* was involved in a grapevine-specific root decay and that these root lesions may provide access to the root vascular system, possibly an important step in the colonisation process. Similarly, Rodriguez-Palenzuela *et al.* (1991) demonstrated that polygalacturonase production was also linked with colonisation of wound sites and tumorigenicity. Other possible adaptations of *A. vitis* that may contribute to the systemic survival of the bacteria were investigated in this chapter.

Plant defence mechanisms restrict bacterial colonisation in two main ways, either by physical obstruction or by biochemical means. Two major biochemical responses of plants to injury and microbial invasion are a rapid increase in oxidase activity and the production of phytoalexins (Hammerschmidt *et al.*, 1982; Smith, 1982). In grapevine cell cultures, production of the phytoalexin resveratrol can be induced by a cellulase elicitor from *Trichoderma viride* (Calderón *et al.*, 1993). As *A. vitis* has been reported to produce cellulase (Ophel & Kerr, unpublished data), it is possible that resveratrol is generated in response to *A. vitis*. Tolerance of *A. vitis* to resveratrol may account for the higher numbers of *A. vitis* K377 compared with *A. rhizogenes* K27 recovered from the grapevine cane-segment bioassay.

The sensitivity of *Agrobacterium* strains to resveratrol was tested by incubating the bacteria in a buffered solution containing the phytoalexin for

24 hours but no inhibitory effect on any strain was detected. It is interesting that *A. rhizogenes* K27 was also tolerant of resveratrol, a grapevine phytoalexin, as *A. rhizogenes* has never been isolated from grapevine. Tolerance of phytoalexins is fairly common in phytopathogenic fungi where genes for phytoalexin detoxification are closely linked to pathogenicity (Van Etten *et al.*, 1989). One explanation for the tolerance of *A. rhizogenes* to the grapevine phytoalexin might be that phytoalexin detoxification enzymes can have a wide spectrum of effect, such as the *Nectria haematococca* cytochrome P-450 monooxygenase involved in pisatin demethylation (Matthews & Van Etten, 1983; Nebert & Gonzalez, 1987). It is possible that detoxification enzymes employed by *A. rhizogenes* against its natural host are functional against the grapevine phytoalexin resveratrol.

Along with phytoalexin production, increased oxidase activity is another important biochemical defence response. Oxidase activity results from activation of polyphenol oxidase and peroxidase complexes that catalyse the oxidation of simple phenolic compounds into highly reactive quinones (Farkas & Király, 1962; Mayer, 1987). The toxic nature of quinones suggests that they have the potential to inhibit bacterial colonisation (Hilderbrand & Schroth, 1964). *Agrobacterium* strains tested were insensitive to catechol and chlorogenic acid but bacterial numbers were greatly reduced in the presence of benzoquinone. In many cases, 24 hours incubation with 10 µg/ml benzoquinone reduced bacterial numbers to below the level of detection. Seventy-six percent of *A. vitis* strains showed a degree of tolerance to benzoquinone, with 57% of *A. tumefaciens* and 25% of *A. rhizogenes* strains showing tolerance. Although the number of *A. tumefaciens* strains with some tolerance is relatively high, 75% of that total were *A. tumefaciens* strains that had been isolated from grapevine sap and these *A. tumefaciens* grapevine isolates were amongst the most tolerant to

benzoquinone of all the *Agrobacterium* strains tested. In summary, a greater proportion of agrobacteria isolated from grapevine vascular fluid than those isolated from plant rhizospheres showed some tolerance to benzoquinone.

This tolerance to benzoquinone may aid survival in the grapevine vascular system. Tolerance to the oxidation products of phenolic compounds has been demonstrated in other phytopathogens. Lovrekovich *et al.* (1967) observed that *Erwinia carotovora* produced cell-bound hydrogenases that inhibited the oxidation of phenols in rotted potato tuber tissue. Similar detoxifying mechanisms were also found in pathogenic *Pseudomonas* and *Xanthomonas* species (Moustafa {Darweish} & Whittenbury, 1970). *Botrytis cinerea*, on the other hand, produced several forms of a laccase enzyme in response to phenolics and the phytoalexin resveratrol (Marbach *et al.*, 1984; Hoos & Blaich, 1990). Laccases are most likely function by oxidative detoxification of phenolics and phytoalexins to phenol-protein conjugates and polymers while hydrogenases reduce toxic quinones to phenolic monomers (Lyr, 1962). A survey of laccase and hydrogenase activity in *Agrobacterium* isolates might clarify the relative importance of these enzymes in agrobacteria-host interactions.

Addition of ascorbic acid, an anti-oxidant (Larson, 1988), to the benzoquinone treatment completely alleviated the toxic effect of benzoquinone on the *Agrobacterium* strains during a 24 hour incubation. Consequently, the effect of ascorbic acid on the recovery of *A. vitis* K377 and *A. rhizogenes* K27 in the grapevine cane-segment bioassay was tested. Amendment of ascorbic acid to incubation buffer offered no significant increase in survival of strain K27 in the bioassay. This leads to the conclusion that either inhibition by quinones is unimportant in bacterial survival in the bioassay, that the level of ascorbic acid tested was not

sufficient to neutralise the bacteriocidal effects of quinones during the 14 day incubation in the bioassay or that additional factors were also involved.

Along with the biochemical defence responses, such as induction of phytoalexins and peroxidases, there are also physical mechanisms that plants use to localise the spread of invading organisms. As mentioned earlier, an important localisation mechanism in *V. vinifera* xylem vessels is production of pectin gel. The hypothesis was put forward that bacterial polygalacturonase production hydrolyses pectin gel and improves bacterial mobility and survival.

McGuire *et al.* (1991) observed polygalacturonase (Peh) activity in all *A. vitis* strains tested but not in other *Agrobacterium* species. Polygalacturonase activity in *A. vitis* was discovered through the close correlation of *A. vitis* and a grapevine-specific root decay (Burr *et al.*, 1987a). It was later established that Peh activity was directly involved with the root decay when a Peh⁻ *A. vitis* strain failed to induce root decay on grapevine roots (Rodriguez-Palenzuela *et al.*, 1991). This finding was supported by the work of Ophel & Kerr (unpublished data) who found that activity from a combination of cloned polygalacturonase and endoglucanase genes conferred the ability to cause grapevine-specific root decay on an *A. tumefaciens* strain previously unable to do so. When testing the hypothesis that Peh⁺ activity conveys a colonisation advantage *in planta*, it was important to establish that any differences between the *in planta* performance of Peh⁻ strains and Peh⁺ strains was due solely to the Peh⁺ phenotype. A Tn5 Peh⁻ mutant (CG50) was obtained and when tested in an *in vitro* fitness comparison against the Peh⁺ wild-type strain (CG49), was found to be significantly less fit. Consequently, strain CG50 was complemented with the cloned *pehA* gene resulting in strain K1336 (Peh⁺)

whose fitness coefficient was lower but not significantly different from strain CG50. Comparisons between CG50 and K1336 were preferred rather than between CG50 and CG49 due to the similar *in vitro* fitness of CG50 and K1336. When *A. rhizogenes* and *A. tumefaciens* strains were transformed with the *pehA* clone, the fitness of the resulting Peh⁺ transconjugants was significantly lower than the corresponding wild-type strains. However, this was considered acceptable as the fitness of the Peh⁺ strains was below that of the Peh⁻ strains rather than above.

In the grapevine cane-segment bioassay, ten-fold lower numbers of the Peh⁻ mutant (CG50) were recovered than the *pehA* complemented mutant (K1336). The significantly reduced survival of the Peh⁻ mutant in the bioassay indicated that bacterial polygalacturonase production can increase bacterial colonisation in the vascular system. Experiments using an *A. tumefaciens* Peh⁺ transconjugant (K1329) supported the Peh⁻ results, as the transconjugant was detected at four-fold higher levels than the wild-type (Peh⁻) after incubation in the bioassay. However, recovery from the bioassay of another *A. tumefaciens* Peh⁺ transconjugant (K1341) and an *A. rhizogenes* Peh⁺ transconjugant (K1312) was not significantly different to that of the corresponding wild-type strains, illustrating that there are factors determining bacterial survival in the vascular system other than Peh activity. This work linking Peh activity with improved vascular colonisation has not been demonstrated before and strengthens the hypothesis put forward by Hopkins (1985) who, in studies on *Xylella fastidiosa* in grapevine vessels, speculated that pectolytic enzymes may improve bacterial movement through and between vessels. In addition, as pectin has sugars incorporated into its matrix, it is likely that depolymerisation of pectin would release these sugars that could be used as a carbon source by bacteria.

Although polygalacturonase enzymes may provide certain advantages for penetrating and colonising grapevine xylem vessels by *A. vitis*, the liberation of oligosaccharide fragments by this enzyme could be eliciting plant defences. A pectinase produced by *Erwinia carotovora* induced polyphenol oxidase formation on potato tuber (Lovrekovich *et al.*, 1967). The polyphenols were subsequently oxidised to quinones, resulting in a black infection barrier. Further research by Nothnagel *et al.* (1983) found that a fraction of partially hydrolysed pectin contained fragments, oligogalacturonides, that elicited the production and accumulation of phytoalexin in soybean cotyledons. An enzyme, polygalacturonic acid lyase, capable of releasing oligogalacturonides from plant cell walls was purified from the culture filtrate of *Erwinia carotovora* grown on media containing pectin (Davis *et al.*, 1984). The fragments released by this polygalacturonic acid lyase activated phytoalexin production in soybeans while heat inactivation of the enzyme destroyed its ability to release oligogalacturonides and prevented the accumulation of phytoalexins. Considering the phenomenon of pectinase liberated fragments eliciting a host response, it is possible that the oligogalacturonides released by the polygalacturonase of *A. vitis* stimulate a similar response in grapevines. In which case, the addition of polygalacturonase activity to *A. tumefaciens* and *A. rhizogenes* strains may be eliciting a host response that is adversely affecting bacterial survival. In fact, strains sensitive to the anti-microbial compound benzoquinone (ie. *A. tumefaciens* HLB2 and *A. rhizogenes* K27) did not show improved recovery from the grapevine cane-segment bioassay when expressing a polygalacturonase clone, whereas the strains with some tolerance to benzoquinone (ie. *A. vitis* CG49 and *A. tumefaciens* C58) did.

Furthermore, certain quinones have been shown to inhibit polygalacturonase activity (Cole, 1958). If quinones are produced in sufficient quantities in

grapevine cane segments, they may be capable of inhibiting the activity of *A. vitis* polygalacturonase enzymes. Assuming that benzoquinone tolerance (this study) is due to the reduction of quinones back to less toxic phenols by hydrogenases, then it is quite possible that reduced quinone levels would result in improved pectinase activity.

In conclusion, two factors, polygalacturonase activity and tolerance to benzoquinone, were identified that may play a role in bacterial colonisation of the grapevine vascular system but neither was solely responsible for the higher recovery of *A. vitis* cells than those of *A. rhizogenes* or *A. tumefaciens* in the grapevine cane-segment bioassay. It would be interesting to investigate the interaction of multiple defence factors on bacterial colonisation, although interpretation of such experiments would be difficult.

CHAPTER SIX

CHARACTERISATION OF BACTERIOCIN PRODUCING STRAINS INHIBITORY TO *AGROBACTERIUM VITIS*

6.1 INTRODUCTION

Research into mechanisms of biological control of plant pathogens by non-pathogenic bacteria has focused on *Pseudomonas* species and non-pathogenic *Agrobacterium* as biocontrol agents. A range of mechanisms that contribute to antagonism have been identified. For instance, *Pseudomonas fluorescens* has been shown to produce a number of compounds: pyoverdines, or pseudobactins, that function as siderophores and bind to iron, making it unavailable to other rhizosphere microbes (Loper, 1988; Loper & Buyer, 1991); hydrogen cyanide, a compound that is toxic to a broad range of organisms (Askeland & Morrison, 1983; Voisard *et al.*, 1989); and extracellular enzymes such as chitinase and proteinase, that reduce the invasiveness of plant pathogens (Lam *et al.*, 1994; Borowicz & Pietr, 1994).

In both *Agrobacterium* and *Pseudomonas*, the production of antibiotics contributes significantly towards controlling pathogens (Arima *et al.*, 1964; Kerr & Htay, 1974). However, it is likely that successful biocontrol requires more than the production of inhibitory compounds. In the antibiotic producing biocontrol strain *A. rhizogenes* K84, transfer of a plasmid containing the genes for antibiotic production to another strain of

Agrobacterium resulted in antibiotic production in the recipient strain but did not always confer biocontrol ability (Ellis *et al.*, 1979). An explanation suggested for this phenomenon was that direct competition by non-pathogenic bacteria for specific binding sites in the plant cell wall, first demonstrated by Lippincott & Lippincott (1969), may inhibit the induction of gall formation by pathogenic bacteria.

Selection of potential biocontrol strains for grapevine crown gall has focused on isolation of bacterial strains inhibitory to *A. vitis* on Stonier's medium (Stonier, 1960; Staphorst *et al.*, 1985; Webster *et al.*, 1986; Chen & Xiang, 1986; Liang *et al.*, 1990). In this chapter the competitiveness and inhibitory properties of several bacteriocin-producing strains were characterised using a number of methods. Results from the screening techniques described in this chapter were compared and will be correlated with the glasshouse biocontrol experiment in the following chapter.

6.2 EXPERIMENTS

6.2.1 Growth and inhibitory properties of antagonistic strains

Some basic properties of four antagonistic strains were evaluated in preparation for subsequent studies on population dynamics. The inhibitory properties of these bacteriocin producing strains was confirmed by testing them against a range of pathogenic *A. vitis* isolates and the growth characteristics of the antagonistic strains were determined.

6.2.1.1 Inhibition of A. vitis strains on solid media

Four diverse bacterial strains that had previously been reported to inhibit *A. vitis* when tested *in vitro* were collected (Table 14). The strains were tested

Table 14. Bacterial strains used for inhibition studies in chapter six and chapter seven.

Strain	Species	Pathogen	Genetic modification ¹	Inhibitory to K1345 ²	Origin
K1345 ³	<i>A. vitis</i>	yes	Tn501	no	Australia
K1069	<i>A. vitis</i>	no	-	no	Australia
E26	<i>A. vitis</i>	no	-	yes	China
F2/5	<i>A. vitis</i>	no	-	yes	S. Africa
K315	<i>P. fluorescens</i>	no	-	yes	Australia
HLB2	<i>A. tumefaciens</i>	no	-	yes	China
K1341	<i>A. tumefaciens</i>	no	pVK102::P9P3	yes	This study

¹ For details on the transposon Tn501 and the *pehA* clone pVK102::P9P3 refer to Materials & Methods, sections 3.3.2.1 and 3.3.1.2 respectively.

² Antagonistic strain results in a zone of inhibition of *A. vitis* K1345 on Stonier's medium (Appendix A.).

³ A Tn501 transposon derivative of *A. vitis* K377.

for bacteriocin production on solid media (Materials & Methods, sections 3.2.3.1-2) by measuring the diameter of the zone of inhibition (Fig 16). The number of *A. vitis* isolates that were sensitive to a particular antagonistic strain (Table 15) indicates the spectrum of activity of the bacteriocin produced by that strain. *A. tumefaciens* HLB2 had the largest average zone of inhibition (63.3 mm), followed by *P. fluorescens* K315 (46.8), *A. vitis* E26 (42.5) and *A. vitis* F2/5 (14.3). *A. vitis* K1069 did not inhibit any of the strains tested.

6.2.1.2 Growth rate of antagonistic strains

Growth rates (Materials & Methods, section 3.4.1.1) of the antagonistic strains (Table 14) were compared in TY broth (Fig. 17). Four replicates per strain were sampled. Curves were fitted to the data and because the data were taken during the log growth phase the best fit was a linear model. The slope of each replicate was calculated and a mean taken for each strain. An analysis of variance indicated a significant difference ($p < 0.05$) between the means. The slope of *P. fluorescens* K315 (0.259 log CFU/ml/hour) was higher than the other strains; *A. vitis* F2/5 (0.224), *A. tumefaciens* HLB2 (0.177), *A. vitis* E26 (0.176) and *A. vitis* K1069 (0.169), but the means could not be separated using Tukey's statistic (0.092) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

6.2.1.3 Death rate of antagonistic strains

The rate at which cells of the antagonistic strains became non-viable during incubation in nutrient-free culture was determined; this procedure was referred to as death rate (Materials & Methods, section 3.4.1.2). Six replicates for each strain were used. An analysis of variance performed on the slopes of curves fitted to the data indicated a significant difference ($p < 0.001$) between the slopes of different antagonistic strains. To rank the

Figure 16. Inhibition of *A. vitis* K377 by *A. tumefaciens* HLB2 on Stonier's medium (Appendix A). Strain K377 was suspended in soft buffered agar (Appendix A) and poured over a plate of Stonier's medium that strain HLB2 had been previously grown on and then removed. The plate with the strain K377 overlay was incubated for 48 hours. The arrow (◄) indicates the zone of inhibition.

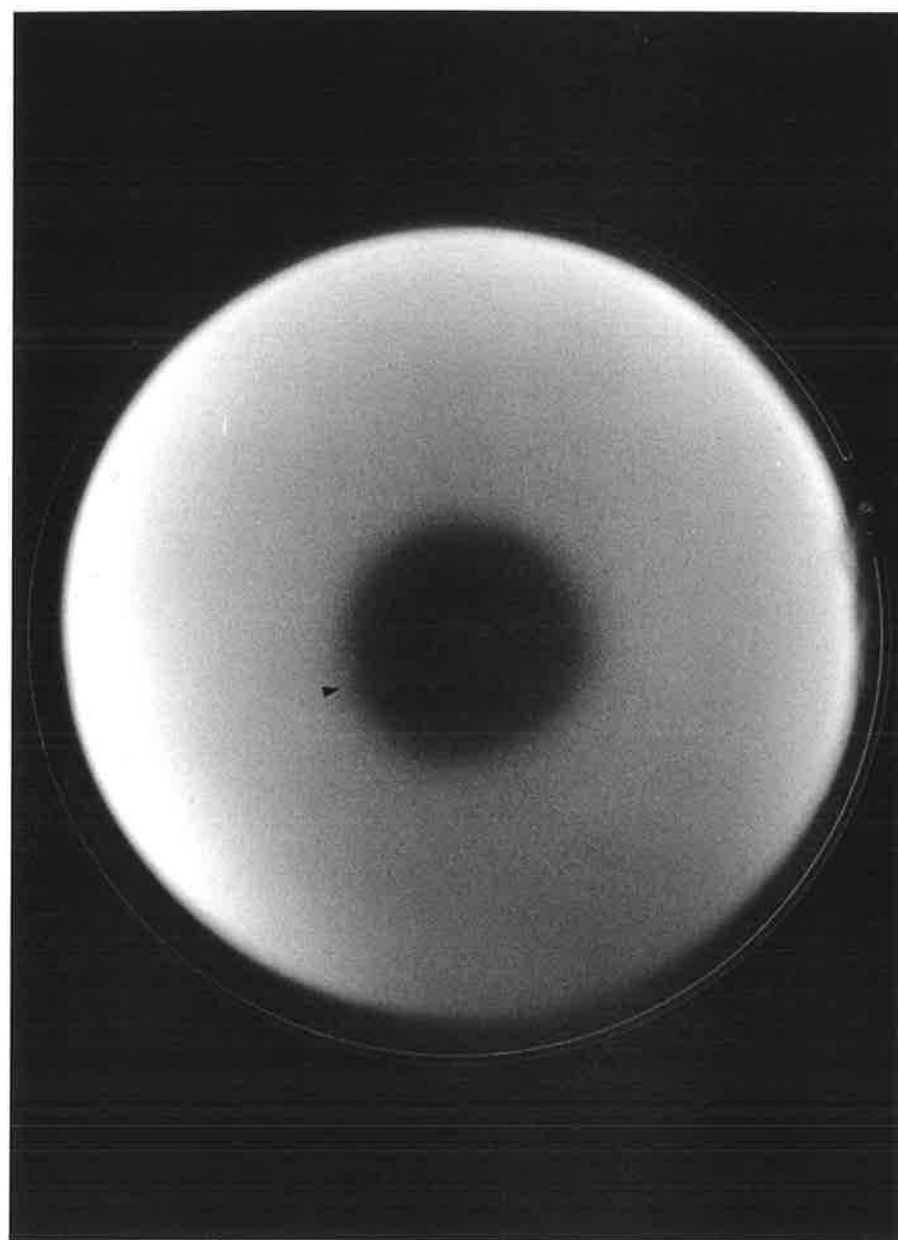


Table 15. Survey of *A. vitis* isolates for sensitivity to antagonistic strains.

<i>A. vitis</i> strain	Pathogenic	Diameter of inhibition zone ¹				
		(mm)				
		<i>A. vitis</i> K1069 ²	<i>A. vitis</i> E26 ²	<i>A. vitis</i> F2/5 ²	<i>P. fluorescens</i> K315 ²	<i>A. tumefaciens</i> HLB2 ²
K306	yes	0	22	5	30	50
K374	yes	0	43	18	32	85
K375	yes	0	85	31	85	85
K376	yes	0	37	28	63	62
K377	yes	0	70	24	62	85
K1070	yes	0	25	0	13	45
K1072	yes	0	26	0	31	47
#675	no	0	58	14	70	71
#676	no	0	42	13	55	57
#678	no	0	40	15	38	55
#680	no	0	19	10	36	54
		0 ³	42.5 ³	14.4 ³	46.8 ³	63.3 ³

Table 15. Continued.

- ¹ Refers to the zone of inhibition of a sensitive strain when the sensitive strain has been suspended in soft buffered agar (Appendix A.) and poured over a plate of Stonier's medium (Appendix A.) that has previously grown an antagonistic strain.
- ² These strains were grown on Stonier's medium for 48 hours, killed and removed; following this, a strain to be tested for sensitivity was added as explained above.
- ³ Average of diameter of inhibition values for each antagonist strain.

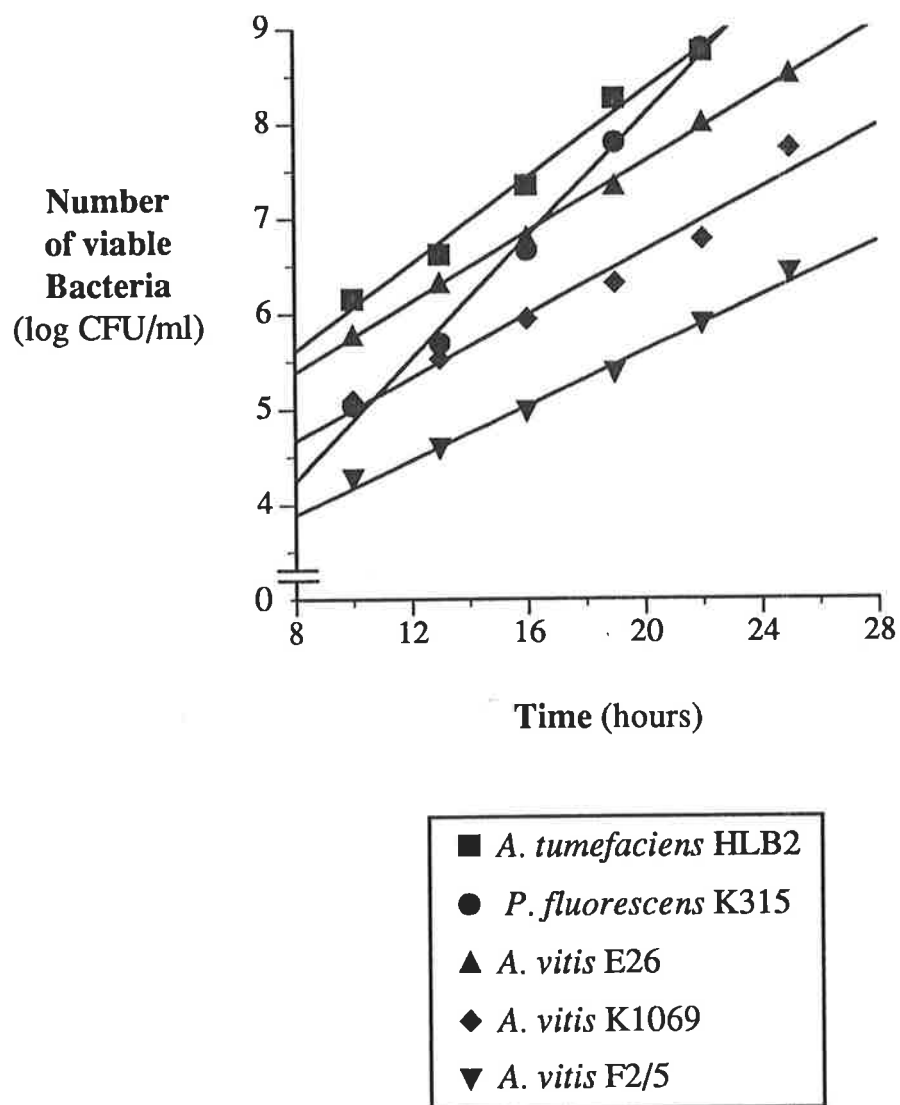


Figure 17. Growth of the bacterial strains *A. tumefaciens* HLB2, *P. fluorescens* K315, *A. vitis* E26, *A. vitis* K1069 and *A. vitis* F2/5 when incubated in TY broth at 28°C. The slope of the fitted line indicates growth rate. An analysis of variance indicated a significant difference ($p < 0.05$) between the means of the slopes.

slope of each antagonistic strain, Tukey's statistic was calculated (Tukey's WSD; Materials & Methods, section 3.6.1.5) and means differing by more than this value (0.049) were considered significantly different. *P. fluorescens* K315 had the lowest death rate (1.129 log CFU/ml/day), followed by *A. tumefaciens* HLB2 (1.030), *A. vitis* E26 (0.993), *A. vitis* F2/5 (0.986) while *A. vitis* K1069 (0.962) had a greater death rate, although this value was not significantly different from the other *A. vitis* strains (Fig. 18).

6.2.1.4 Screening antagonistic strains for polygalacturonase activity and tolerance to benzoquinone

In the previous chapter, polygalacturonase activity was correlated with improved colonisation of grapevine segments and tolerance to benzoquinone was shown to be more predominant in grapevine vascular colonising bacteria than in rhizosphere colonising bacteria. It is likely that biocontrol agents with these colonisation factors will be more competitive under *in planta* conditions than those without. Consequently, the antagonistic strains were screened for polygalacturonase activity (Materials & Methods, section 3.2.2.1) and tolerance to benzoquinone (Materials & Methods, sections 3.4.2.1-2). A summary of the characteristics of the antagonist strains is given in Table 16.

6.2.2 Population dynamics of a transposon-tagged *A. vitis* pathogen in the presence of antagonistic strains

To study the population dynamics of a pathogenic strain cultivated with an antagonistic strain, it is necessary to distinguish between the two strains. This was achieved by introducing a transposon conferring mercury resistance, into a pathogenic *A. vitis* strain. The effect of inhibitory and non-inhibitory bacterial strains on the population of the transposon-tagged *A.*

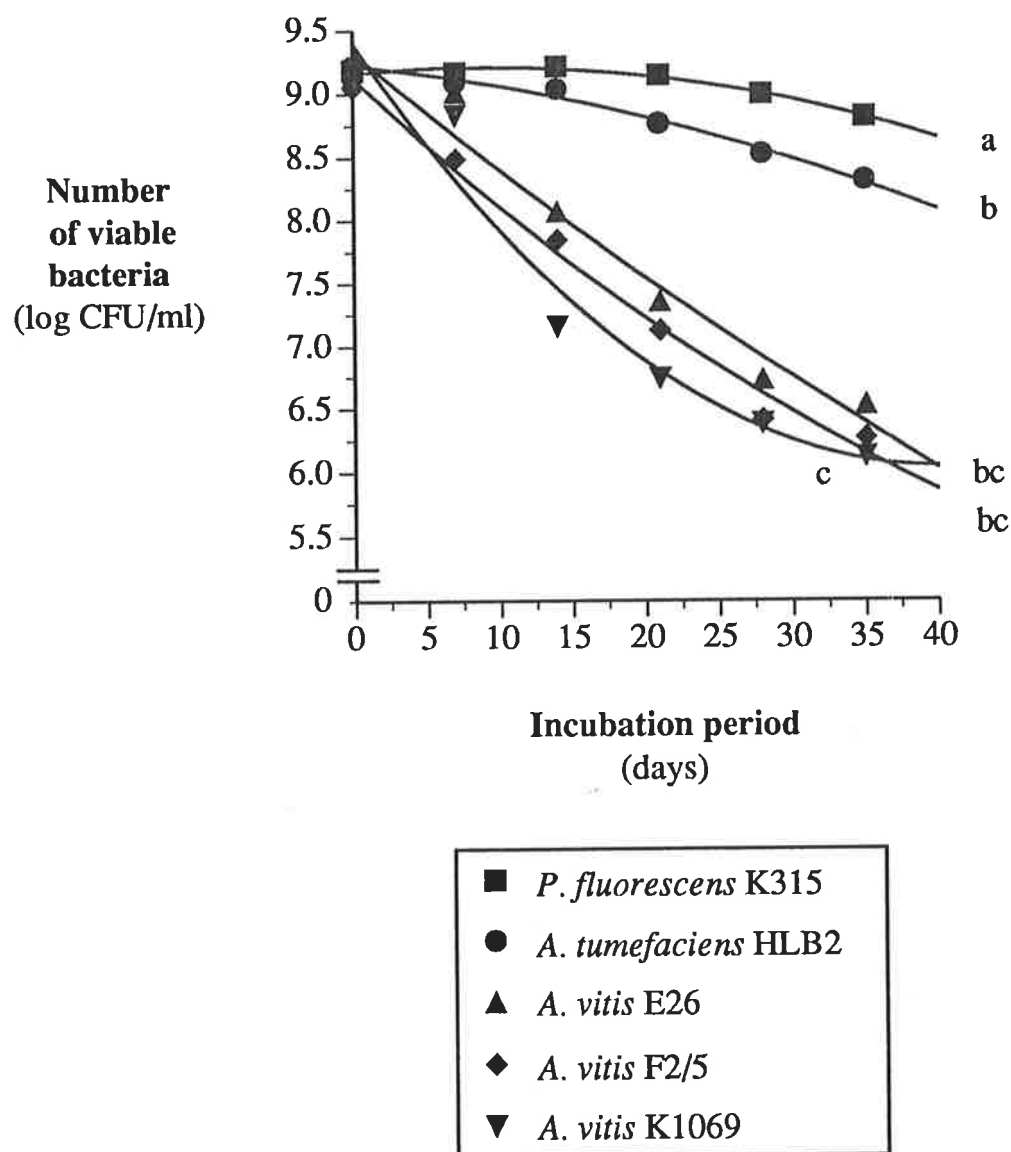


Figure 18. Survival of the bacterial strains *A. tumefaciens* HLB2, *P. fluorescens* K315, *A. vitis* E26, *A. vitis* K1069 and *A. vitis* F2/5 when incubated in MSALTS solution (Appendix C) at 25°C. The slope of the fitted curve represents death rate. Different letters (a-c) indicate slopes that differ by more than Tukey's statistic (0.049) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

Table 16. Characteristics of antagonistic strains.

Species	Strain	Bacteriocin production ¹	Growth rate advantage ²	Death rate disadvantage ³	Peh activity ⁴	Tolerance to benzoquinone ⁵
<i>A. vitis</i>	K1069	-	-	1	+	-
<i>A. vitis</i>	E26	+	-	2	+	-
<i>A. vitis</i>	F2/5	+	-	2	+	+
<i>A. tumefaciens</i>	HLB2	+	-	3	-	-
<i>A. tumefaciens</i>	K1341	+	-	3	+	-
<i>P. fluorescens</i>	K315	+	+	4	-	-

¹ Antagonistic strain inhibits *A. vitis* K377 on Stonier's medium (Appendix A).

² Growth rate of cells in TY broth (Appendix A). Plus (+) indicates a significantly faster growth rate.

³ Death rate of cells in MSALTS buffer (Appendix B). Smaller number indicates faster death rate.

⁴ Polygalacturonase activity refers to clear zones in polypectate agar plates as described by Ried and Collmer (1985).

⁵ Bacteria able to form colonies on TY media (Appendix A) following a 24 hour incubation in buffer containing 10 µg/ml benzoquinone.

vitis strain was monitored in TY broth, Stonier's broth and in the grapevine cane-segment bioassay.

6.2.2.1 *Recovery of transposon-tagged A. vitis K1345 on media containing mercury*

The recovery (Materials & Methods, section 3.4.1.3) of a Tn501 transposon-tagged strain, *A. vitis* K1345 (Materials & Methods, section 3.3.2.1), and antagonistic strains (Table 14) was determined on media containing mercuric chloride. The number of viable bacteria recovered on TY and RS media containing different levels of mercuric chloride was compared with that on TY media containing no mercuric chloride (Table 17). Five replicates per treatment were used. It was found that no strains other than *A. vitis* K1345 grew even at the lowest concentrations tested, suggesting that 3 mg/ml of mercuric chloride in TY and 1 mg/ml of mercuric chloride in RS is sufficient to ensure only the transposon-tagged strain can grow.

6.2.2.2 *Fitness of the transposon-tagged A. vitis strain K1345*

To determine the effect of Tn501 on the growth of *A. vitis* K1345, the fitness (Material & Methods, sections 3.4.3.1-2) of this strain was compared with *A. vitis* K377, the wild-type parent (Fig. 19). The two strains were co-inoculated into TY broth and incubated for 48 hours. An aliquot of the bacterial suspension was transferred to fresh TY broth. This serial transfer process was repeated seven times and each time the bacterial populations were enumerated. The relative change in population numbers of the transconjugant and wild-type populations was plotted, enabling a linear model to be fitted. The residual error plot was not completely random thus indicating a slight problem with the assumption of normal distribution but this was not considered severe enough to reject the model. Calculating the slope of this line gave the fitness coefficient of transposon-tagged strain

Table 17. Recovery of bacterial strains on TY and RS media amended with different concentrations of mercuric chloride.

Bacterial strain	Percentage recovery of bacteria on different media containing mercuric chloride ¹					
	TY ² (3 mg/ml) ³	TY (6 mg/ml)	TY (9 mg/ml)	RS ⁴ (1 mg/ml)	RS (2 mg/ml)	RS (3 mg/ml)
<i>A. vitis</i> K1345	30.1	28.9	39.7	19.9	16.7	13.6
<i>A. vitis</i> K377	0	0	0	0	0	0
<i>A. vitis</i> K1069	0	0	0	0	0	0
<i>A. vitis</i> E26	0	0	0	0	0	0
<i>A. vitis</i> F2/5	0	0	0	0	0	0
<i>P. fluorescens</i> K315	0	0	0	0	0	0
<i>A. tumefaciens</i> HLB2	0	0	0	0	0	0

¹ Percentage recovery is calculated by dividing number of CFU's on media containing mercuric chloride by the number of CFU's on TY media and multiplying by one hundred.

² TY is undefined media (Beringer, 1974)

³ Concentration of mercuric chloride in media.

⁴ RS media is semi-selective for *A. vitis* (Roy & Sasser, 1983).

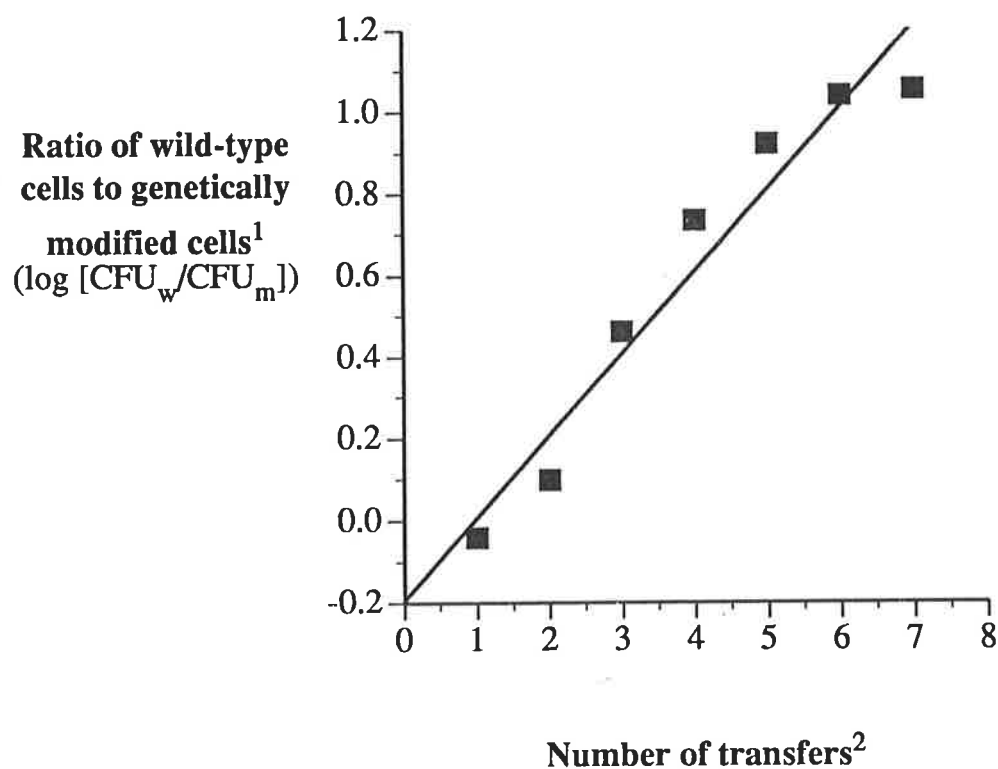


Figure 19. Fitness co-efficients of the transposon-tagged (Tn501) *A. vitis* K1345 when competing against its wild-type parent *A. vitis* K377. ¹ The rate at which the log of the ratio of wild-type cells (CFU_w) to genetically modified cells (CFU_m) increases is the fitness co-efficient.

² Genetically modified and wild-type parent cells were co-inoculated to TY broth and incubated for 48 hours, the dual culture was then transferred to fresh TY broth. The slope (0.200) was greater than two times the standard error (0.022), so it was significantly greater than zero.

K1345. The slope (0.200) was greater than two times the standard error (0.022); so it was significantly greater than zero, indicating that the transposon imposed a noticeable burden on the strain. However, when comparing the performance of the antagonistic strains any effects of the transposon-tagged pathogen would be constant in all treatments.

6.2.2.3 *Pathogen displacement by antagonistic strains in TY broth*

Determining the extent to which each antagonistic strain can reduce the number of pathogenic *A. vitis* cells, enables the efficacy of the antagonistic strains to be compared. Equivalent numbers of *A. vitis* K1345 and each antagonistic strain (Table 14) were co-inoculated into TY broth and incubated for 48 hours (Materials & Methods, sections 3.4.4.1-2). *A. vitis* K1345 was co-inoculated with *A. vitis* K377, its wild-type progenitor, as a control treatment. After incubation, the surviving cells of strain K1345 were selected on TY agar containing 3 mg/ml of mercuric chloride (Fig. 20). Four replicates per treatment were used. An analysis of variance indicated that the means were significantly ($p < 0.001$) different. To rank the means of pathogen numbers in the presence of each antagonistic strain, Tukey's statistic was calculated (Tukey's WSD; Materials & Methods, section 3.6.1.5) and means differing by more than this value (0.315) were considered significantly different. Antagonistic strain *P. fluorescens* K315 reduced numbers of the mercury resistant *A. vitis* pathogen (K1345) by the largest margin, followed by the *A. tumefaciens* strains HLB2 and K1341. Antagonistic strains *A. vitis* E26 and *A. vitis* F2/5 and non-antagonistic strain K1069 did not reduce pathogen numbers below that of the control (*A. vitis* K377).

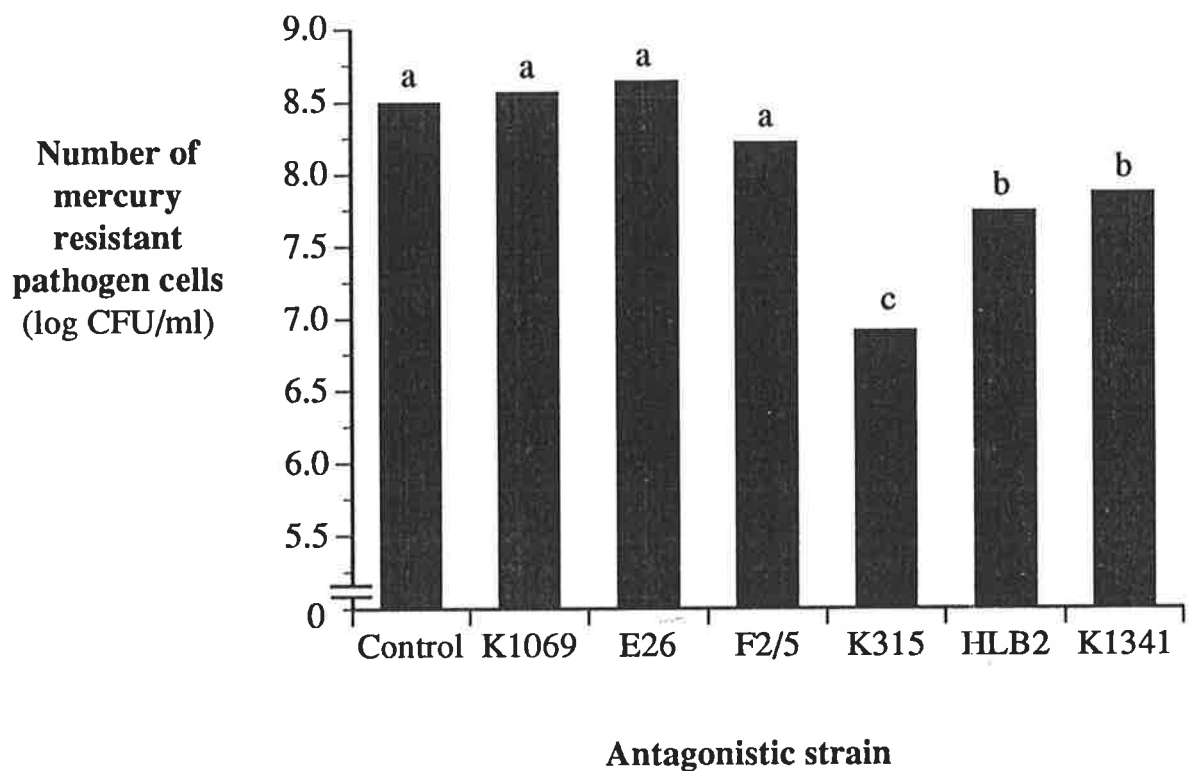


Figure 20. Pathogen displacement in TY broth when *A. vitis* K1345 was co-inoculated with antagonistic strains *A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 and *A. tumefaciens* K1341 and incubated for 48 hours. In the control treatment transposon-tagged *A. vitis* K1345 was co-inoculated with its wild-type parent *A. vitis* K377. Different letters (a-c) indicate means that differ by more than Tukey's statistic (0.315) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

6.2.2.4 *Pathogen displacement by antagonistic strains in Stonier's broth*

Studying pathogen displacement in Stonier's broth, a medium designed to promote bacteriocin production, and comparing with pathogen displacement in TY broth, enables the relative effects of the bacteriocins to be assessed. It also indicates differences in bacteriocin production and/or sensitivity under different conditions when compared to pathogen displacement in TY broth. *A. vitis* strain K1345 was co-inoculated with antagonistic strains (Table 14) into Stonier's broth (Appendix A) and incubated for 48 hours (Materials & Methods, sections 3.4.4.1-2). *A. vitis* K1345 was co-inoculated with *A. vitis* K377 as a control treatment. Numbers of mercury resistant *A. vitis* K1345 cells were determined for each treatment (Fig. 21). An analysis of variance on the data indicated significant ($p < 0.001$) variation between the means. Tukey's statistic was calculated (Tukey's WSD; Materials & Methods, section 3.6.1.5) to rank the means of pathogen numbers in the presence of each antagonistic strain. Means differing by more than this value (1.169) were considered significantly different. Pathogen numbers were reduced most by *A. vitis* E26, followed by *P. fluorescens* K315, *A. vitis* F2/5, *A. tumefaciens* HLB2 and *A. tumefaciens* K1341. Non-antagonistic strain *A. vitis* K1069 was not significantly different from the control.

6.2.2.5 *Pathogen displacement by antagonistic strains in the grapevine cane-segment bioassay*

Antagonism can be observed in defined media and depends on the composition of the medium. In the grapevine vascular system, factors may be present that alter the performance of antagonistic strains. Antagonistic strains (Table 14) were co-inoculated with a mercury-resistant *A. vitis* pathogen, strain K1345, and incubated in the grapevine cane-segment

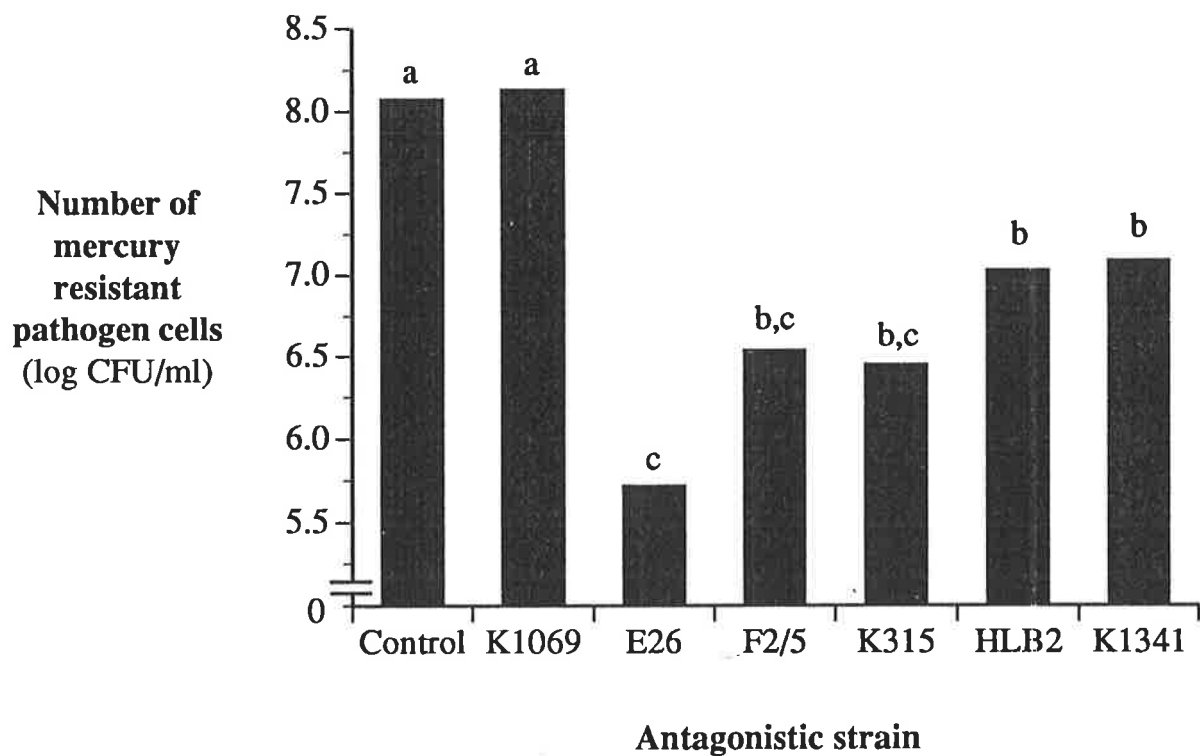


Figure 21. Pathogen displacement in Stonier's broth when *A. vitis* K1345 was co-inoculated with antagonistic strains *A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 and *A. tumefaciens* K1341 and incubated for 48 hours. In the control treatment transposon-tagged *A. vitis* K1345 was co-inoculated with its wild-type parent *A. vitis* K377. Different letters (a-c) indicate means that differ by more than Tukey's statistic (1.169) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

bioassay for fourteen days (Materials & Methods, sections 3.6.1.1-4). *A. vitis* K1345 was co-inoculated with *A. vitis* K377 as a control treatment. Following incubation, the number of the mercury resistant pathogens was determined (Table 18) on RS medium amended with 1 mg/ml of mercuric chloride. An analysis of variance of the data indicated that the means were significantly ($p < 0.001$) different. Tukey's statistic (Tukey's WSD; Materials & Methods, section 3.6.1.5) was calculated (0.282) to rank the means. Antagonistic strains *A. vitis* E26, *A. vitis* F2/5 and *A. tumefaciens* HLB2 reduced the population of pathogenic *A. vitis* K1345 cells significantly below that of the control treatment, whereas *P. fluorescens* K315 and *A. vitis* K1069 had no significant antagonistic effect.

6.2.3 Bacterial antagonism in artificial wound sites on grapevine stems

In previous studies the ability of the antagonistic strains to reduce pathogen numbers in different media and in the grapevine cane-segment bioassay was examined. This indicated the extent to which the antagonistic strains inhibited a pathogen population but gave no indication of their ability to prevent the induction of crown gall formation. Consequently, this experiment compares wound colonisation by antagonistic strains and a pathogen by observing gall formation at artificial wound sites co-inoculated with antagonistic and pathogenic bacteria.

6.2.3.1 Preventing gall formation by *A. vitis* K377 at wound sites with antagonistic strains

Bacterial suspensions were inoculated into artificial wounds on grapevine stems, with sterile MSALTS used as a control treatment (Materials & Methods, sections 3.6.2.1-2). Two inoculation treatments were used, a co-

Table 18. Effect of antagonistic strains on the recovery of Tn501-tagged *A. vitis* K1345 from the grapevine cane-segment system bioassay.

Antagonistic strain	Inhibition on Stonier's medium ¹	No. of <i>A. vitis</i> K1345 cells (log CFU/ml)	
		Inoculated ²	Recovered ³
Control	-	8.61	7.08 ^a
<i>A. vitis</i> K1069	-	8.61	7.10 ^a
<i>A. vitis</i> E26	+	8.61	6.68 ^b
<i>A. vitis</i> F2/5	+	8.61	6.68 ^b
<i>P. fluorescens</i> K315	+	8.61	7.06 ^a
<i>A. tumefaciens</i> HLB2	+	8.61	6.67 ^b
<i>A. tumefaciens</i> K1341	+	8.61	6.64 ^b

¹ Antagonistic strain leads to a zone of inhibition of *A. vitis* K1345 on Stonier's medium (Appendix A).

² The number of viable cells inoculated to grapevine cane segments, as determined by CFU's on TY media (Appendix A).

³ The number of viable cells recovered from the grapevine cane-segment bioassay, as determined by CFU's on RS media (Appendix A) amended with 1 mg/ml of mercuric chloride and adjusted for percentage recovery of the selective media (Chapter 6, section 6.2.2.1).

^{a-b} Different letters indicate means that differ by more than Tukey's statistic (0.282) (Tukey's WSD; Materials & Methods, section 3.6.1.5).

inoculation and a pre-inoculation. In the co-inoculation treatment, the antagonistic strain was added first and the pathogen was added approximately 60 seconds later. In the pre-inoculation treatment the antagonistic strain was added 24 hours before the pathogen. Each treatment had a minimum of six replicates, consisting of three grapevines with two wounds per grapevine. The experimental layout was completely randomised with seven bacterial strain treatments and two inoculation methods.

After an eight week incubation period the wound sites were observed for gall formation (Fig. 22). The data were recorded as tumour size (mm²) using both perpendicular and parallel diameter measurements. Due to the presence of zeros in the data, the residual plot was skewed; so a log transformation ($\log\{\text{area}+1\}$) was performed on the data. An analysis of variance indicated that the means were significantly ($p<0.001$) different from each other. The means were ranked using the average Standard Error Difference (SED) and means differing by more than this margin (0.793) are indicated by different letters (Figures 23 & 24). Tumour size was affected by different antagonistic strains and by the timing of the inoculation method. Pre-inoculation with *A. vitis* E26 reduced gall size more than when this strain was co-inoculated while *A. vitis* K1069 and *P. fluorescens* K315 gave better protection when co-inoculated rather than when pre-inoculated. Other strains performed similarly in co-inoculation and pre-inoculation treatments. The only strain that did not significantly reduce gall formation in either inoculation treatment was *A. tumefaciens* K1341, the HLB2 strain containing a *pehA* clone. The best protection came from strain *A. vitis* F2/5 which completely prevented gall formation in both inoculation treatments.

Figure 22. Tumour formation at artificial wound sites co-inoculated with *A. tumefaciens* HLB2 and *A. vitis* K377. The arrows (◄) indicate artificial wound sites. The upper wound site has no gall formation whereas the lower wound site is galled.



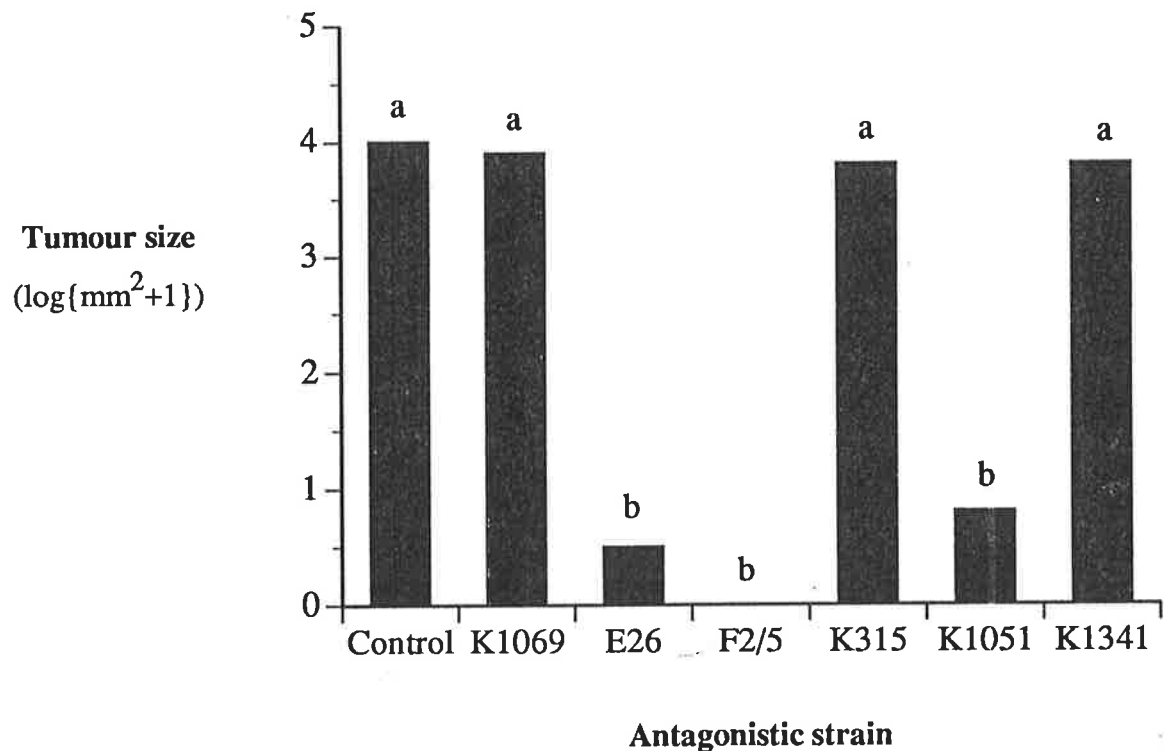


Figure 23. Effect on tumour formation at artificial wound sites on grapevine stems when sites are pre-inoculated with an antagonistic strain (*A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 or *A. tumefaciens* K1341) 24 hours before inoculation with an equal volume of a suspension of *A. vitis* K1345. The control treatment was inoculated with sterile MSALTS (Appendix C). The average standard error difference was 0.793 and means differing by more than this margin are indicated by different letters.

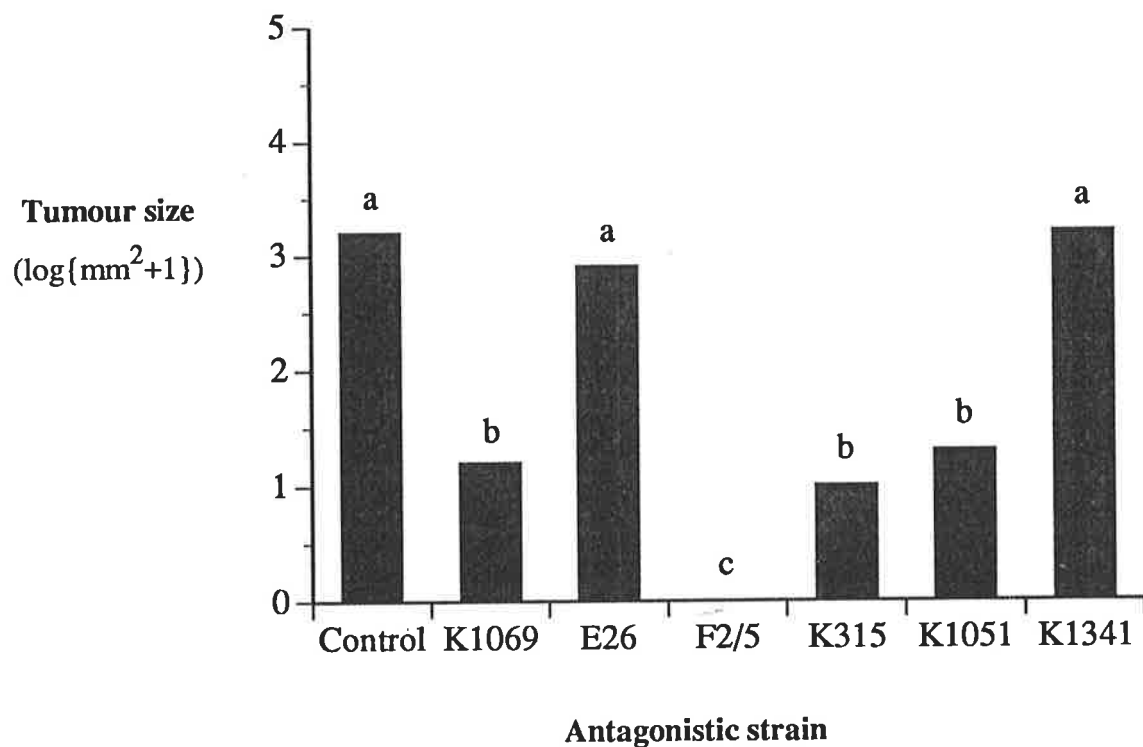


Figure 24. Effect on tumour formation at artificial wound sites on grapevine stems when sites are co-inoculated with an antagonistic strain (*A. vitis* K1069, *A. vitis* E26, *A. vitis* F2/5, *P. fluorescens* K315, *A. tumefaciens* HLB2 or *A. tumefaciens* K1341) and an equal volume of a suspension of *A. vitis* K1345. The control treatment was inoculated with sterile MSALTS (Appendix C). The average standard error difference was 0.793 and means differing by more than this margin are indicated by different letters.

6.3 DISCUSSION

A number of previously documented antagonistic bacteria were collected (Table 14) and characterised. The ability of these strains, with the exception of *A. vitis* K1069, to produce bacteriocins *in vitro* was confirmed on Stonier's media. The inhibitory effect of the antagonistic strains was tested against a range of *A. vitis* pathogens, with the antagonistic strain *A. tumefaciens* HLB2 having the largest average zone of inhibition. Pu & Goodman (1993b) also found HLB2 was inhibitory to many *A. vitis* strains, from a range of geographical locations. Strain *A. vitis* K1069 did not inhibit any of the pathogens tested and was used as a control strain in later experiments. Only *A. vitis* F2/5 failed to inhibit all the *A. vitis* strains tested and had the smallest average zone of inhibition.

Bacteriocin production is an important factor in bacterial antagonism and the amount of bacteriocin produced is related to the number of bacteria present at the site of antagonism. For this reason, the capacity of a bacterium to perform efficiently under *in vitro* conditions of adequate nutrition and inadequate nutrition was studied. A strain that grows quickly with adequate nutrition and dies slowly with inadequate nutrition should be well positioned to effectively compete in an ecological niche. When the rate of growth was determined for the antagonistic strains in TY broth, *P. fluorescens* K315 had a faster growth rate than the other strains. A faster growth rate may give strain K315 an advantage under conditions where nutrients are non-limiting. To examine bacterial survival under conditions where nutrients were limiting, antagonistic strains were incubated in nutrient-free MSALTS and the decrease in viable cell counts was plotted over time. The slope of fitted curves was calculated and this value called the death rate. *A. vitis* strains had

the highest death rates, followed by *A. tumefaciens* HLB2 and then *P. fluorescens* K315. These experiments suggest that *P. fluorescens* K315 would be the most competitive of the antagonistic strains under conditions of adequate and inadequate nutrition, while the *A. vitis* strains were the least competitive.

To determine the relative abilities of the different antagonistic strains to compete against *A. vitis* pathogens, the population dynamics of an *A. vitis* pathogen was studied when co-inoculated with the antagonistic strains (See Table 19 for a summary of the performance of the antagonistic strains in *in vitro* and *in planta* assays). To do this, a transposon known as Tn501 was introduced into *A. vitis* K377 and a mercury resistant mutant (strain K1345) was selected. The *A. vitis* K377 pathogen was chosen for further study, as it was sensitive to the four antagonistic strains when tested on Stonier's media. Although *A. vitis* K1345 was resistant to mercury on TY media and RS selective media, the viability of strain K1345 cells decreased as the concentration of mercury was increased. For this reason, the lowest concentration of mercury necessary to kill bacteria not containing Tn501 was used in subsequent experiments. The wild-type strain was more competitive than the transposon-tagged strain during serial transfers but this is not unexpected as integration of a transposon into the bacterial genome can reduce population fitness (Condit *et al.*, 1988). Even though the fitness of the tagged pathogen cells was compromised, any effects should be constant when the tagged strain is compared with the antagonistic strains.

The population dynamics of the mercury-resistant pathogen *A. vitis* K1345 was studied when grown in direct competition with antagonistic strains. This was done in two types of liquid culture and in the grapevine cane-segment bioassay. In TY broth, antagonistic strain *P. fluorescens* K315 was the most

Table 19. Performance of antagonistic strains in pathogen displacement studies and in the artificial wound assay.

Species	Strain	Pathogen displacement ¹			Protection in wound assay ⁵	
		TY broth ²	Stonier's broth ³	Cane segment bioassay ⁴	pre-inoculated ⁶	co-inoculated ⁷
<i>A. vitis</i>	K1069	-	-	-	-	+
<i>A. vitis</i>	E26	-	+	+	+	-
<i>A. vitis</i>	F2/5	-	+	+	+	+
<i>P. fluorescens</i>	K315	+	+	-	-	+
<i>A. tumefaciens</i>	HLB2	+	+	+	+	+
<i>A. tumefaciens</i>	K1341	+	+	+	-	-

Table 19. Continued.

- ¹ Tn501 tagged pathogen (*A. vitis* K1345) co-inoculated with antagonistic strains and enumerated on TY or RS media (Appendix A) containing mercuric chloride.
- ² Plus (+) indicates that pathogen numbers were significantly lower than control after incubation in TY broth.
- ³ Plus (+) indicates that pathogen numbers were significantly lower than control after incubation in Stonier's broth.
- ⁴ Plus (+) indicates that pathogen numbers were significantly lower than control after incubation in the grapevine cane-segment bioassay.
- ⁵ Prevention of gall formation on artificially wounded grapevine stems inoculated with antagonistic strains and an *A. vitis* pathogen (K377).
- ⁶ Plus (+) indicates that antagonistic strains inoculated to wound site 24 hours before the pathogen reduced gall formation to a level significantly below that of the control.
- ⁷ Plus (+) indicates that antagonistic strains inoculated to wound site 60 seconds before the pathogen reduced gall formation to a level significantly below that of the control.

effective at reducing pathogen numbers, followed by *A. tumefaciens* HLB2 and *A. tumefaciens* K1341. In contrast, all bacterial strains except K1069, the non-antagonistic control strain, significantly reduced pathogen numbers in Stonier's broth. It is important to note that both *A. vitis* antagonistic strains, E26 and F2/5, varied significantly in their ability to reduce pathogen numbers in the different media types. The difference in antagonism of the strains between the different media could be due to increased bacteriocin production, increased sensitivity of the pathogen to the bacteriocins or a combination of both. This work supports the findings of others who suggested that media composition could effect the antagonism of agrocin 84. Dhanvantari (1983) noted that amending Stonier's medium with vitamins and replacing ammonium nitrate with L-glutamic acid as the nitrogen source could render previously insensitive *A. vitis* strains sensitive to *A. rhizogenes* K84. Similarly, Cooksey & Moore (1980) demonstrated that addition of glucose to an overlay also led to insensitive *Agrobacterium* pathogens becoming sensitive to strain K84. It was further suggested that the sensitivity of *Agrobacterium* to agrocin 84 may be altered by root exudates and other plant factors present *in situ*.

When the antagonistic strains were tested in the grapevine cane-segment bioassay, the non-antagonist *A. vitis* K1069 and the antagonist *P. fluorescens* K315 did not significantly decrease pathogen numbers. As suggested by Cooksey & Moore (1980), plant factors may be contributing to the reduced antagonism of strain K315. Tests in TY and Stonier's broth would not detect this. The lack of correlation between the pathogen displacement studies indicates that bacterial antagonism, most likely due to bacteriocin production and sensitivity of the pathogen to these bacteriocins, varies under different environmental conditions. This implies that extrapolation of antagonism results obtained under defined *in vitro*

conditions to what may happen *in planta* should be done with caution.

The ability of the antagonistic strains to reduce the induction of gall formation by a pathogen was tested using artificial wound sites on grapevine stems as described by Burr & Reid (1994). This type of 'wound assay' encompasses not only bacteria-bacteria interactions but the ability of antagonistic strains to prevent the induction of gall formation. Comparing results obtained with *in vitro* screening experiments with results from an *in planta* wound assay may give some insight into the merits of different screening techniques.

Different antagonistic strains and the timing of antagonistic strain inoculation with pathogen inoculation were examined using gall formation at artificial wound sites as an indicator of disease incidence. The inoculation treatments included a pre-inoculation treatment, with the antagonistic strain inoculated 24 hours before the pathogen; and a co-inoculation treatment, with the antagonistic strain inoculated 60 seconds before the pathogen. The timing of inoculation significantly affected the prevention of gall formation by antagonistic strains. The antagonistic strain *P. fluorescens* K315 reduced the size and incidence of tumour formation below that of the control when co-inoculated with the pathogen but not when pre-inoculated. An explanation for this could be that the bacteria do not persist in high numbers in the wound site and so are most effective for a brief period immediately following inoculation. This is unexpected, as *P. fluorescens* K315 had a faster *in vitro* growth rate and a slower *in vitro* death rate than the other antagonistic strains. *A. vitis* K1069 did not reduce gall formation when pre-inoculated but gave significant control when co-inoculated with the pathogen. The non-pathogenic strain *A. vitis* K1069 did not inhibit *A. vitis* K377 *in vitro* but reduced gall formation by strain K377 *in planta*. This indicates that

mechanisms other than bacteriocin production could be contributing to reduced gall formation; for example, mechanisms such as competition for plant binding sites as demonstrated by Lippincott & Lippincott (1969). In contrast, *A. vitis* E26 provided a significant reduction in gall formation when pre-inoculated rather than when co-inoculated. This indicates that strain E26 cells can persist at the wound site but may be poor competitors when co-inoculated with other bacteria. However, the timing of the inoculation treatment had no significant effect on strains *A. vitis* F2/5 and *A. tumefaciens* HLB2. Both gave significant protection against gall formation in co-inoculation and pre-inoculation treatments. Even though both strains performed well regardless of the timing of inoculation, there was some gall formation with HLB2 whereas strains F2/5 completely prevented gall formation. It would appear that *A. vitis* F2/5 is a very effective wound coloniser.

Polygalacturonase activity in *A. vitis* has been linked with improved wound colonisation, root attachment and tumorigenicity, as an *A. vitis* mutant with no Peh activity had a reduction in these functions (Brisset *et al.*, 1991; Rodriguez-Palenzuela *et al.*, 1991). Peh activity has also been implicated with improved colonisation of the grapevine vascular system (Chapter Five, section 5.2.3). Consequently, the hypothesis that the addition of Peh activity could improve antagonism of a non-pectinolytic bacteriocin producing strain was tested. The addition of a plasmid conveying Peh activity to *A. tumefaciens* HLB2 (transconjugant strain, K1341) significantly increased gall formation in comparison with HLB2. That Peh activity did not improve antagonism of K1341 and even increased gall formation compared to HLB2 suggests that any beneficial effects of Peh activity on wound colonisation, such as making binding sites more accessible, do not give any advantage to the Peh producing *A. tumefaciens* strain.

Another factor implicated with systemic colonisation (Chapter Five, section 5.2.1) was tolerance to benzoquinone, an oxidation product of phenolic compounds. There is evidence that phenolic compounds are liberated from plant wounds (Stachel *et al.*, 1985b), as are polyphenol oxidases (Mayer, 1987). This combination normally results in the formation of quinone compounds that are toxic to bacteria (Geiger, 1946). Consequently, tolerance to quinones is likely to enable a colonisation advantage at wound sites, allowing antagonistic strains to persist in a location where the induction of gall formation process takes place. Notably, the only antagonistic strain with some tolerance to benzoquinone was *A. vitis* F2/5 and this strain completely prevented gall formation in the wound assay.

In summary, there was low correlation between *in vitro* growth characteristics of the antagonistic strains and prevention of gall formation in the wound assay. *In vitro* inhibition of a pathogen by bacteriocin-producing antagonistic strains varied with media composition and did not correspond with antagonism in the grapevine cane-segment bioassay. *In vitro* antagonism was not the only factor involved in prevention of gall formation in the wound assay, as a non-inhibitory strain also prevented gall formation while some inhibitory strains failed to do so. This indicates that plant factors can influence the efficiency of antagonism and that factors other than inhibition by bacteriocins can interfere with the process of crown gall induction. Others have observed similar results (Cooksey & Moore, 1980; Staphorst *et al.*, 1985; and Burr & Reid, 1994). This suggests that *in vitro* antagonism assays need to be performed in conjunction with *in planta* gall formation assays.

CHAPTER SEVEN

GLASSHOUSE TESTING OF THERMOTHERAPY AND BIOLOGICAL CONTROL AGENTS AS A CONTROL STRATEGY FOR GRAPEVINE CROWN GALL

7.1 INTRODUCTION

Crown gall of grapevine is caused by *A. vitis* strains harbouring a tumour-inducing (Ti) plasmid (Knauf *et al.*, 1982). The bacterium, an endophyte, can reside latently in propagating material; consequently, *A. vitis* is widespread in Australia and other vine growing regions of the world (Ophel *et al.*, 1988).

Control strategies have focused on obtaining *A. vitis*-free grapevines using shoot-tip culture and thermotherapy of dormant cuttings (Burr *et al.*, 1987c; Burr *et al.*, 1989). Such methods have proven successful in eliminating *A. vitis* from propagation material but concerns about re-infestation have arisen with evidence that *A. vitis*-free grapevines became recontaminated after sixteen months in infected vineyard soil (Goodman *et al.*, 1993).

Chemical treatments, such as the bacteriocide Gallex™, have certain disadvantages when used to control crown gall: the cost, the need for frequent applications and the potential environmental hazards. Such limitations have driven research for alternative control measures. Successful control of crown gall on stone fruit and rose crops has been achieved using biocontrol agents K84 and K1026 (Jones & Kerr, 1989). However, K84 was unable to inhibit the *A. vitis* strains tested or to prevent crown gall induction

on tomato seedlings by these strains (Kerr & Panagopoulos, 1977). Subsequently, screening for potential biocontrol agents of grapevine crown gall has occurred in a number of laboratories.

There were three main aims in this chapter; firstly, to determine the effectiveness of thermotherapy in reducing systemic *A. vitis* cells in dormant grapevine cuttings. This was tested by planting heat treated cuttings into uninfested soil and growing them for a range of times. The grapevines were then artificially wounded and observed for gall formation, the appearance of which indicated that systemic agrobacteria had survived the thermotherapy. The second experiment was designed to examine gall formation and infestation of heat treated grapevines planted into soil containing *A. vitis* pathogens. A number of antagonistic strains were tested for their capacity to prevent gall formation on these grapevines and to protect the grapevines from systemic colonisation by the soil-borne *A. vitis* pathogens. Finally, various biocontrol screening assays performed in the previous chapter were compared with results from a pot experiment assessing the same biocontrol strains in this chapter.

7.2 EXPERIMENTS

7.2.1 Impact of thermotherapy on gall formation by endogenous *A. vitis*

Most grapevines are propagated vegetatively using dormant canes taken from existing grapevines as new planting or grafting material. However, as many existing grapevines are systemically infested with *A. vitis*, this means of propagation has distributed *A. vitis* cells widely and thus become a major source of potential new infection. In this experiment, the effectiveness of

dipping dormant grapevines into a hot water (50°C) bath for 30 minutes to reduce endogenous *A. vitis* populations was investigated. This was done because it is thought that although thermotherapy reduces the number of *A. vitis* cells beyond detection, it may not completely eradicate *A. vitis* populations. Consequently, *A. vitis* cells surviving thermotherapy could, over time, multiply to levels capable of inducing gall formation. This was measured by assessing gall formation following artificial wounding of potted grapevines previously treated at different times with thermotherapy.

7.2.1.1 Gall formation following artificial wounding of thermotherapy treated cuttings

Grapevine cuttings were treated with thermotherapy (Materials & Methods, section 3.5.1.2) then grown in potting soil that had been steam-treated for 100 minutes. When the heat treated grapevines were artificially wounded (Materials & Methods, section 3.6.2.1) they had been growing in steam-treated soil for various periods (15, 23 and 41 months). The wounds were inoculated with either sterile MSALTS, a pathogen-free treatment or with the pathogen *A. vitis* K377 (Materials & Methods, section 3.6.2.2) as a positive control treatment. Only one type of treatment per grapevine was used, with two wound sites per grapevine.

After an eight week incubation period the wound sites were observed for gall formation. Tumour size was measured using two diameters to calculate the area in mm² (Table 20). When the results were analysed using a chi-squared model, they indicated that grapevines that had the longest period (41 months) between thermotherapy and artificial wounding had a significantly ($p < 0.01$) higher incidence of gall formation (three galls) than those with shorter periods (15 and 23 months) in which no galls formed.

Table 20. Incidence and size of gall formation at artificial wound sites on grapevines that have been previously treated with thermotherapy.

Months since thermotherapy	Inoculation solution ¹	Total No. of wound sites	No. wound sites with galls	Mean gall area (mm ²)
15	<i>A. vitis</i> K377 ²	2	2	42
15	MSALTS ³	12	0	0 ^a
23	<i>A. vitis</i> K377	2	2	58
23	MSALTS	80	0	0 ^a
41	<i>A. vitis</i> K377	2	2	72
41	MSALTS	26	3	1.2 ^b

¹ Inoculation solutions were added to wound sites in 80µl aliquots.

² Cell density of *A. vitis* K377 was 9.01 log CFU/ml.

³ MSALTS is a sterile buffer solution (Appendix C).

^{a-b} Different letters indicate means significantly different from each other (Chi-squared $p < 0.01$).

7.2.2 Protection of thermotherapy treated cuttings from crown gall using antagonistic bacteria

Grapevine cuttings that have been heat treated to reduce the possibility of systemic infestation are still susceptible to crown gall if they are planted into soil infested with *A. vitis* (Goodman *et al.*, 1993). This study investigated the incidence of gall formation and systemic colonisation of heat treated grapevines by a soil-borne, genetically tagged *A. vitis* pathogen. The ability of five antagonistic bacteria and one non-inhibitory *A. vitis* strain to control the disease under these conditions was tested. The experiment explored two aspects of biological control. First, the antagonistic strains were compared with each other and second, the way in which the antagonistic strains were applied was compared.

7.2.2.1 Experimental design

The experiment was a 7x2x2 factorial design: there were five bacterial strains inhibitory to the pathogen, one non-antagonistic strain and a control treatment (TY broth) in the biocontrol treatments; there were two methods of applying the biocontrol treatments, and two methods of applying the genetically tagged pathogen (Materials & Methods, sections 3.6.3.1-3). The potential biocontrol strains were applied to the cuttings either before they were planted into sand (a medium to promote root formation) or one month later to rooted grapevines, just before planting into infested soil. The pathogen was applied either to the soil that the biocontrol treated grapevines were planted into or the biocontrol treated grapevines were dipped into a suspension of the pathogen before being planted into soil. Unless stated otherwise, the results presented were from the grapevines that had been planted into soil containing the pathogen, as this is a better approximation of

natural pathogen levels than is dipping the grapevines into a suspension of the pathogen. There were 28 treatments with eight replicates per treatment, giving a total of 224 pots. The pots were arranged randomly in eight trays with one tray per replicate.

7.2.2.2 *Incidence of gall formation on grapevines treated with antagonistic strains*

Twenty weeks after the grapevines were inoculated the root system of each plant was closely examined. The incidence of gall formation was recorded (Fig. 25). An overall analysis of variance on percentage gall formation indicated that differences between biocontrol inoculum treatments were significant ($p < 0.05$) with no significant differences between the methods of applying the biocontrol strains. Restricted analysis of variance comparing biocontrol treatments to the control treatment found that all strains, even non-inhibitory *A. vitis* K1069, resulted in significantly ($p < 0.05$) less gall formation than the control treatment.

7.2.2.3 *Pathogen numbers in the soil around grapevines treated with antagonistic strains*

Mercury resistant *A. vitis* soil populations were determined by taking a soil sample from the base of the stem where the roots emerge (Materials & Methods, section 3.6.3.4). An analysis of variance on pathogen numbers in the soil indicated no significant interaction ($p = 0.053$) between treatments. As the significance level lay just beyond what is considered significant, ie. $p < 0.05$, a restricted analysis of variance was performed whereby the biocontrol treatments were compared to the control treatment. No biocontrol treatments were significantly different from the control treatment (Fig. 26).

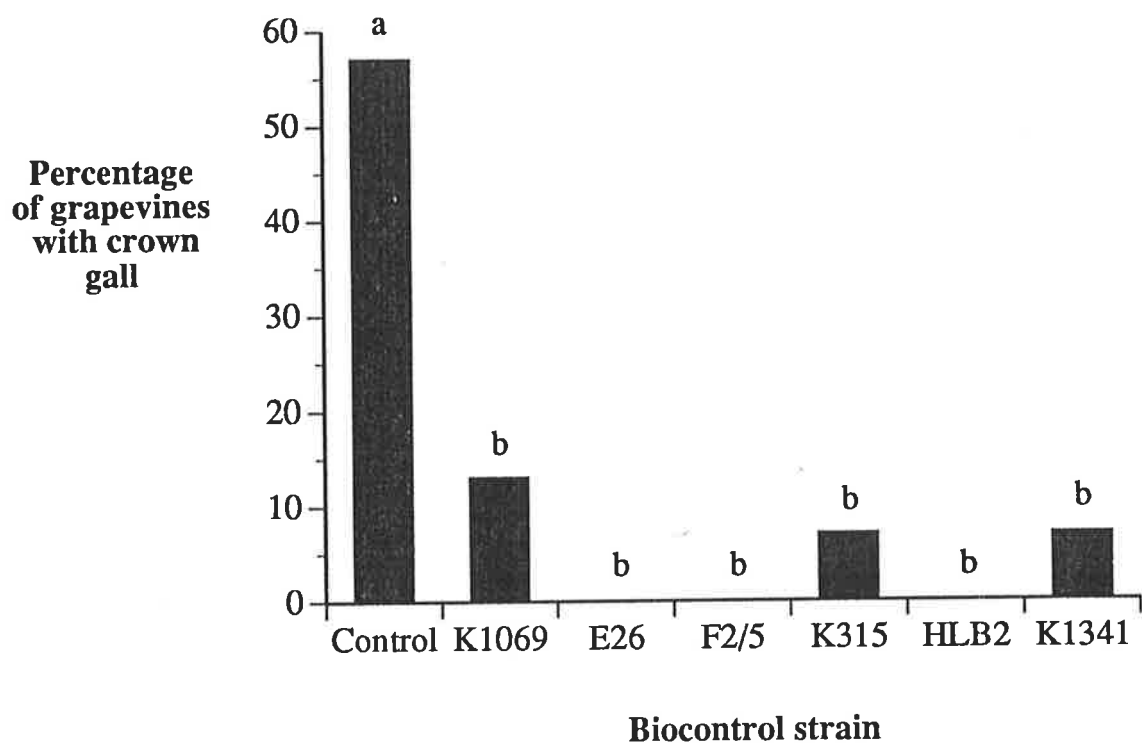


Figure 25. Effect of different biocontrol strains on the percentage of gall formation. Control treatment was TY broth. Different letters indicate means significantly ($p < 0.05$) different from each other. All strains have means significantly lower than the control mean.

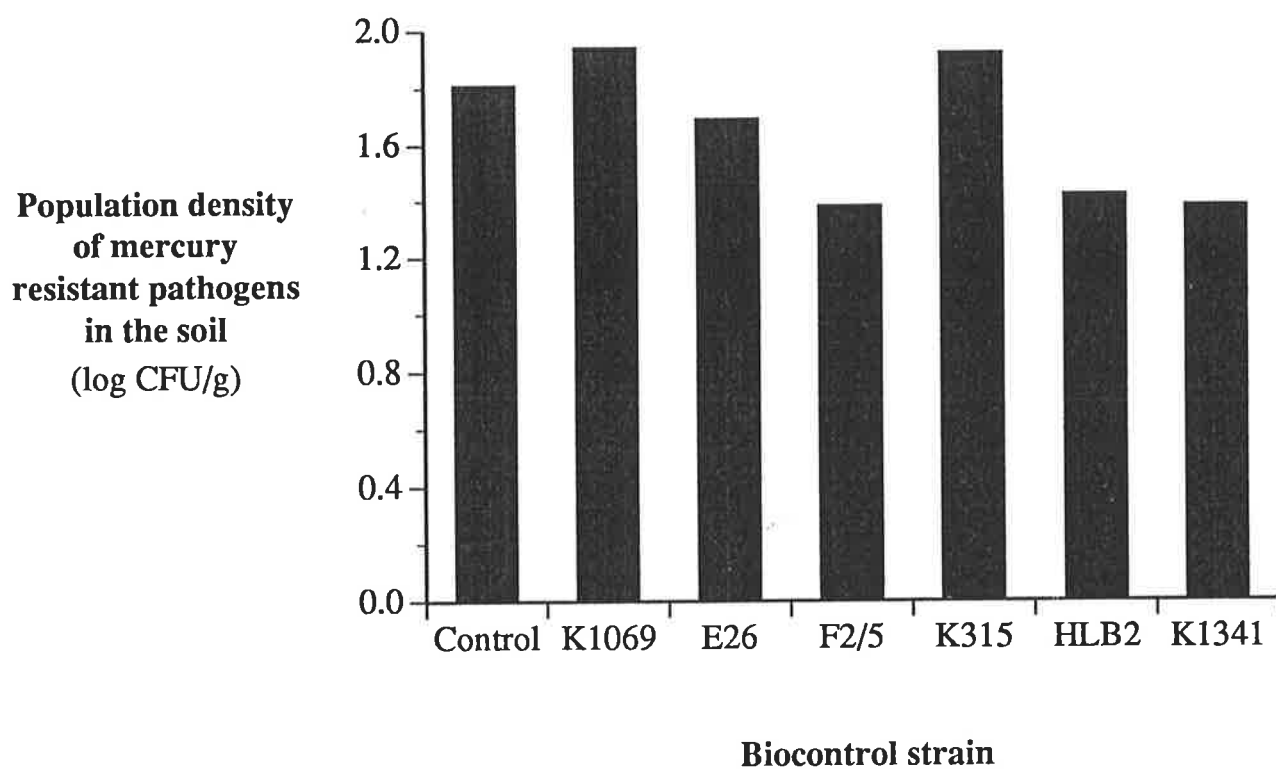


Figure 26. Effect of different biocontrol strains on numbers of the mercury resistant pathogens (*A. vitis* K1345) in the soil. The control treatment was TY broth. There was no significant difference between the means.

7.2.2.4 *Pathogen numbers in the vascular fluid of grapevines treated with antagonistic strains*

Systemic colonisation of grapevines by the mercury resistant pathogen *A. vitis* K1345 was assessed by enumerating viable bacterial cells in vascular fluid collected from grapevine segments (Materials & Methods, section 3.6.3.5). The observed values of pathogen numbers (log CFU/cm) detected in the vascular fluid of stem segments had zeros in the data set which skewed the residual error plots. As a consequence, the data were reclassified as a score. The score referred to the number of observed values in each of four defined categories; 0 (obs=0), 1 (0.01<obs<0.1), 2 (0.1<obs<1.0) and 3 (obs>1.0). A contingency table for strain, method of application and score was then formed by counting the number of values that fell into each class. A log-linear Poisson model was fitted and a regression analysis on the reclassified means indicated that numbers of mercury resistant pathogens varied significantly ($p<0.001$) in the different biocontrol treatments. A restricted analysis of variance comparing each biocontrol treatment with the control treatment found that all biocontrol treatments resulted in significantly lower pathogen numbers except *A. vitis* strain K1069 (Fig. 27). However, it should be noted that there was a significant difference between the method of application for the biocontrol strains *A. vitis* F2/5 and *A. vitis* E26 (Table 21). Both strains resulted in considerably lower pathogen numbers when co-inoculated with the pathogen rather than when pre-inoculated.

7.2.2.5 *Re-analysis of results to include data from grapevines that were dipped into a pathogen suspension before planting*

The mercury resistant pathogen was applied in one of two ways (section 7.2.2.1), either integrated with the soil to emulate natural conditions or the biocontrol-treated grapevines were dipped into a suspension (10^9 cells/ml)

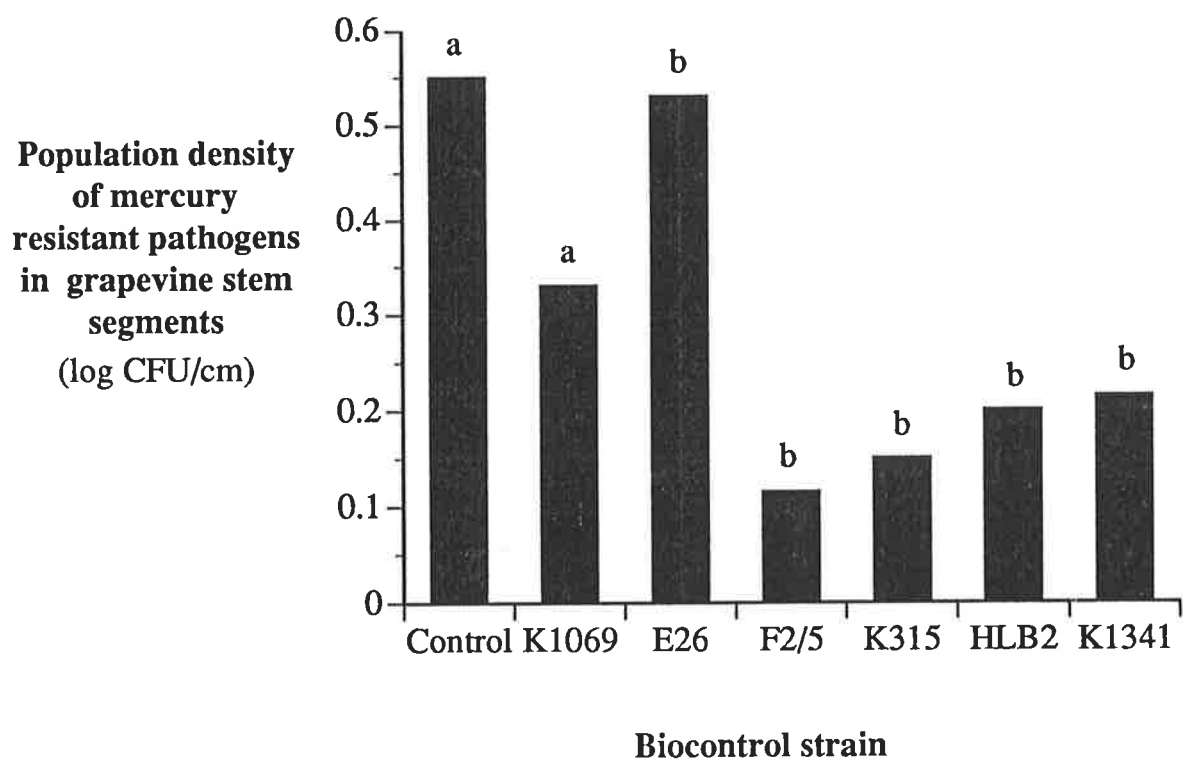


Figure 27. Effect of different biocontrol strains on numbers of mercury resistant pathogens (*A. vitis* K1345) in xylem fluid. The two methods of inoculation have been grouped to give a single mean for each strain. The control treatment was TY broth. Means are significantly different ($p < 0.01$) from each other and letters different from the letter on the control, indicate means significantly different ($p < 0.05$) from the control.

Table 21. Effect of different pathogen application methods on performance of biocontrol strains in restricting numbers of mercury resistant pathogens in grapevine xylem fluid.

Biocontrol strain	Mercury resistant pathogens in vascular fluid of grapevine stem segments ¹			
	Biocontrol inoculated pre-root ²		Biocontrol inoculated post-root ³	
	number ⁴ (log CFU/cm)	incidence ⁵ (%)	number ⁴ (log CFU/cm)	incidence ⁵ (%)
Control	0.25	100	0.85	100
K1069	0.22	100	0.44	100
E26	1.02	88	0.04 [†]	50
F2/5	0.22	100	0.01 [†]	25
K315	0.16	100	0.14	88
HLB2	0.36	75	0.04	33
K1341	0.21	43	0.22	83

Table 21. Continued.

- ¹ The number of viable cells on RS medium (Roy & Sasser, 1983) containing 1 mg/ml of mercuric chloride recovered from vascular washings of grapevine cane-segments.
- ² Biocontrol strain applied to grapevine cuttings four weeks before grapevine planted into infested soil.
- ³ Biocontrol strain applied to grapevine roots ten minutes prior to the grapevine being planted into infested soil.
- ⁴ Number of bacteria (log CFU/cm) detected in vascular fluid samples from three stem sections per vine.
- ⁵ Proportion (%) of vines in which bacteria were detected in the vascular fluid samples.
- [†] Indicates that means were significantly different when antagonistic strain applied before root formation compared with being applied after root formation.

of pathogenic bacteria. The dipping of grapevines into a pathogen suspension was used to indicate biocontrol performance under extreme conditions. Data from grapevines dipped in the pathogen suspension were incorporated with data from grapevines that were planted into soil amended with a pathogen suspension and the pooled data were re-analysed.

An analysis of variance for incidence of gall formation gave the same result as the data from grapevines planted into pathogen infested soil. All biocontrol strains, including the *in vitro* non-antagonist *A. vitis* K1069, resulted in significantly less gall formation than the control treatment (Table 22). The only difference was that only *A. vitis* F2/5 and *A. vitis* E26 prevented gall formation when the positive control data was included in the analysis whereas *A. vitis* F2/5, *A. vitis* E26 and *A. tumefaciens* HLB2 prevented gall formation when the positive control was not included in the analysis.

An analysis of variance performed on the number of mercury resistant pathogens in the soil indicated that there was a significant interaction ($p=0.05$) between antagonistic strains. A restricted analysis of variance comparing the control to other strains found that pathogen numbers were significantly ($p<0.05$) lower than the control when *A. vitis* F2/5 was present (Table 22).

The data for pathogen numbers in the grapevine vascular system were analysed using a Poisson model fitted to data reclassified as a score (section 7.2.2.3). An analysis of variance indicated a significant ($p<0.001$) difference between antagonistic strains but the analysis did not allow comparisons between the strains (Table 22). Treatment with *A. vitis* F2/5 resulted in the lowest number of pathogens in the vascular system.

Table 22. Performance of antagonistic strains when data from grapevines dipped into a pathogen suspension is included with data from grapevines planted into pathogen infested soil.

Biocontrol strain	Incidence of gall formation ¹	Number of mercury resistant pathogens ²	
		Soil ³	Vascular system ⁴
	(%)	(logCFU/g)	(logCFU/cm)
Control	47 ^a	1.84 ^a	0.36
<i>A. vitis</i> K1069	9 ^b	1.86 ^a	0.17
<i>A. vitis</i> E26	0 ^b	1.60 ^a	0.88
<i>A. vitis</i> F2/5	0 ^b	1.23 ^b	0.08
<i>P. fluorescens</i> K315	3 ^b	1.91 ^a	0.09
<i>A. tumefaciens</i> HLB2	6 ^b	1.70 ^a	0.24
<i>A. tumefaciens</i> K1341	6 ^b	1.40 ^a	0.60

Table 22. Continued.

- ¹ Proportion (%) of grapevines that gall formation was observed on.
 - ² The number of viable cells on RS medium (Roy & Sasser, 1983) containing 1 mg/ml of mercuric chloride.
 - ³ Number of bacteria (log CFU/g) detected in washings of a soil sample taken from the base of the grapevine cutting.
 - ⁴ Number of bacteria (log CFU/cm) detected in vascular fluid samples from three stem sections per grapevine. Means were significantly ($p < 0.001$) different.
- ^{a-b} Different letters indicate means significantly different from each other ($p < 0.05$).

7.3 DISCUSSION

Thermotherapy of grapevine cuttings is a cost effective, rapid method of disinfesting infected material (Ophel *et al.*, 1990a). Treatment at 50°C for 30 minutes can reduce *A. vitis* numbers beyond detection but may not eradicate the bacteria (Burr *et al.*, 1989). If viable bacteria remain in the heat treated cuttings it is possible that over time the numbers could increase to a level sufficient to induce gall formation. This possibility was tested by artificially wounding grapevines that had been heat treated before planting. No gall formation occurred in grapevines when the time between thermotherapy and wounding was relatively short, ie. 15 to 23 months, but small galls formed in the grapevines when the period between thermotherapy and wounding was 41 months. The treatment with the longest period between thermotherapy and wounding had the highest incidence of gall formation so it seems possible that some bacteria survived the heat treatment and increased in numbers to a level capable of causing gall formation. This experiment does not preclude the gall formation being caused by bacteria from another source such as from the soil, water or wind. However, the soil was steam treated, the water is heavily chlorinated and the grapevines were grown in glasshouses, so it is unlikely that tumorigenic bacteria originated from these sources.

It is possible that the galls observed in the heat treated grapevines were formed not by *A. vitis* but by oncogenic *A. tumefaciens*. Rarely are tumorigenic *A. tumefaciens* isolated from grapevines (Bien *et al.*, 1990) but there have been exceptions (Thies *et al.*, 1991). This in combination with the fact that *A. tumefaciens* is thermo-tolerant and can survive heat treatments at 50°C for 30 minutes (Burr *et al.*, 1989) means that gall formation due to tumorigenic *A. tumefaciens* cannot be discounted. Regardless of what

species may be causing the gall formation, thermotherapy at 50°C for 30 minutes is not sufficient to prevent gall formation under certain circumstances. In areas where complete eradication of *A. vitis* is preferable, such as cold frost-prone regions, shoot-tip tissue culture (Burr *et al.*, 1987c) would be a better choice than thermotherapy.

Grapevines free of *A. vitis* can become galled and systemically infested when planted into vineyard sites that have contained diseased grapevines (Goodman *et al.*, 1993). A logical progression to prevent heat treated grapevines from infection and re-infestation by soil-borne *A. vitis* is biological control. Five antagonistic bacterial strains were investigated and found to inhibit the pathogen *A. vitis* K377 on Stonier's medium (Chapter 6). The pathogen was marked with Tn501, a transposon conferring mercury resistance, to enable the marked strain to be distinguished from other *A. vitis* cells. The mercury resistant pathogen, *A. vitis* K1345, was used to investigate the effect of antagonistic strains on disease incidence and pathogen populations in heat treated grapevines grown in a glasshouse.

All bacterial strains (even *A. vitis* K1069 which was not antagonistic *in vitro*) reduced the incidence of gall formation to levels significantly lower than the control treatment. The antagonistic strains *A. vitis* E26, *A. vitis* F2/5 and *A. tumefaciens* HLB2 prevented any galls from developing and warrant further testing in field trials. *A. vitis* strain K1069 was not expected to significantly reduce gall formation. This strain showed no inhibitory action towards the tagged pathogen on Stonier's medium so it is interesting that it reduced pathogen numbers to levels significantly less than the control. A possible explanation for this is that K1069 has inhibitory properties against K1345 *in vivo* that were not detected *in vitro*. This unforeseen biocontrol could be due either to the onset of bacteriocin production by K1069 or to the

increased sensitivity of the pathogen to a bacteriocin *in vivo* but this seems unlikely as K1069 was not inhibitory to a wide range of *A. vitis* pathogens (this study). However, evidence that biocontrol can occur when *in vitro* evidence suggests otherwise was presented by Staphorst *et al.* (1985) who found that gall formation was reduced when agrocin-producing non pathogens were co-inoculated with pathogens that were insensitive to an agrocin produced by the non pathogen *in vitro*. Although in both these experiments it cannot be discounted that other mechanisms, unrelated to bacteriocin production, are involved in reducing gall formation. One possibility, alluded to in the previous chapter, is that K1069 can displace the pathogen from infection sites. Lippincott & Lippincott (1969) described reduced gall formation when non pathogens were inoculated onto wound sites before pathogens and proposed lack of access of pathogens to binding sites as the mechanism.

While the incidence of crown gall was significantly reduced from the control level in all biocontrol strain treatments, the number of mercury resistant pathogens in the soil was relatively high and was not significantly different to the control. Consequently, it would appear that prevention of crown gall using antagonistic strains can be achieved when significant pathogen populations are present in the soil. This has important implications for using biocontrol agents to prevent infection of clean vines when planted into pathogen infested soils.

When pathogens are present in the soil it is possible that the grapevines can become systemically infested. Although the population density of pathogens in the vascular system presumably has less relevance to the incidence of gall formation at the crown or roots, it may well influence the incidence of trunk gall (in association with freezing or grafting injury) and transmission of

latently infected propagating material. It was for this reason the effect of biocontrol strains on systemic colonisation of the tagged pathogen was monitored. All biocontrol strains resulted in pathogen numbers significantly lower than the control, except strain *A. vitis* K1069. *A. vitis* strains F2/5 and E26 resulted in significantly fewer pathogens when applied after, rather than before root formation. *A. tumefaciens* HLB2 also resulted in substantially fewer systemic pathogens when applied after root formation rather than before root formation but the values were not significantly different. It is likely that applying biocontrol strains after the grapevine roots have formed gives better coverage and thus better protection.

The only other experimental evidence regarding prevention of reinfestation of grapevines using antagonistic bacteria comes from Pu & Goodman (1993a). They treated indexed Chancellor grapevines with *A. tumefaciens* HLB2 before planting into diseased vineyard soil. HLB2 reduced the incidence of gall formation from 100% to 22% and the incidence of reinfection from 90% to 50%; similarly in this study, gall formation was reduced from 57% to 0% and reinfection from 100% to 54%. The results are comparable, with better protection against gall formation observed in this study. However, this is not unexpected as the experiment of Pu and Goodman (1993a) was conducted in the field where pathogen sensitivity to the bacteriocin of HLB2 was unknown while this study dealt with a pathogen that was known to be sensitive to HLB2. The effectiveness of biocontrol agents to prevent reinfestation of grapevines in the field may depend on whether the predominant pathogen population of a proposed planting site is sensitive to bacteriocins produced by the antagonistic strain(s) to be used for biocontrol.

In the biocontrol pot experiment, there were two methods of applying the transposon-tagged pathogen; the pathogen was either added to the soil or the grapevines were dipped directly into a suspension of the pathogen. The results discussed to this point have been from grapevines where the pathogen was added to the soil. Data from grapevines dipped into a pathogen suspension to simulate a more severe pathogen load were merged with data from grapevines that were planted into pathogen infested soil and re-analysed. When this was done, the incidence of crown gall was reduced by all bacterial strains, with *A. vitis* F2/5 and *A. vitis* E26 preventing any gall formation. In the soil, only *A. vitis* F2/5 significantly reduced pathogen numbers beyond that of the control treatment. There were also significant differences in the ability of antagonistic strains to reduce pathogen numbers in the grapevine vascular system and although the means could not be statistically ranked, *A. vitis* F2/5 resulted in the lowest number of pathogens. So even when the level of pathogenic inoculum was high, where grapevines were dipped into a pathogen suspension, *A. vitis* F2/5 was capable of significantly reducing the level of disease severity.

When the results from the glasshouse experiment were compared with results from *in vitro* antagonism tests, antagonism in the grapevine cane-segment bioassay and an *in planta* wound assay (previous chapter), the assay that best predicted biocontrol of grapevine crown gall was determined. The incidence of gall formation in potted grapevines was not accurately predicted by *in vitro* growth characteristics of the antagonistic strains, as *A. vitis* strains grew uncompetitively *in vitro* but provided amongst the best disease control. Again, the incidence of gall formation in pot trials was not accurately predicted by *in vitro* bacteriocin production, as a non-inhibitory strain was as effective at reducing gall formation on potted grapevines as the inhibitory strains. However, there was good correlation between *in vitro* bacteriocin

production by an antagonist on Stonier's medium and a reduction in the number of *A. vitis* pathogens that infested the grapevine vascular system in the presence of that antagonist. The *in planta* wound assay did not have good correlation with prevention of gall formation in potted grapevines, suggesting that even *in planta* assays cannot take into account all the variables that affect biological control. An assay to detect bacteriocin production on the plant may clarify the relative importance of bacteriocins in biocontrol.

In summary, the biocontrol strains *A. vitis* F2/5 and *A. vitis* E26 prevented gall formation even when grapevines were dipped into a pathogen suspension. *A. vitis* F2/5 significantly decreased the incidence and number of pathogens in the vascular system. These strains need to be tested under field conditions for a full evaluation of their potential. Biocontrol by *A. tumefaciens* K1341, the HLB2 strain with Peh activity, was not significantly different to HLB2. Therefore, Peh activity was not an advantage for preventing gall formation. Reduced re-infestation of the grapevine vascular system was achieved when the biocontrol strains were applied after root development, just before planting into contaminated soil, as opposed to being applied when the unrooted cuttings were first planted into sand. The method of application affected the level of re-infestation; therefore, it may be an important consideration in the biocontrol strategy. For example, if the sand is pathogen-free then the grapevines would only need to be dipped into the biocontrol suspension before planting into soil but if sand was suspected to be contaminated then the grapevines would need to be dipped before planting into the sand and before planting into soil.

CHAPTER EIGHT

GENERAL CONCLUSIONS

Control of plant diseases using biological agents has many advantages; not least, the specific nature of biochemical antagonism towards target organisms. Such specificity produces little environmental and ecological disturbance and usually allows precise control of a particular pathogen. However, the price paid for specificity of individual biocontrol agents is the need for a diverse range of these antagonists to control the broad spectrum of pathogens and diseases present and evolving in the environment. Consequently, rapid and effective screening techniques for biocontrol agents are required to keep pace with evolving pathogen populations. To develop effective screening techniques an understanding of what characteristics define a competent biocontrol agent is beneficial. The focus of this thesis was to gain knowledge about the interactions of *A. vitis* with its host and of *A. vitis* with antagonistic bacteria. The relevance of these interactions to biological control of grapevine crown gall was then investigated.

An aspect of screening for biocontrol agents that deserves consideration but is often overlooked is ecological competence. Poorly competitive biocontrol strains may not colonise in sufficient numbers to effectively antagonise aggressive pathogen populations. This study addressed several facets of screening for biocontrol agents of grapevine crown gall, including; investigation of the *A. vitis* ecological niche, a comparison of biocontrol screening techniques and trials of a number of potential biocontrol strains.

The specificity of *A. vitis* for grapevine hosts and the systemic nature of the association suggest that *A. vitis* has certain adaptations for successful colonisation of grapevines. To test whether the grapevine vascular system was readily colonised by bacteria isolated from different ecological backgrounds a bioassay was developed using grapevine cane segments. The recovery of *A. vitis* K377 cells from the grapevine cane-segment bioassay during a fourteen day incubation was compared with that of *A. rhizogenes* K27 cells. The number of strain K27 cells recovered from the bioassay was significantly lower than strain K377 cells and considerably lower than could be accounted for by the death rate of strain K27 cells in nutrient-free media, indicating that the number of viable strain K27 cells was being actively reduced in the bioassay. The hypothesis was put forward that *A. rhizogenes* K27 lacks certain mechanisms that enable *A. vitis* K377 to more effectively colonise the vascular system of grapevine cane segments.

The nature of the putative mechanisms associated with improved vascular colonisation by *A. vitis* was subsequently investigated. A review of plant processes likely to be associated with bacterial colonisation of grapevine xylem vessels indicated several plant defence mechanisms that could be involved. Experiments were conducted to determine what adaptations *A. vitis* might use to counter conditions when defence mechanisms of the grapevine vascular system are activated.

One defence mechanism in the vascular system of *V. vinifera* grapevines is the production of pectin gels. The hypothesis that the ability to dissolve pectin gels using polygalacturonase enzymes may improve vascular colonisation was tested. An *A. vitis* mutant defective in polygalacturonase production was recovered at lower levels from the grapevine cane-segment bioassay than the same mutant with restored polygalacturonase activity.

Furthermore, when *A. tumefaciens* strain C58 was transformed with a plasmid conferring polygalacturonase activity it was recovered at higher levels than the wild-type strain. However, polygalacturonase activity did not confer an advantage to two other *Agrobacterium* strains tested, suggesting that polygalacturonase activity is only one factor in a multi-component system.

Polygalacturonase activity, such as that produced by *A. vitis* strains, can liberate plant cell wall fragments that elicit plant defence reactions, such as phytoalexin production or the oxidation of phenolic compounds to quinones which are bacteriocidal. Tolerance of *Agrobacterium* strains to the grapevine phytoalexin resveratrol was similar for all strains tested but *A. vitis* strains and *A. tumefaciens* strains isolated from the grapevine vascular system were more tolerant to benzoquinone than other strains. Although only a limited number of strains were tested in the grapevine cane-segment bioassay, a correlation between polygalacturonase production and tolerance to benzoquinone indicated that both traits were necessary for a colonisation advantage to be detected in the bioassay.

Screening assays can be used to save time and reduce costs when screening for biological control agents. Several *in vitro* assays were tested, including a plate assay for bacteriocin production and a pathogen displacement assay in TY broth or Stonier's broth. Pathogen displacement in a grapevine cane-segment bioassay was also tested. None of these assays were very useful for predicting the prevention of gall formation in pot trials, although *in vitro* bacteriocin production by antagonistic strains did correlate with their ability to reduce the level of systemic infection of grapevines from pathogen infested soil. An *in planta* wound assay that investigated gall formation at artificial wound sites did not correlate well with gall formation on potted grapevines. The *in vitro* and *in planta* assays tested in this study were not

capable of accurately predicting biocontrol of potted grapevines.

Two traits, polygalacturonase activity and tolerance to benzoquinone, that were shown to be associated with improved vascular colonisation, were assessed in relation to biocontrol. The addition of a Peh clone to the antagonist *A. tumefaciens* HLB2 detrimentally affected this strains ability to prevent gall formation, although when this strain was pre-inoculated onto grapevine cuttings it resulted in the lowest incidence of vascular re-infestation by a pathogenic *A. vitis* strain. Tolerance to benzoquinone was only exhibited by one antagonist strain, *A. vitis* F2/5, and although this strain had the smallest average zone of inhibition against pathogens on Stonier's medium, it had the best overall performance as a biocontrol agent. Such correlations are interesting but further work is needed to confirm the role these traits play in plant-bacteria interactions and their impact on biocontrol.

A finding from this study with particular practical relevance was that grapevines grown in soil heavily infested with pathogenic *A. vitis* were protected from crown gall when grapevine cuttings were treated with a biocontrol agent before planting. Of the biocontrol strains tested, *A. vitis* F2/5, *A. vitis* E26 and *A. tumefaciens* HLB2 completely prevented gall formation. When grapevines were dipped into a suspension of the pathogen to simulate a heavy pathogen load, only strains F2/5 and E26 resulted in zero gall formation. No strains significantly reduced pathogen numbers in the rhizosphere except when grapevines were dipped into a pathogen suspension before planting. When this was done, strain F2/5 reduced pathogen numbers below that of the control treatment. In the vascular system, the incidence and number of pathogens was most reduced by *A. vitis* F2/5. The timing of inoculation of biocontrols affected the ability of biocontrol agents to reduce systemic infection. Application of *A. vitis* F2/5

and *A. vitis* E26 to grapevine cuttings with roots, as opposed to application to cuttings before root formation, resulted in the lowest number of systemic pathogens. These results are promising and indicate that good disease control is possible in the glasshouse. Testing of these biocontrol strains, particularly *A. vitis* F2/5, under field conditions seems warranted.

APPENDICES

APPENDIX A: Culture media

1. TY medium (Beringer, 1974)

agar	15.00 g/L
CaCl ₂ .2H ₂ O	0.90 g/L
tryptone	5.00 g/L
yeast extract	3.00 g/L

2. LB medium (Miller, 1972)

agar	15.0 g/L
NaCl	10.0 g/L
tryptone	10.0 g/L
yeast extract	5.0 g/L

3. Bergersen's medium (modified from Bergersen, 1961)

agar	15.00 g/L
biotin	1.00 mg/L
CaCl ₂ .2H ₂ O	0.04 g/L
calcium pantothenate	200 µg/L
FeCl ₃	0.02 g/L
glutamic acid	1 mg/L
K ₂ HPO ₄	0.45 g/L
MgSO ₄ .7H ₂ O	0.10 g/L
(NH ₄) ₂ SO ₄	2.00 g/L
nicotinic acid	200 µg/L
thiamine	1.00 mg/L

4. Stonier's medium (modified from Stonier, 1960)

agar	15.00 g/L
biotin	2.0 mg/L
calcium pantothenate	200 µg/L
CaSO ₄	0.10 g/L
Fe(Na ₂ EDTA) ₃	5.0 mg/L
K ₂ HPO ₄	0.88 g/L
MgSO ₄ ·7H ₂ O	0.20 g/L
MnCl ₂	0.1 mg/L
NaH ₂ PO ₄	0.30 g/L
NH ₄ NO ₃	2.70 g/L
NaCl	0.20 g/L
nicotinic acid	200 µg/L
potassium citrate	10.00 g/L
sodium glutamate	2.00 g/L
thiamine	1.00 mg/L
ZnCl ₂	0.10 mg/L

5. Soft buffered agar overlay (modified from Stonier, 1960)

agar	7.00 g/L
NaH ₂ PO ₄	1.22 g/L
Na ₂ HPO ₄	4.37 g/L

6. Polygalacturonase test agar (Reid & Collmer, 1985)

PG buffer (Appendix B)	100 ml
Agarose	1.0 g
Polygalacturonic acid (Nutritional Biochemical Corp.)	0.1 g

7. RS medium, selective for *A. vitis* (Roy & Sasser, 1983)

adonitol	4.00 g/L
agar	15.00 g/L
boric acid	1.00 g/L
K ₂ HPO ₄	0.90 g/L
KH ₂ PO ₄	0.70 g/L
MgSO ₄ ·7H ₂ O	0.20 g/L
NaCl	0.20 g/L
yeast extract	0.14 g/L

autoclave, cool, then add antibiotics (100 µl stock/100 ml media) from filter sterilised stocks;

D-cycloserine	0.02 g/ml (stock)
trimethoprin (dilute acid to dissolve)	0.02 g/ml (stock)
triphenyl tetrazolium chloride	0.08 g/ml (stock)

8. 1A medium, selective for *A. tumefaciens* (Brisbane & Kerr, 1983)

agar	15.00 g/L
erythritol	3.05 g/L
MgSO ₄ ·7H ₂ O	0.25 g/L
K ₂ HPO ₄	1.04 g/L
KH ₂ PO ₄	0.54 g/L
malachite green (0.1 %)	5.00 ml/L
NH ₄ NO ₃	0.16 g/L
sodium taurocholate	0.29 g/L
yeast extract (1.0 %)	1.00 ml/L

autoclave, cool, then add antibiotics (100 µl stock/100 ml media) from filter sterilised stocks;

sodium selenite	1.0 g/100 ml (stock)
-----------------	----------------------

9. 2E medium, selective for *A. rhizogenes* (Brisbane & Kerr, 1983)

agar	15.00 g/L
arabitol (L-)	3.04 g/L
crystal violet (0.1 %)	5.00 ml/L
K ₂ HPO ₄	1.04 g/L
KH ₂ PO ₄	0.54 g/L
MgSO ₄ ·7H ₂ O	0.25 g/L
NH ₄ NO ₃	0.16 g/L
sodium taurocholate	0.29 g/L

autoclave, cool, then add antibiotics (100 µl stock/100 ml media) from filter sterilised stocks;

sodium selenite	1.0 g/100 ml (stock)
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APPENDIX B: Antibiotics and other anti-microbial compounds

Compound ¹	Abbreviation	Solvent	Stock (mg/ml)	Concentration in media	
				<i>Agrobacterium</i> (µg/ml)	<i>E. coli</i> (µg/ml)
Ampicillin	Ap	H ₂ O	100	-	50
Carbenicillin	Cb	H ₂ O	100	500	-
Cycloheximide	Cx	DMSO (25%)	25	125	125
Kanamycin	Km	H ₂ O	50	50	25
Mercuric choride	Hg	H ₂ O	12	6	6
Tetracycline	Tc	EtOH (50%)	10	10	10

¹ All antibiotics were supplied by Sigma Chemical Co.

APPENDIX C: Miscellaneous buffers

1. PG buffer (Reid and Collmer, 1985)

DDW	400ml
EDTA sodium salt	1.49g
KOAc	1.96g
pH (with HCl)	4.5

2. MSALTS (modified from Bergersen, 1961)

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.04 g/L
FeCl_3	0.02 g/L
K_2HPO_4	0.45 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.10 g/L

2. Phosphate buffer (0.02 M)

0.2 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	3.9 ml/L
0.2 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	6.1 ml/L
pH	7.3

3. Tris buffer

Tris (Sigma Chemical Co.)	12.1 g/L
pH (using 1M HCl)	6.0

APPENDIX D: Publications from this thesis.

Kerr A., Biggs J. & Ophel K. (1994). Biological Control of Crown Gall. In: Proceedings of International Symposium on Biological Control of Plant Diseases. (Ed. S.H. Ohh) pp. 11-26. Korean Society of Plant Pathology, Suwon.

Biggs J.G., McClure N.C., Murphy P.J., Ryder M.H. & Kerr A. (1994). Monitoring effects of different potential biocontrol strains on gall formation and population density of a transposon-tagged *Agrobacterium vitis* pathogen. In: Improving Plant Productivity with Rhizosphere Bacteria. (Eds M.H. Ryder, P.M. Stephens & G.D. Bowen) pp. 195-197. CSIRO Division of Soils, Adelaide.

APPENDIX E. Data from figures and tables.

Fig. 8 data												
	Day 0		Day 2		Day 4		Day 7		Day 10		Day 14	
Rep	K377	K27	K377	K27	K377	K27	K377	K27	K377	K27	K377	K27
1	8.360	9.217	8.477	8.318	7.650	8.199	7.863	7.533	7.578	5.485	7.546	5.184
2	8.560	9.092	8.186	8.282	7.898	7.848	7.761	6.331	8.052	6.807	6.883	5.485
3	8.883	8.013	8.376	8.294	7.736	8.071	7.761	7.350	7.734	7.226	7.469	5.184
4	8.184	8.967	8.178	8.379	7.862	8.136	7.982	6.850	8.206	5.485	6.837	5.786
5	8.800	8.712	8.417	8.398	7.579	8.033	8.000	7.080	8.083	5.485	6.980	5.485
6	8.643	8.858	8.254	8.185	7.977	7.900	7.921	6.559	7.859	6.786	7.114	5.184
7	8.861	8.967	8.327	8.341	7.489	8.239	7.758	7.462	7.635	5.485	7.205	5.485
8	8.485	8.858	8.536	8.308	7.989	7.668	7.973	7.834	7.573	5.786	6.813	6.388
9	8.712	9.013	8.455	8.375	8.280	8.041	7.746	7.465	7.898	5.786	6.962	6.087
10	8.560	9.243	8.291	8.307	7.972	7.840	8.040	7.221	7.658	6.932	7.485	5.485
Average	8.605	8.894	8.350	8.319	7.843	7.998	7.881	7.169	7.828	6.126	7.129	5.575

Fig. 9 data

	Time	10 hours	13 hours	16 hours	19 hours	22 hours	25 hours			
Rep	Strain									
1	K27	6.000	6.146	6.724	7.117	7.575	8.000			
2	K27	4.756	5.083	5.699	5.903	6.176	7.000			
3	K27	6.544	6.991	7.431	8.079	8.613	8.934			
4	K27	6.447	6.756	7.029	7.225	8.114	8.724			
5	K27	6.613	6.826	7.004	7.179	7.699	7.954			
Average		6.072	6.360	6.778	7.101	7.635	8.123			
	Time	8 hours	10 hours	12 hours	14 hours	16 hours	18 hours	20 hours	22 hours	24 hours
Rep	Strain									
1	K377	5.863	6.193	6.613	6.949	7.312	7.792	8.134	8.322	8.708
2	K377	5.857	6.196	6.556	6.898	7.320	7.724	8.017	8.316	8.613
3	K377	5.785	6.127	6.556	6.954	7.320	7.716	8.121	8.367	8.699
4	K377	5.663	6.164	6.477	7.017	7.270	7.623	8.013	8.322	8.556
5	K377	5.643	6.196	6.556	6.964	7.283	7.699	8.093	8.274	8.477
6	K377	5.806	6.114	6.613	6.875	7.292	7.580	8.004	8.303	8.633
Average		5.770	6.165	6.562	6.943	7.300	7.689	8.064	8.318	8.614

Fig. 10 data							
	Time	0 days	7 days	14 days	21 days	28 days	35 days
Strain	Rep						
K377	1	9.212	8.633	8.083	7.863	7.505	7.279
K377	2	9.270	8.653	8.104	7.740	7.881	7.505
K377	3	9.100	8.591	7.987	7.633	7.716	7.322
K377	4	9.276	8.602	8.004	7.591	6.653	7.380
K377	5	9.265	8.505	7.978	7.908	7.724	7.431
K377	6	9.288	8.462	8.121	7.580	6.851	7.415
K377	7	9.207	8.462	7.968	7.653	6.785	7.431
K377	8	9.288	8.613	8.161	7.799	7.839	7.322
K377	9	9.230	8.633	7.968	7.663	7.672	7.342
K377	10	9.228	8.556	8.021	7.771	7.799	7.362
Average		9.236	8.571	8.040	7.720	7.443	7.379
K27	1	9.170	8.756	8.182	7.949	7.892	7.813
K27	2	9.179	8.756	8.250	8.072	8.004	7.898
K27	3	9.193	8.785	8.170	8.097	7.944	8.013
K27	4	9.199	8.602	8.299	8.053	7.892	7.982
K27	5	9.196	8.771	8.262	7.968	7.982	7.875
K27	6	9.158	8.799	8.292	8.100	7.959	7.987
K27	7	9.185	8.653	8.352	8.068	7.959	7.792
K27	8	9.210	8.771	8.276	8.049	7.978	7.898
K27	9	9.158	8.699	8.217	8.117	7.944	7.959
K27	10	9.233	8.633	8.161	7.996	8.013	7.968
Average		9.188	8.723	8.246	8.047	7.957	7.919

Fig. 11 data				
	Control		Thermotherapy	
Rep	K377	K27	K377	K27
1	7.953	6.419	8.214	6.991
2	8.001	6.096	8.087	6.444
3	8.047	6.140	8.012	6.636
4	7.712	6.263	7.997	7.322
5	7.813	6.496	7.952	7.301
6	7.667	6.429	8.166	7.228
7	7.748	6.320	8.208	6.845
8	7.829	6.517	8.050	6.712
9	7.963	6.008	8.140	6.217
10	7.888	6.042	8.220	6.805
Average	7.862	6.273	8.104	6.850
Fig. 12 data				
	Node		Internode	
Rep	K377	K27	K377	K27
1	7.953	6.419	8.068	6.972
2	8.001	6.096	7.482	6.314
3	8.047	6.140	8.095	6.518
4	7.712	6.263	7.904	6.335
5	7.813	6.496	7.870	6.490
6	7.667	6.429	7.800	6.411
7	7.748	6.320	7.557	7.077
8	7.829	6.517	7.900	6.916
9	7.963	6.008	7.992	7.251
10	7.888	6.042	7.990	6.569
Average	7.862	6.273	7.866	6.685

Fig. 15 data (CG50)

Rep	Transfer 1			Transfer 2			Transfer 3		
	CG50	CG49	fitness	CG50	CG49	fitness	CG50	CG49	fitness
1	8.978	8.623	-0.354	8.886	9.367	0.481	8.591	9.584	0.993
2	8.978	8.623	-0.354	8.799	9.502	0.703	8.255	9.633	1.378
3	8.978	8.623	-0.354	8.968	9.453	0.485	8.380	9.603	1.223
4	8.978	8.623	-0.354	8.991	9.364	0.372	8.176	9.621	1.445
5	8.978	8.623	-0.354	8.934	9.398	0.463	8.230	9.594	1.364
6	8.978	8.623	-0.354	8.708	9.436	0.729	8.000	9.554	1.554
Average	8.978	8.623	-0.354	8.881	9.420	0.539	8.272	9.598	1.326
Rep	Transfer 4			Transfer 5			Transfer 6		
	CG50	CG49	fitness	CG50	CG49	fitness	CG50	CG49	fitness
1	7.778	9.801	2.023	6.845	9.688	2.843	6.699	9.404	2.705
2	7.602	9.694	2.092	6.699	9.673	2.975	6.301	9.595	3.294
3	7.477	9.790	2.313	6.301	9.677	3.376	6.477	9.563	3.086
4	7.778	9.739	1.961	7.041	9.614	2.572	6.000	9.544	3.544
5	7.477	9.775	2.297	6.602	9.582	2.980	6.301	9.554	3.253
6	7.477	9.758	2.281	6.477	9.651	3.174	6.602	9.494	2.892
Average	7.598	9.759	2.161	6.661	9.648	2.987	6.397	9.526	3.129

Fig. 15 data (K1336)

Fig. 15 data (K1336)									
	Transfer 1			Transfer 2			Transfer 3		
Rep	K1336	CG49	fitness	K1336	CG49	fitness	K1336	CG49	fitness
1	8.982	8.690	-0.292	8.643	9.576	0.933	7.845	9.601	1.756
2	8.982	8.690	-0.292	8.813	9.501	0.688	8.079	9.620	1.541
3	8.982	8.690	-0.292	8.785	9.505	0.720	7.845	9.537	1.691
4	8.982	8.690	-0.292	8.886	9.498	0.612	8.000	9.575	1.575
5	8.982	8.690	-0.292	8.740	9.501	0.761	7.301	9.594	2.293
6	8.982	8.690	-0.292	8.892	9.401	0.509	8.146	9.585	1.439
Average	8.982	8.690	-0.292	8.793	9.497	0.704	7.869	9.585	1.716
	Transfer 4			Transfer 5			Transfer 6		
Rep	K1336	CG49	fitness	K1336	CG49	fitness	K1336	CG49	fitness
1	7.000	9.754	2.754	6.000	9.600	3.600	4.582	5.983	1.401
2	7.000	9.741	2.741	6.699	9.488	2.789	3.766	6.698	2.932
3	7.000	9.698	2.698	6.602	9.551	2.949	3.929	6.601	2.672
4	7.301	9.768	2.467	6.000	9.568	3.568	4.549	5.984	1.435
5	7.000	9.786	2.786	6.477	9.602	3.125	4.107	6.475	2.368
6	7.477	9.760	2.283	6.000	9.477	3.477	4.454	5.987	1.534
Average	7.130	9.751	2.621	6.296	9.548	3.251	4.231	6.288	2.057

Fig. 15 data (K1312)

Transfer 1			Transfer 2			Transfer 3			
Rep	K1312	K27	fitness	K1312	K27	fitness	K1312	K27	fitness
1	9.100	8.949	-0.151	8.987	9.375	0.388	9.025	9.471	0.446
2	9.100	8.949	-0.151	8.869	9.430	0.561	9.045	9.484	0.439
3	9.100	8.949	-0.151	9.021	9.438	0.417	9.076	9.431	0.356
4	9.100	8.949	-0.151	8.898	9.455	0.557	8.973	9.483	0.510
5	9.100	8.949	-0.151	8.959	9.340	0.381	9.064	9.394	0.330
6	9.100	8.949	-0.151	8.919	9.375	0.456	8.944	9.354	0.410
Average	9.100	8.949	-0.151	8.942	9.402	0.460	9.021	9.436	0.415
Transfer 4			Transfer 5			Transfer 6			
Rep	K1312	K27	fitness	K1312	K27	fitness	K1312	K27	fitness
1	8.857	9.369	0.512	8.833	9.358	0.525	8.613	9.452	0.839
2	8.806	9.236	0.429	8.771	9.303	0.532	8.602	9.435	0.833
3	8.914	9.330	0.417	8.690	9.320	0.630	8.681	9.462	0.781
4	8.898	9.348	0.451	8.681	9.377	0.695	8.633	9.223	0.589
5	8.820	9.292	0.473	8.792	9.204	0.412	8.398	9.286	0.888
6	8.886	9.294	0.408	8.663	9.342	0.680	8.531	9.418	0.887
Average	8.863	9.312	0.448	8.738	9.317	0.579	8.576	9.379	0.803

Fig. 15 (K1329)

Fig. 15 (K1329)									
Transfer 1			Transfer 2			Transfer 3			
Rep	K1329	C58	fitness	K1329	C58	fitness	K1329	C58	fitness
1	9.021	9.228	0.207	8.799	9.369	0.570	8.633	9.525	0.892
2	9.021	9.228	0.207	8.851	9.417	0.565	8.591	9.556	0.965
3	9.021	9.228	0.207	8.756	9.394	0.639	8.681	9.562	0.881
4	9.021	9.228	0.207	8.771	9.389	0.618	8.724	9.602	0.878
5	9.021	9.228	0.207	8.919	9.422	0.503	8.740	9.524	0.783
6	9.021	9.228	0.207	8.875	9.403	0.528	8.681	9.576	0.895
Average	9.021	9.228	0.207	8.829	9.399	0.570	8.675	9.558	0.882
Transfer 4			Transfer 5			Transfer 6			
Rep	K1329	C58	fitness	K1329	C58	fitness	K1329	C58	fitness
1	8.415	9.615	1.200	8.373	9.604	1.231	8.270	9.578	1.308
2	8.431	9.628	1.197	8.461	9.650	1.190	8.303	9.611	1.307
3	8.568	9.649	1.081	8.479	9.653	1.175	8.350	9.632	1.282
4	8.643	9.626	0.983	8.436	9.629	1.193	8.375	9.653	1.278
5	8.602	9.537	0.934	8.405	9.579	1.174	8.340	9.624	1.284
6	8.531	9.612	1.080	8.365	9.617	1.251	8.246	9.562	1.316
Average	8.532	9.611	1.079	8.420	9.622	1.202	8.314	9.610	1.296

Fig. 15 data (K1341)

	Transfer 1			Transfer 2			Transfer 3		
Rep	K1341	HLB2	fitness	K1341	HLB2	fitness	K1341	HLB2	fitness
1	8.991	9.130	0.139	8.748	9.396	0.648	8.663	9.425	0.762
2	8.991	9.130	0.139	8.799	9.398	0.599	8.681	9.417	0.735
3	8.991	9.130	0.139	8.724	9.373	0.649	8.699	9.597	0.898
4	8.991	9.130	0.139	8.799	9.386	0.586	8.792	9.624	0.832
5	8.991	9.130	0.139	8.740	9.394	0.654	8.708	9.430	0.722
6	8.991	9.130	0.139	9.041	9.292	0.251	8.740	9.387	0.647
Average	8.991	9.130	0.139	8.809	9.373	0.564	8.714	9.480	0.766
	Transfer 4			Transfer 5			Transfer 6		
Rep	K1341	HLB2	fitness	K1341	HLB2	fitness	K1341	HLB2	fitness
1	8.544	9.615	1.071	8.427	9.561	1.135	8.612	9.479	0.867
2	8.591	9.675	1.084	8.562	9.530	0.967	8.193	9.200	1.007
3	8.491	9.512	1.021	8.428	9.503	1.075	8.625	9.623	0.998
4	8.623	9.597	0.973	8.350	9.437	1.087	7.908	9.401	1.493
5	8.447	9.513	1.066	8.389	9.451	1.062	8.633	9.685	1.049
6	8.556	9.560	1.004	8.474	9.419	0.944	8.470	9.564	1.094
Average	8.542	9.579	1.036	8.438	9.483	1.045	8.407	9.492	1.085

Fig. 17 data							
Rep	Strain	10 hours	13 hours	16 hours	19 hours	21 hours	24 hours
1	K1069	4.681	5.061	5.301	5.602	6.255	7.301
2	K1069	5.560	6.176	6.580	6.908	7.391	8.431
3	K1069	5.009	5.477	5.903	6.447	6.851	7.699
4	K1069	4.903	5.090	5.699	6.041	6.398	7.477
5	K1069	5.292	5.845	6.230	6.591	6.991	7.778
Average		5.089	5.530	5.943	6.318	6.777	7.737
1	E26	5.477	6.079	6.778	7.318	7.699	8.362
2	E26	5.778	6.362	6.716	7.134	7.954	8.431
3	E26	5.845	6.380	6.763	7.364	7.954	8.398
4	E26	6.079	6.462	6.898	7.462	8.230	8.763
5	E26	5.699	6.322	6.820	7.439	8.176	8.633
Average		5.776	6.321	6.795	7.343	8.003	8.518
1	F2/5	4.000	4.462	4.959	5.477	5.954	6.462
2	F2/5	4.301	4.556	4.914	5.477	6.041	6.633
3	F2/5	4.447	4.613	4.978	5.301	5.778	6.301
4	F2/5	4.176	4.505	4.851	5.000	5.602	6.041
5	F2/5	4.398	4.785	5.143	5.602	6.000	6.613
Average		4.264	4.584	4.969	5.371	5.875	6.410
1	K315	5.477	6.041	7.217	8.255	8.949	
2	K315	5.778	6.041	7.199	8.279	9.064	
3	K315	4.851	5.602	6.556	7.845	9.064	
4	K315	4.431	5.301	6.041	7.301	8.279	
5	K315	4.653	5.477	6.301	7.301	8.556	
Average		5.038	5.693	6.663	7.796	8.783	
1	HLB2	6.255	6.699	7.461	8.255	8.820	
2	HLB2	6.230	6.653	7.342	8.230	8.740	
3	HLB2	6.000	6.477	7.207	8.176	8.672	
4	HLB2	6.079	6.544	7.250	8.230	8.708	
5	HLB2	6.176	6.708	7.476	8.398	8.826	
Average		6.148	6.616	7.347	8.258	8.753	

Fig. 18 data							
Strain	Rep	0 days	7 days	14 days	21 days	28 days	35 days
K1069	1	9.182	8.833	7.000	6.699	6.462	6.114
K1069	2	9.217	8.934	7.000	6.672	6.362	5.845
K1069	3	9.193	8.954	7.301	6.785	6.462	6.279
K1069	4	9.210	8.813	7.301	6.763	6.301	6.362
K1069	5	9.196	8.681	7.301	6.716	6.398	5.954
K1069	6	9.137	8.763	7.000	6.806	6.279	6.176
Average		9.189	8.830	7.151	6.740	6.377	6.122
E26	1	9.086	9.045	8.176	7.336	6.716	6.613
E26	2	9.182	9.053	8.114	7.400	6.898	6.544
E26	3	9.124	8.934	7.954	7.328	6.633	6.653
E26	4	9.130	8.968	8.176	7.299	6.690	6.462
E26	5	9.114	9.009	8.079	7.354	6.591	6.398
E26	6	9.170	9.049	7.903	7.364	6.826	6.491
Average		9.134	9.010	8.067	7.347	6.726	6.527
F2/5	1	9.064	8.544	7.903	7.079	6.398	6.079
F2/5	2	9.053	8.544	7.903	7.190	6.580	6.204
F2/5	3	9.068	8.380	7.845	7.143	6.322	6.505
F2/5	4	9.000	8.568	7.903	7.083	6.380	6.398
F2/5	5	9.053	8.447	7.602	7.167	6.505	6.279
F2/5	6	9.049	8.447	7.903	7.076	6.279	6.146
Average		9.048	8.488	7.843	7.123	6.411	6.269
K315	1	9.146	9.072	9.204	9.158	9.033	8.785
K315	2	9.215	9.185	9.167	9.158	9.013	8.724
K315	3	9.134	9.155	9.272	9.155	9.029	8.869
K315	4	9.233	9.164	9.161	9.107	8.949	8.875
K315	5	9.152	9.233	9.220	9.134	9.057	8.919
K315	6	9.199	9.167	9.236	9.124	8.851	8.690
Average		9.180	9.163	9.210	9.139	8.989	8.811
HLB2	1	9.310	9.068	9.045	8.653	8.716	8.041
HLB2	2	9.204	9.117	9.029	8.898	8.613	8.568
HLB2	3	9.255	9.124	9.037	8.785	8.519	8.362
HLB2	4	9.170	9.097	9.072	8.740	8.591	8.415
HLB2	5	9.204	9.045	9.061	8.663	8.415	8.230
HLB2	6	9.185	9.090	8.964	8.845	8.279	8.255
Average		9.221	9.090	9.035	8.764	8.522	8.312
K1341	1	9.358	9.017	8.991	8.708	8.362	8.544
K1341	2	9.301	9.149	9.041	8.857	8.568	8.362
K1341	3	9.330	9.033	8.934	8.699	8.519	8.279
K1341	4	9.288	9.021	8.875	8.806	8.462	8.146
K1341	5	9.299	9.130	8.886	8.833	8.431	8.462
K1341	6	9.292	9.072	8.949	8.792	8.477	8.041
Average		9.311	9.071	8.946	8.782	8.470	8.306

Fig. 19 data

Fig. 19 data												
Transfer 1				Transfer 2			Transfer 3			Transfer 4		
Rep	K1345	K377	fitness	K1345	K377	fitness	K1345	K377	fitness	K1345	K377	fitness
1	9.086	9.029	-0.057	9.100	9.182	0.081	8.991	9.260	0.269	9.004	9.375	0.370
2	9.086	9.029	-0.057	9.253	9.107	-0.146	9.137	9.104	-0.033	8.924	9.365	0.441
3	9.086	9.029	-0.057	9.283	9.100	-0.183	9.104	9.228	0.124	8.826	9.250	0.424
4	9.086	9.029	-0.057	9.161	9.179	0.018	9.013	9.314	0.301	8.778	9.348	0.570
5	9.086	9.029	-0.057	9.190	9.176	-0.014	9.173	9.134	-0.040	8.987	9.401	0.415
6	9.086	9.029	-0.057	9.100	9.093	-0.007	9.258	9.230	-0.027	8.740	9.283	0.543
Average	9.086	9.029	-0.057	9.181	9.140	-0.042	9.113	9.212	0.099	8.877	9.337	0.461
Transfer 5				Transfer 6			Transfer 7			Transfer 8		
Rep	K1345	K377	fitness	K1345	K377	fitness	K1345	K377	fitness	K1345	K377	fitness
1	8.643	9.258	0.614	8.493	9.382	0.889	8.338	9.373	1.035	8.179	9.338	1.159
2	8.633	9.344	0.711	8.591	9.464	0.873	8.253	9.405	1.152	8.324	9.245	0.921
3	8.653	9.415	0.762	8.441	9.366	0.925	8.276	9.267	0.991	8.246	9.345	1.100
4	8.690	9.423	0.733	8.365	9.378	1.013	8.334	9.351	1.017	8.185	9.340	1.155
5	8.623	9.360	0.737	8.540	9.360	0.820	8.272	9.337	1.065	8.299	9.281	0.982
6	8.708	9.548	0.840	8.384	9.387	1.003	8.338	9.318	0.980	8.316	9.319	1.003
Average	8.659	9.391	0.733	8.469	9.390	0.921	8.302	9.342	1.040	8.258	9.311	1.053

Fig. 20 data							
Rep	Control	K1069	E26	F2/5	K315	HLB2	K1341
1	8.398	8.556	8.491	8.146	6.944	7.602	8.000
2	8.568	8.591	8.653	8.380	6.792	7.903	7.903
3	8.556	8.462	8.708	8.041	6.857	7.477	7.845
4	8.447	8.633	8.681	8.279	7.013	7.954	7.699
Average	8.492	8.561	8.633	8.212	6.902	7.734	7.862
Fig. 21 data							
Rep	Control	K1069	E26	F2/5	K315	HLB2	K1341
1	8.898	8.903	5.513	6.653	6.079	6.699	7.137
2	7.778	7.602	6.431	6.322	6.415	7.303	7.000
3	7.000	7.477	5.569	6.771	6.813	6.949	6.959
4	8.623	8.544	5.352	6.398	6.477	7.155	7.243
Average	8.075	8.132	5.717	6.536	6.446	7.027	7.085

Fig. 23/24 data

[illegible]

Fig. 23/24 data (Continued)

Antagonist	Inoculation	Tumour length (mm)	Tumour diameter (mm)	Tumour area	Antagonist	Inoculation	Tumour length (mm)	Tumour diameter (mm)	Tumour area
F2/5	pre	0	0	0.0					
F2/5	pre	0	0	0.0					
F2/5	pre	0	0	0.0					
F2/5	pre	0	0	0.0					
K315	pre	4	4	50.3	K315	co	0	0	0.0
K315	pre	6	5	95.8	K315	co	0	0	0.0
K315	pre	2	2	12.6	K315	co	3	1	15.7
K315	pre	3	2	20.4	K315	co	0	0	0.0
K315	pre	12	8	326.7	K315	co	0	0	0.0
K315	pre	4	5	64.4	K315	co	3	3	28.3
K1051	pre	0	0	0.0	K1051	co	2	1	7.9
K1051	pre	5	3	53.4	K1051	co	0	0	0.0
K1051	pre	0	0	0.0	K1051	co	2	2	12.6
K1051	pre	1	1	3.1	K1051	co	0	0	0.0
K1051	pre	0	0	0.0	K1051	co	1	1	3.1
K1051	pre	0	0	0.0	K1051	co	1	1	3.1
K1051	pre	1	1	3.1					
K1051	pre	0	0	0.0					
K1051	pre	2	1	7.9					
K1051	pre	0	0	0.0					
K1051	pre	1	1	3.1					
K1341	pre	10	5	196.3	K1341	co	6	2	62.8
K1341	pre	1	1	3.1	K1341	co	7	5	116.2
K1341	pre	0	0	0.0	K1341	co	4	2	31.4

Fig. 23/24 data (Continued)

Antagonist	Inoculation	Tumour length (mm)	Tumour diameter (mm)	Tumour area	Antagonist	Inoculation	Tumour length (mm)	Tumour diameter (mm)	Tumour area
K1341	pre	0	0	0.0	K1341	co	0	0	0.0
K1341	pre	0	0	0.0	K1341	co	3	2	20.4
K1341	pre	4	3	39.3	K1341	co	5	3	53.4
K1341	pre	1	1	3.1					
K1341	pre	0	0	0.0					
K1341	pre	0	0	0.0					
K1341	pre	9	8	227.8					
K1341	pre	10	8	257.6					
K1341	pre	7	6	133.5					

Table 4 data			
Strain	Rep	TY media	RS media
K377	1	57	20
K377	2	51	24
K377	3	51	28
K377	4	41	26
K377	5	43	29
Average		48.6	25.4
% recovery		52.3	
Strain	Rep	TY media	2E media
K27	1	44	49
K27	2	48	45
K27	3	51	59
K27	4	60	41
K27	5	46	47
Average		49.8	48.2
% recovery		96.8	
Table 5 data			
	Control	Thermotherapy	
rep	<i>A. vitis</i>	<i>A. vitis</i>	
1	4.623	0	
2	5.477	0	
3	4.756	0	
4	6.255	0	
5	6.342	0	
6	5.778	0	
7	5.000	0	
8	6.531	0	
9	6.000	0	
10	5.903	0	
Average	5.667	0	

Table 6 data				
	Control		Co-inoculated	
Rep	K377	K27	K377	K27
1	7.728	6.636	7.560	6.268
2	7.728	6.720	7.696	6.268
3	7.235	6.967	7.696	6.054
4	7.915	6.910	7.786	6.737
5	7.485	6.720	7.997	6.604
6	7.603	6.753	7.962	6.557
7	7.800	6.626	7.696	6.189
8	7.582	6.839	7.813	6.444
9	7.360	6.189	7.924	6.335
10	7.825	6.784	7.395	6.268
Average	7.626	6.714	7.753	6.372
Table 7 data				
Rep	Uninoculated	Miss377	Miss27	
1	0	7.515	5.694	
2	0	7.108	5.355	
3	0	6.847	5.314	
4	0	6.415	4.615	
5	0	7.035	5.593	
6	0	5.661	5.393	
7	0	5.962	5.694	
8	0	6.564	5.845	
9	0	6.615	5.490	
10	0	5.661	5.729	
Average	0	6.538	5.472	

Table 8 data

Strain	Rep	Control	Catechol	Chlorogenic acid	Resveratrol	Benzoquinone2	Benzoquinone23	Benzoquinone5	Benzoquinone25	quinone/ascorbi
K377	1	6.839	7.137	7.190	7.320	6.519	1.903	2.415	0.000	7.093
K377	2	7.061	7.176	7.217	7.190	5.477	1.301	2.079	0.000	7.086
K377	3	6.903	7.152	7.267	7.185	4.806	3.500	1.602	0.000	6.204
K377	4	6.898	7.130	7.238	7.276	5.954	2.447	2.230	0.000	6.949
K377	5	7.021	7.143	7.250	7.215	6.204	2.079	2.322	0.000	6.756
Average		6.944	7.148	7.233	7.237	5.792	2.246	2.130	0.000	6.818
Strain	Rep									
C58	1	7.401	7.511	7.371	7.262	7.500	1.602	0.000	0.000	7.526
C58	2	7.344	7.547	7.433	7.310	6.708	0.000	0.000	0.000	7.455
C58	3	7.294	7.507	7.371	7.458	7.398	0.000	0.000	0.000	7.533
C58	4	7.367	7.465	7.391	7.288	7.072	0.000	0.000	0.000	7.412
C58	5	7.336	7.538	7.428	7.474	7.241	0.000	0.000	0.000	7.502
Average		7.349	7.513	7.399	7.358	7.184	0.320	0.000	0.000	7.486
Strain	Rep									
K27	1	7.272	7.079	7.188	7.104	7.307	0.000	0.000	0.000	7.528
K27	2	7.375	6.973	7.193	7.248	7.286	1.301	1.000	0.000	7.461
K27	3	7.307	7.382	7.246	7.155	7.476	0.000	0.000	0.000	7.407
K27	4	7.238	7.253	7.272	7.233	7.330	1.000	1.301	0.000	7.436
K27	5	7.354	7.316	7.173	7.377	7.375	0.000	0.000	0.000	7.391
Average		7.309	7.201	7.214	7.223	7.355	0.460	0.460	0.000	7.444

Table 9 data							
Species	Strain	Control	rep 1	rep 2	rep 3	rep 4	Average
<i>A. vitis</i>	Ag57	6.93	1.000	2.176	1.845	2.079	1.775
<i>A. vitis</i>	CG49	6.83	1.602	3.173	2.176	2.447	2.350
<i>A. vitis</i>	E26	7.39	1.602	3.173	2.176	2.447	2.350
<i>A. vitis</i>	F2/5	6.77	1.477	1.000	0.000	1.301	0.945
<i>A. vitis</i>	K306	6.98	0.000	1.000	0.000	1.301	0.575
<i>A. vitis</i>	K374	6.9	0.000	0.000	0.000	0.000	0.000
<i>A. vitis</i>	K375	6.94	1.477	1.903	1.000	1.602	1.496
<i>A. vitis</i>	K376	6.83	1.301	1.699	1.301	0.000	1.075
<i>A. vitis</i>	K377	6.92	1.301	1.699	0.000	1.000	1.000
<i>A. vitis</i>	K1069	7.03	0.000	0.000	0.000	0.000	0.000
<i>A. vitis</i>	K1070	6.74	0.000	1.954	1.699	1.778	1.358
<i>A. vitis</i>	K1072	6.86	1.301	1.845	1.845	1.602	1.648
<i>A. vitis</i>	#673	6.92	0.000	0.000	1.477	0.000	0.369
<i>A. vitis</i>	#675	7.05	0.000	0.000	0.000	0.000	0.000
<i>A. vitis</i>	#676	6.85	0.000	1.000	0.000	0.000	0.250
<i>A. vitis</i>	#678	6.83	1.477	0.000	1.301	1.699	1.119
<i>A. vitis</i>	#680	6.99	2.431	2.672	3.276	2.580	2.740
<i>A. tumefaciens</i>	C58	6.94	1.000	1.000	1.602	0.000	0.901
<i>A. tumefaciens</i>	HLB2	7.1	0.000	0.000	0.000	0.000	0.000
<i>A. tumefaciens</i>	K42	6.97	0.000	0.000	0.000	0.000	0.000
<i>A. tumefaciens</i>	K122	6.97	0.000	0.000	0.000	0.000	0.000
<i>A. tumefaciens</i>	K1073	7.12	3.272	2.929	2.826	2.732	2.940
<i>A. tumefaciens</i>	K1074	7.27	1.301	1.778	2.041	2.431	1.888
<i>A. tumefaciens</i>	K1075	7.44	2.929	3.193	3.444	2.934	3.125
<i>A. rhizogenes</i>	CPBTR10	6.93	0.000	0.000	0.000	0.000	0.000
<i>A. rhizogenes</i>	K27	6.95	0.000	0.000	0.000	0.000	0.000
<i>A. rhizogenes</i>	K118	6.88	1.000	2.114	1.954	1.301	1.592

Table 10 data								
	Ascorbic acid (0)		Ascorbic acid (0.01)		Ascorbic acid (0.1)			
Rep	K377	K27	K377	K27	K377	K27		
1	7.743	6.335	7.485	6.569	7.395	6.375		
2	7.395	6.490	7.679	6.694	7.934	6.819		
3	7.457	6.074	7.582	6.544	7.837	6.243		
4	7.536	6.544	7.661	6.355	7.661	6.676		
5	7.758	6.460	7.837	6.544	7.582	5.977		
6	7.235	6.103	7.582	6.544	6.696	6.636		
7	8.126	5.518	7.623	6.217	7.184	5.986		
8	7.322	6.017	6.758	6.475	7.360	6.444		
9	7.603	6.444	7.511	6.296	7.184	6.694		
10	7.059	5.504	7.511	6.228	7.126	6.504		
Average	7.524	6.149	7.523	6.447	7.396	6.435		
Table 12 data								
Strain	CG49		CG50		K1336		K27	
Media	TY	RS	TY	RS Km	TY	RS TcKm	TY	2E
Rep								
1	310	117	320	111	360	93	44	49
2	390	97	460	112	330	71	48	45
3	440	103	410	93	320	79	51	59
4	260	114	430	95	410	85	60	41
5	350	108	370	104	390	87	46	47
Average	350	107.8	398	103	362	83	49.8	48.2
% recov	30.8		25.9		22.9		96.8	
Strain	K1312		C58		K1329			
Media	TY	2E Tc	TY	1A	TY	1A Tc		
Rep								
1	39	44	49	41	39	35		
2	33	32	58	57	53	45		
3	45	35	62	43	42	42		
4	38	37	51	47	46	44		
5	41	33	54	60	55	32		
Average	39.2	36.2	54.8	49.6	47	39.6		
% recov	92.3		90.5		84.3			

Table 13 data									
	<i>A. vitis</i>	<i>A. vitis</i>	<i>A. vitis</i>	<i>A. rhizogenes</i>	<i>A. rhizogenes</i>	<i>A. tumefaciens</i>	<i>A. tumefaciens</i>	<i>A. tumefaciens</i>	<i>A. tumefaciens</i>
Rep	CG49	CG50	K1336	K27	K1312	C58	K1329	HLB2	K1341
1	8.186	7.147	8.235	6.636	6.624	5.041	6.252	6.598	5.377
2	7.920	5.892	7.643	6.615	6.953	5.944	5.854	6.272	6.398
3	8.118	6.591	7.643	6.569	6.496	5.643	5.775	6.439	5.854
4	8.195	6.591	8.005	6.876	6.334	5.643	6.437	5.820	6.280
5	8.290	6.737	7.685	6.798	6.539	5.342	5.775	6.384	6.377
6	7.651	6.494	7.790	6.962	6.356	5.820	6.189	6.504	5.377
7	8.024	5.591	7.986	6.531	6.696	5.041	6.473	6.805	7.076
8	7.982	6.193	7.489	6.864	6.840	5.041	6.117	6.874	5.775
9	8.177	6.068	7.685	6.812	7.094	5.041	6.030	5.996	7.015
10	8.269	6.369	7.757	6.870	6.812	5.886	6.030	6.893	5.678
Average	8.081	6.367	7.792	6.753	6.674	5.445	6.093	6.458	6.120
Table 16 data									
Species	Strain	Control	rep 1	rep 2	rep 3	rep 4	Average		
<i>A. vitis</i>	K1069	7.03	0.000	0.000	0.000	0.000	0		
<i>A. vitis</i>	E26	7.39	0.000	0.000	0.000	0.000	0		
<i>A. vitis</i>	F2/5	6.77	1.477	1.000	0.000	1.301	0.94		
<i>A. tumefaciens</i>	HLB2	7.1	0.000	0.000	0.000	0.000	0		
<i>A. tumefaciens</i>	HLB2	7.15	0.000	0.000	0.000	0.000	0		
<i>P. fluorescens</i>	K315	6.83	0.000	0.000	0.000	0.000	0		

Table 17 data									
	Media	TY	TY	TY	TY	TY	RS	RS	RS
	Mercury	0	3	6	9	0	1	2	3
Rep	Strain								
1	K1345	139	48	41	42	117	23	30	17
2	K1345	116	48	49	32	97	25	12	7
3	K1345	86	33	31	53	115	17	9	22
4	K1345	123	29	38	47	106	21	18	11
5	K1345	141	25	18	65	93	19	19	15
Average		121	36.6	35.4	47.8	105.6	21	17.6	14.4
% recovery			30.2	29.3	39.5		19.9	16.7	13.6
Rep	Strain								
1	K377	172	0	0	0	0	0	0	0
2	K377	151	0	0	0	0	0	0	0
3	K377	193	0	0	0	0	0	0	0
4	K377	189	0	0	0	0	0	0	0
5	K377	147	0	0	0	0	0	0	0
Average		170.4	0	0	0	0	0	0	0
Rep	Strain								
1	K1069	176	0	0	0	0	0	0	0
2	K1069	203	0	0	0	0	0	0	0
3	K1069	184	0	0	0	0	0	0	0
4	K1069	198	0	0	0	0	0	0	0
5	K1069	167	0	0	0	0	0	0	0
Average		185.6	0	0	0	0	0	0	0
Rep	Strain								
1	E26	143	0	0	0	0	0	0	0
2	E26	147	0	0	0	0	0	0	0
3	E26	178	0	0	0	0	0	0	0
4	E26	165	0	0	0	0	0	0	0
5	E26	159	0	0	0	0	0	0	0
Average		158.4	0	0	0	0	0	0	0

Table 17 data									
	Media	TY	TY	TY	TY	TY	RS	RS	RS
	Mercury	0	3	6	9	0	1	2	3
Rep	Strain								
1	F2/5	156	0	0	0	0	0	0	0
2	F2/5	173	0	0	0	0	0	0	0
3	F2/5	139	0	0	0	0	0	0	0
4	F2/5	157	0	0	0	0	0	0	0
5	F2/5	155	0	0	0	0	0	0	0
Average		156	0	0	0	0	0	0	0
Rep	Strain								
1	K315	212	0	0	0	0	0	0	0
2	K315	228	0	0	0	0	0	0	0
3	K315	197	0	0	0	0	0	0	0
4	K315	221	0	0	0	0	0	0	0
5	K315	218	0	0	0	0	0	0	0
Average		215.2	0	0	0	0	0	0	0
Rep	Strain								
1	HLB2	187	0	0	0	0	0	0	0
2	HLB2	234	0	0	0	0	0	0	0
3	HLB2	204	0	0	0	0	0	0	0
4	HLB2	215	0	0	0	0	0	0	0
5	HLB2	226	0	0	0	0	0	0	0
Average		213.2	0	0	0	0	0	0	0

Table 18 data							
Rep	K1345 (K377)	K1345 (K1069)	K1345 (E26)	K1345 (F2/5)	K1345 (K315)	K1345 (HLB2)	K1345 (K1341)
1	6.802	7.373	6.593	6.317	7.101	6.934	6.599
2	7.340	7.351	6.586	6.810	7.111	6.664	6.724
3	7.039	7.340	6.738	6.790	7.066	6.586	6.738
4	6.937	7.122	6.560	6.825	7.133	6.760	6.832
5	7.394	7.451	6.854	6.593	6.847	6.573	6.729
6	7.292	7.266	6.630	6.612	6.951	6.680	6.158
7	7.175	6.606	6.913	6.939	7.167	6.329	6.362
8	7.061	7.039	6.840	6.685	7.028	6.881	6.840
9	6.907	7.016	6.624	6.539	7.172	6.442	6.476
10	6.840	6.459	6.509	6.685	7.044	6.850	6.973
Average	7.079	7.102	6.685	6.679	7.062	6.670	6.643

Table 20. data				
Months	Inoculum	Vine	Area of gall Site 1	Area of gall Site 2
15	K377	1	64.4	-
15	K377	2	20.4	-
15	MSALTS	3	0	0
15	MSALTS	4	0	0
15	MSALTS	5	0	0
15	MSALTS	6	0	0
15	MSALTS	7	0	0
15	MSALTS	8	0	0
Average			0	0
23	K377	1	20.4	-
23	K377	2	95.8	-
23	MSALTS	3	0	0
23	MSALTS	4	0	0
23	MSALTS	5	0	0
23	MSALTS	6	0	0
23	MSALTS	7	0	0
23	MSALTS	8	0	0
23	MSALTS	9	0	0
23	MSALTS	10	0	0
23	MSALTS	11	0	0
23	MSALTS	12	0	0
23	MSALTS	13	0	0
23	MSALTS	14	0	0
23	MSALTS	15	0	0
23	MSALTS	16	0	0
23	MSALTS	17	0	0
23	MSALTS	18	0	0
23	MSALTS	19	0	0
23	MSALTS	20	0	0
23	MSALTS	21	0	0
23	MSALTS	22	0	0
23	MSALTS	23	0	0
23	MSALTS	24	0	0
23	MSALTS	25	0	0
23	MSALTS	26	0	0

Table 20. data				
Months	Inoculum	Vine	Area of gall Site 1	Area of gall Site 2
23	MSALTS	27	0	0
23	MSALTS	28	0	0
23	MSALTS	29	0	0
23	MSALTS	30	0	0
23	MSALTS	31	0	0
23	MSALTS	32	0	0
23	MSALTS	33	0	0
23	MSALTS	34	0	0
23	MSALTS	35	0	0
23	MSALTS	36	0	0
23	MSALTS	37	0	0
23	MSALTS	38	0	0
23	MSALTS	39	0	0
23	MSALTS	40	0	0
23	MSALTS	41	0	0
23	MSALTS	42	0	0
Average			0	0
control	K377	1	28.3	-
control	K377	2	116.2	-
41	MSALTS	3	0	0
41	MSALTS	4	0	7.9
41	MSALTS	5	0	0
41	MSALTS	6	0	15.7
41	MSALTS	7	0	0
41	MSALTS	8	0	7.9
41	MSALTS	9	0	0
41	MSALTS	10	0	0
41	MSALTS	11	0	0
41	MSALTS	12	0	0
41	MSALTS	13	0	0
41	MSALTS	14	0	0
41	MSALTS	15	0	0
Average			0	2.4230769

Tables 21/22 data, Figures 25/26/27 data									
ID	strain	inoc	path	stem A	stem B	stem C	ΣABC	soil	gall
37	control	1	1	0.079	0.000	0.000	0.079	1.187	
72	control	1	1	0.114	0.017	0.000	0.131	2.924	G
93	control	1	1	0.028	0.079	0.000	0.107	1.938	G
160	control	1	1	0.067	0.000	0.000	0.067	2.738	G
189	control	1	1	0.024	0.045	0.000	0.069	0.900	
238	control	1	1	0.176	0.028	0.000	0.204	1.995	G
319	control	1	1	0.054	0.100	0.000	0.154	2.659	
22	control	1	2	0.091	0.000	0.000	0.091	2.559	
117	control	1	2	0.028	0.091	0.000	0.119	0.425	G
159	control	1	2	0.585	0.011	0.000	0.597	2.235	G
191	control	1	2	0.072	0.000	0.000	0.072	2.425	
221	control	1	2	0.093	0.007	0.000	0.100	1.174	
255	control	1	2	0.092	0.474	0.000	0.566	2.221	G
289	control	1	2	0.204	0.000	0.000	0.204	3.390	G
6	control	2	1	0.025	0.007	0.000	0.032	3.282	
63	control	2	1	0.181	0.000	0.000	0.181	2.534	
114	control	2	1	0.146	0.221	0.192	0.559	0.662	G
132	control	2	1	0.063	0.097	0.000	0.159	1.910	
186	control	2	1	0.061	0.000	0.000	0.061	3.414	
202	control	2	1	0.204	0.157	0.000	0.361	0.000	G
285	control	2	1	0.010	0.000	0.000	0.010	0.000	
10	control	2	2	0.826	0.648	0.041	1.515	2.775	
58	control	2	2	0.146	0.010	0.009	0.164	1.418	G
130	control	2	2	1.575	0.325	0.117	2.017	1.795	G
171	control	2	2	0.556	0.000	0.063	0.618	2.839	G
208	control	2	2	0.953	0.079	0.010	1.041	0.247	G
270	control	2	2	0.009	0.152	0.000	0.161	1.455	G
301	control	2	2	0.327	0.079	0.021	0.427	0.403	
38	K1069	1	1	0.000	0.021	0.000	0.021	1.523	
69	K1069	1	1	0.010	0.000	0.000	0.010	2.146	
118	K1069	1	1	0.000	0.000	0.000	0.000	2.038	
122	K1069	1	1	0.000	0.008	0.017	0.025	2.829	
182	K1069	1	1	0.000	0.000	0.000	0.000	2.295	
222	K1069	1	1	0.000	0.010	0.000	0.010	1.167	G
258	K1069	1	1	0.000	0.000	0.000	0.000	0.417	
303	K1069	1	1	0.000	0.000	0.000	0.000	2.102	
17	K1069	1	2	0.012	0.015	0.000	0.027	1.401	
59	K1069	1	2	0.000	0.021	0.000	0.021	2.433	
113	K1069	1	2	0.021	0.121	0.000	0.143	2.854	
164	K1069	1	2	0.436	0.265	0.351	1.052	3.054	
236	K1069	1	2	0.152	0.037	0.000	0.189	0.648	
259	K1069	1	2	0.061	0.006	0.000	0.066	1.322	
317	K1069	1	2	0.010	0.000	0.000	0.010	3.405	
61	K1069	2	1	0.008	0.028	0.011	0.047	2.111	

Tables 21/22 data, Figures 25/26/27 data									
ID	strain	inoc	path	stem A	stem B	stem C	ΣABC	soil	gall
86	K1069	2	1	0.000	0.000	0.000	0.000	0.927	
154	K1069	2	1	0.000	0.000	0.000	0.000	2.603	
190	K1069	2	1	0.000	0.028	0.000	0.028	2.621	
227	K1069	2	1	0.000	0.000	0.000	0.000	0.000	
248	K1069	2	1	0.000	0.019	0.021	0.040	1.592	
308	K1069	2	1	0.000	0.000	0.000	0.000	2.363	G
12	K1069	2	2	0.209	0.000	0.000	0.209	3.133	
57	K1069	2	2	0.139	0.010	0.008	0.156	0.000	
106	K1069	2	2	2.309	0.140	0.010	2.459	2.618	
176	K1069	2	2	0.000	0.015	0.000	0.015	3.200	G
212	K1069	2	2	0.105	0.008	0.000	0.113	0.000	
249	K1069	2	2	0.018	0.052	0.023	0.094	0.000	
316	K1069	2	2	0.000	0.006	0.000	0.006	2.988	
40	K315	1	1	0.000	0.000	0.000	0.000	2.694	
42	K315	1	1	0.093	0.000	0.000	0.093	3.316	
96	K315	1	1	0.000	0.000	0.000	0.000	1.971	
133	K315	1	1	0.000	0.000	0.000	0.000	1.992	
185	K315	1	1	0.011	0.000	0.000	0.011	2.908	
225	K315	1	1	0.000	0.000	0.000	0.000	0.000	
254	K315	1	1	0.325	0.000	0.000	0.325	0.486	
293	K315	1	1	0.000	0.000	0.000	0.000	3.075	
2	K315	1	2	0.031	0.007	0.000	0.038	1.424	
74	K315	1	2	0.000	0.086	0.000	0.086	2.346	
85	K315	1	2	0.138	0.030	0.000	0.168	1.672	
145	K315	1	2	0.028	0.041	0.000	0.069	2.755	
200	K315	1	2	0.041	0.010	0.000	0.051	1.884	
278	K315	1	2	0.226	0.000	0.000	0.226	0.761	
310	K315	1	2	0.437	0.041	0.033	0.512	1.380	
11	K315	2	1	0.012	0.000	0.000	0.012	3.228	
45	K315	2	1	0.054	0.000	0.000	0.054	1.187	
92	K315	2	1	0.000	0.000	0.000	0.000	1.531	
147	K315	2	1	0.000	0.000	0.000	0.000	2.180	
178	K315	2	1	0.000	0.000	0.000	0.000	2.793	
229	K315	2	1	0.000	0.000	0.000	0.000	0.956	
274	K315	2	1	0.000	0.000	0.000	0.000	0.000	
288	K315	2	1	0.000	0.000	0.000	0.000	2.783	
8	K315	2	2	0.000	0.010	0.017	0.027	2.876	
51	K315	2	2	0.614	0.031	0.017	0.662	1.123	
112	K315	2	2	0.047	0.000	0.000	0.047	1.394	
135	K315	2	2	0.234	0.012	0.010	0.255	3.164	
194	K315	2	2	0.010	0.000	0.000	0.010	2.389	
226	K315	2	2	0.058	0.008	0.026	0.092	0.721	G
242	K315	2	2	0.028	0.000	0.000	0.028	2.274	
287	K315	2	2	0.000	0.000	0.000	0.000	2.786	

Tables 21/22 data, Figures 25/26/27 data									
ID	strain	inoc	path	stem A	stem B	stem C	ΣABC	soil	gall
5	HLB2	1	1	0.159	0.000	0.007	0.166	1.374	
49	HLB2	1	1	0.428	0.079	0.092	0.599	1.542	G
89	HLB2	1	1	0.058	0.000	0.000	0.058	2.834	
131	HLB2	1	1	0.011	0.047	0.000	0.058	2.299	
173	HLB2	1	1	0.058	0.000	0.028	0.086	2.187	G
237	HLB2	1	1	0.000	0.008	0.000	0.008	1.456	
279	HLB2	1	1	0.219	0.023	0.423	0.665	0.000	
311	HLB2	1	1	0.024	0.064	0.041	0.130	3.058	
26	HLB2	1	2	0.225	0.000	0.000	0.225	0.000	
62	HLB2	1	2	0.000	0.000	0.000	0.000	2.590	
98	HLB2	1	2	1.490	0.012	0.010	1.512	1.592	
123	HLB2	1	2	0.017	0.017	0.000	0.034	2.915	
180	HLB2	1	2	0.009	0.000	0.006	0.015	3.266	
203	HLB2	1	2	0.247	0.100	0.189	0.535	0.352	
277	HLB2	1	2	0.317	0.089	0.125	0.531	1.230	
298	HLB2	1	2	0.000	0.000	0.000	0.000	1.824	
14	HLB2	2	1	0.278	0.011	0.000	0.289	3.727	
81	HLB2	2	1	0.109	0.037	0.007	0.152	2.586	
143	HLB2	2	1	0.067	0.091	0.000	0.158	2.243	
161	HLB2	2	1	0.000	0.000	0.000	0.000	3.758	
215	HLB2	2	1	0.967	0.230	0.000	1.197	0.000	
297	HLB2	2	1	0.133	0.000	0.000	0.133	0.411	
33	HLB2	2	2	0.000	0.011	0.010	0.020	0.315	
88	HLB2	2	2	0.000	0.000	0.000	0.000	1.092	
137	HLB2	2	2	0.213	0.000	0.000	0.213	2.354	
196	HLB2	2	2	0.000	0.000	0.000	0.000	2.973	
272	HLB2	2	2	0.000	0.000	0.000	0.000	0.000	
296	HLB2	2	2	0.000	0.000	0.000	0.000	0.000	
16	K1341	1	1	0.079	0.021	0.084	0.184	0.000	
41	K1341	1	1	0.170	0.473	0.763	1.405	3.011	
103	K1341	1	1	0.628	0.374	0.664	1.666	3.003	
155	K1341	1	1	0.024	0.068	0.006	0.098	2.639	
162	K1341	1	1	0.255	0.000	0.000	0.255	3.856	
206	K1341	1	1	0.326	0.028	0.068	0.422	0.000	
276	K1341	1	1	0.151	0.000	0.000	0.151	0.965	
284	K1341	1	1	0.031	0.011	0.000	0.042	0.000	
19	K1341	1	2	0.000	0.000	0.000	0.000	0.351	
101	K1341	1	2	0.510	0.526	0.000	1.037	0.866	
134	K1341	1	2	0.170	0.000	0.000	0.170	1.271	
170	K1341	1	2	0.146	0.137	0.000	0.283	2.951	
231	K1341	1	2	0.000	0.000	0.000	0.000	1.062	
262	K1341	1	2	0.000	0.000	0.000	0.000	0.000	
307	K1341	1	2	0.000	0.000	0.000	0.000	0.000	
66	K1341	2	1	0.306	0.006	0.000	0.312	0.605	

Tables 21/22 data, Figures 25/26/27 data									
ID	strain	inoc	path	stem A	stem B	stem C	ΣABC	soil	gall
83	K1341	2	1	0.245	0.030	0.000	0.275	2.228	
146	K1341	2	1	1.611	0.146	0.000	1.757	1.579	
165	K1341	2	1	1.082	1.426	0.000	2.508	2.572	G
214	K1341	2	1	2.425	0.496	0.684	3.606	0.000	
271	K1341	2	1	0.000	0.000	0.049	0.049	0.000	
15	K1341	2	2	0.000	0.000	0.000	0.000	1.385	G
80	K1341	2	2	0.844	0.049	0.000	0.893	2.634	
105	K1341	2	2	0.155	0.000	0.000	0.155	2.544	
128	K1341	2	2	0.041	0.000	0.062	0.103	1.908	
175	K1341	2	2	0.000	0.035	0.000	0.035	1.690	
243	K1341	2	2	0.146	0.000	0.000	0.146	0.778	
21	E26	1	1	0.024	0.000	0.095	0.119	2.321	
71	E26	1	1	0.883	0.234	0.120	1.237	2.694	
104	E26	1	1	2.082	0.789	0.851	3.722	2.740	
150	E26	1	1	1.039	0.000	0.000	1.039	1.402	
167	E26	1	1	2.648	1.129	0.928	4.706	2.962	
207	E26	1	1	0.691	0.020	0.541	1.252	0.000	
260	E26	1	1	0.008	0.000	0.000	0.008	0.919	
283	E26	1	1	0.031	0.000	0.000	0.031	0.000	
30	E26	1	2	0.079	0.000	0.000	0.079	1.154	
75	E26	1	2	0.069	0.000	0.000	0.069	1.471	
108	E26	1	2	1.580	0.653	0.690	2.924	2.659	
139	E26	1	2	0.577	0.548	0.000	1.125	2.562	
193	E26	1	2	0.015	0.000	0.000	0.015	2.570	
219	E26	1	2	2.198	0.715	0.866	3.779	0.000	
241	E26	1	2	0.146	0.007	0.000	0.153	1.126	
314	E26	1	2	0.000	0.000	0.000	0.000	1.714	
4	E26	2	1	0.446	0.000	0.000	0.446	1.630	
95	E26	2	1	1.652	0.438	0.000	2.090	1.763	
121	E26	2	1	0.102	0.000	0.000	0.102	2.531	
177	E26	2	1	0.928	0.017	0.000	0.945	2.210	
216	E26	2	1	0.585	0.135	0.000	0.721	0.000	
244	E26	2	1	2.414	0.000	0.000	2.414	1.190	
304	E26	2	1	0.021	0.008	0.000	0.029	0.395	
9	E26	2	2	0.010	0.000	0.000	0.010	2.515	
65	E26	2	2	0.000	0.031	0.010	0.041	1.288	
84	E26	2	2	0.069	0.105	0.041	0.215	0.868	
129	E26	2	2	0.000	0.000	0.000	0.000	1.730	
188	E26	2	2	0.000	0.000	0.000	0.000	1.876	
235	E26	2	2	0.000	0.011	0.000	0.011	1.127	
263	E26	2	2	0.000	0.000	0.000	0.000	0.660	
312	E26	2	2	0.000	0.000	0.000	0.000	3.591	
23	E26	1	1	0.000	0.007	0.000	0.007	0.872	
102	F2/5	1	1	0.021	0.000	0.007	0.028	1.838	

[illegible]

BIBLIOGRAPHY

- Akiyoshi D.E., Klee H., Amasino R.M., Nester E.W. & Gordon M.P. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Academy of Sciences* **81**, 5994-5998.
- Allen O.N. & Holding A.J. (1974). Genus II. *Agrobacterium*. In: *Bergey's Manual of Determinative Bacteriology*, 8th edition. (Eds R.E. Buchanan & Gibbons N.E.) pp. 264-267. Williams & Wilkins Co., Baltimore.
- Allison A.V. & Shalla T.A. (1974). The ultrastructure of local lesions induced by potato virus X: A sequence of cytological events in the course of infection. *Phytopathology* **64**, 783-793.
- Andersen P.C. & Brodbeck B.V. (1991). Influence of fertilization on xylem fluid chemistry of *Vitis rotundifolia* Noble and *Vitis* Hibrid Suwannee. *American Journal of Enology and Viticulture* **42**, 245-251.
- Anderson A.R. & Moore L.W. (1979). Host specificity in the genus *Agrobacterium*. *Phytopathology* **69**, 320-323.
- Ankenbauer R.G. & Nester E.W. (1990). Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *Journal of Bacteriology* **172**, 6442-6446.
- Arima K., Imanaka H., Kousaka M., Fukuda A. & Tamura G. (1964). Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. *Agricultural and Biological Chemistry* **28**, 575-576.
- Ashby A.M., Watson M.D. & Shaw C.H. (1987). A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. *FEMS Microbiology Letters* **41**, 189-192.

- Askeland R.A. & Morrison S.M. (1983). Cyanide production by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **45**, 1802-1807.
- Barry G.F., Rogers S.G., Fralet R.T. & Brand L. (1984). Identification of a cloned cytokinin biosynthesis gene. *Proceedings of the National Academy of Sciences* **81**, 4776-4780.
- Bazzi C. & Rosciglione B. (1982). *Agrobacterium tumefaciens* biotype 3, causal agent of crown gall on *Chrysanthemum* in Itali. *Phytopathologische Zeitschrift* **103**, 280-284.
- Bazzi C., Minardi P., Burr T.J., Katz B.H., Bishop A.L. & Blanchard L.M. (1988). Monoclonal and polyclonal antibodies in a comparative serological study of *Agrobacterium* Conn. biovars. *Phytopathologie Mediteraneae* **27**, 51-56.
- Bazzi C., Piazza C. & Burr T.J. (1987). Detection of *Agrobacterium tumefaciens* in grapevine cuttings. *EPPO Bulletin* **17**, 105-112.
- Bazzi C., Stefani E., Gozzi R., Burr T.J., Moore C.L. & Anaclerio F. (1991). Hot-water treatment of dormant grape cuttings: Its effects on *Agrobacterium tumefaciens* and on grafting and growth of vine. *Vitis* **30**, 177-187.
- Beckman C.H. & Zaroogian G.E. (1967). Origin and composition of vascular gel in infected banana roots. *Phytopathology* **57**, 11-13.
- Beckman C.H. (1964). Host responses to vascular infection. *Annual Reveiw of Phytopathology* **2**, 231-252.
- Beckman C.H. (1989). Recognition and response between host and parasite as determinants in resistance and disease development. In: *Vascular Wilt Diseases of Plants*. (Eds E.C. Tjamos & C.H. Beckman) pp. 154-162. Springer-Verlag, Berlin.
- Bergersen F.J. (1961). The growth of *Rhizobium* in synthetic media. *Australian Journal of Biological Sciences* **14**, 349-360.

- Beringer J.E. (1974). R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology **84**, 188-198.
- Bien E., Lorenz D., Eichhorn K. & Plapp R. (1990). Isolation and characterization of *Agrobacterium tumefaciens* from the German vineregion Rheinpfalz. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz **97**, 313-322.
- Binns A.N. & Thomashow M.F. (1988). Cell biology of *Agrobacterium* infection and transformation of plants. Annual Review of Microbiology **42**, 575-606.
- Bishop A.L., Burr T.J., Mittak V.L. & Katz B.H. (1989). A monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3 and its utilization for indexing grapevine propagating material. Phytopathology **79**, 995-998.
- Bomhoff G., Klapwijk P.M., Kester H.C.M. & Schilperoort R.A. (1976). Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. Molecular and General Genetics **145**, 177-181.
- Bonnard G., Tinland B., Paulus F., Szegedi E. & Otten L. (1989). Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Molecular and General Genetics **216**, 428-438.
- Borowicz J.J. & Pietr S.J. (1994). Secretion of protease by rhizobacteria from the genus *Pseudomonas*. In: Improving Plant Productivity with Rhizosphere Bacteria. (Eds M.H. Ryder, P.M. Stephens & G.D. Bowen) pp. 156-158. CSIRO Division of Soils, Adelaide.
- Bouzar H. & Moore L.W. (1987). Isolation of different *Agrobacterium* biovars from a natural oak savanna and tallgrass prairie. Applied and Environmental Microbiology **53**, 717-721.
- Bouzar H., Jones J.B. & Hodge N.C. (1993). Differential characterization of *Agrobacterium* species using carbon-source utilization patterns and fatty acid profiles. Phytopathology **83**, 733-739.

- Braun A.C. & White P.R. (1942). Bacteriological sterility of tissues derived from secondary crown-gall tumors. *Phytopathology* **33**, 85-100.
- Brisbane P.G. & Kerr A. (1983). Selective media for three biovars of *Agrobacterium*. *Journal of Applied Bacteriology* **54**, 425-431.
- Brisset M-N., Rodriguez-Palenzuela P., Burr T.J. & Collmer A. (1991). Attachment, chemotaxis, and multiplication of *Agrobacterium tumefaciens* biovar 1 and biovar 3 on grapevine and pea. *Applied and Environmental Microbiology* **57**, 3178-3182.
- Buchanan-Wollaston V., Passiatore J.E. & Cannon F. (1987). The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. *Nature* **328**, 172-175.
- Buchholz W.G. & Thomashow M.F. (1984). Host range encoded by the *Agrobacterium tumefaciens* tumor-inducing plasmid pTiAg63 can be expanded by modification of its T-DNA oncogene complement. *Journal of Bacteriology* **160**, 327-332.
- Buchmann I., Marner F.-J., Schröder G., Waffenschmidt S. & Schröder J. (1985). Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. *The EMBO Journal* **4**, 853-859.
- Burr T.J. & Hurwitz B. (1981). Occurrence of *Agrobacterium radiobacter* pv. *tumefaciens* (Smith & Townsend) Conn. biotype 3 on grapevine in New York State. (Abstract) *Phytopathology* **71**, 206.
- Burr T.J. & Katz B.H. (1983). Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. *Phytopathology* **73**, 163-165.
- Burr T.J. & Katz B.H. (1984). Grapevine cuttings as potential sites of survival and means of dissemination of *Agrobacterium tumefaciens*. *Plant Disease* **68**, 976-978.

- Burr T.J. & Reid C.L. (1994). Biological control of grape crown gall with nontumorigenic *Agrobacterium vitis* strain F2/5. *American Journal of Enology and Viticulture* **45**, 213-219.
- Burr T.J. (1978). Crown gall of grapevine. *Vinifera Wine Growers Journal* **5**, 131-133.
- Burr T.J., Bishop A.L., Katz B.H., Blanchard L.M. & Bazzi C. (1987a). A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. *Phytopathology* **77**, 1424-1427.
- Burr T.J., Katz B.H. & Bishop A.L. (1987b). Populations of *Agrobacterium* in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. *Plant Disease* **71**, 617-620.
- Burr T.J., Katz B.H., Bishop A.L., Meyers C.A. & Mittak V.L. (1987c). Effect of shoot age and tip culture propagation of grapes on systemic infestations of *Agrobacterium tumefaciens* biovar 3. *American Journal of Enology and Viticulture* **39**, 66-70.
- Burr T.J., Ophel K., Katz B.H. & Kerr A. (1989). Effect of hot water treatment on systemic *Agrobacterium tumefaciens* biovar 3 in dormant grape cuttings. *Plant Disease* **73**, 242-245.
- Calderón A.A., Zapata J.M., Muños R., Pedreño M.A. & Barceló (1993). Resveratrol production as a part of the hypersensitive-like response of grapevine cells to an elicitor from *Trichoderma viride*. *New Phytologist* **124**, 455-463.
- Cangelosi G.A., Hung L., Puvanesarajah V., Stacey G., Ozga D.A., Leigh J.A. & Nester E.W. (1987). Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *Journal of Bacteriology* **169**, 2086-2091.
- Chamberlain G.C. (1962). The occurrence of aerial crown gall of grapevines in the Niagara Peninsula of Ontario. *Canadian Plant Disease Survey* **42**, 208-211.

- Chen X. & Xiang W. (1986). A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biotype 3 strains of *Agrobacterium tumefaciens* from grapevine. *Acta Microbiologica Sinica* **26**, 193-199.
- Chilton M.-D., Drummond M.H., Merlo D.J. & Sciaky D. (1978). Highly conserved DNA of Ti plasmids overlaps T-DNA maintained in plant tumours. *Nature* **275**, 147-149.
- Chilton M.-D., Drummond M.H., Merlo D.J., Sciaky D., Montoya A.L., Gordon M.P. & Nester E.W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**, 263-271.
- Clare B.G., Kerr A. & Jones D.A. (1990). Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. *Plasmid* **23**, 126-137.
- Close T.J., Rogowsky P.M., Kado C.I., Winans S.C., Yanofsky M.F. & Nester E.W. (1987). Dual control of *Agrobacterium tumefaciens* Ti plasmid virulence genes. *Journal of Bacteriology* **169**, 5113-5118.
- Cole M. (1958). Oxidation products of leuco-anthocyanins as inhibitors of fungal polygalacturonase in rotting apple fruit. *Nature* **181**, 1596-1597.
- Condit R., Stewart F.M. & Levin B.R. (1988). The population biology of bacterial transposons: *a priori* conditions for maintenance as parasitic DNA. *The American Naturalist* **132**, 129-147.
- Conn H.J. (1942). The validity of the genus *Alcaligenes*. *Journal of Bacteriology* **44**, 353-360.
- Cooksey D.A. & Moore L.W. (1980). Biological control of crown gall with fungal and bacterial antagonists. *Phytopathology* **70**, 506-509.
- Cooksey D.A. & Moore L.W. (1982). Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. *Phytopathology* **72**, 919-921.

- Cruickshank I.A.M. & Perrin D.R. (1960). Isolation of a phytoalexin from *Pisum sativum* L. *Nature* **187**, 799-800.
- Darvill A.G. & Albersheim P. (1984). Phytoalexins and their elicitors - a defense against microbial infection in plants. *Annual Review of Plant Physiology* **35**, 243-275.
- Das P.K., Basu M. & Chatterjee G.C. (1978). Studies on the mode of action of agrocin 84. *The Journal of Antibiotics* **31**, 490-492.
- Davis K.R., Lyon G.D., Darvill A.G. & Albersheim P. (1984). Host-pathogen interactions XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiology* **74**, 52-60.
- Davis M.J., Purcell A.H. & Thomson S.V. (1978). Pierce's disease of grapevines: Isolation of the causal bacterium. *Science* **199**, 75-77.
- De Cleene M. & De Ley J. (1976). The host range of crown gall. *The Botanical Review* **42**, 389-466.
- De Ley J., Bernaerts M., Rassel A. & Guilmo J. (1966). Approach to an improved taxonomy of the genus *Agrobacterium*. *Journal of General Microbiology* **43**, 7-17.
- Dhanvantari B.N. (1983). Etiology of grape crown gall in Ontario. *Canadian Journal of Botany* **61**, 2641-2646.
- Ditta G., Stanfield S., Corbin D. & Helinski D. (1980). Broad host range cloning system for gram negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proceedings of the National Academy of Science USA* **77**, 7347-7351.
- Donner S.C., Jones D.A., McClure N.C., Rosewarne G.M., Tate M.E., Kerr A., Fajardo N.N. & Clare B.G. (1993). Agrocin 434, a new plasmid encoded agrocin from the biocontrol *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria. *Physiological and Molecular Plant Pathology* **42**, 185-194.

- Douglas C.J., Halperin W. & Nester E.W. (1982). *Agrobacterium tumefaciens* mutants affected in attachment to plant cells. *Journal of Bacteriology* **152**, 1265-1275.
- Douglas C.J., Staneloni R.J., Rubin R.A. & Nester E.W. (1985). Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *Journal of Bacteriology* **161**, 850-860.
- Dylan T., Ielpi L., Stanfield S., Kashyap L., Douglas C., Yanofsky M., Nester E., Helinski D.R. & Ditta G. (1986). *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences USA* **83**, 4403-4407.
- Eckhardt T. (1978). A rapid method for the identification of deoxyribonucleic acid in bacteria. *Plasmid* **1**, 584-588.
- Ellis J.G. & Kerr A. (1978). Developing biological control agents for soil borne pathogens. In: *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria*. (Ed. M. Ride) pp. 245-250. INRA, Angers.
- Ellis J.G. & Murphy P.J. (1981). Four new opines from crown gall tumours - their detection and properties. *Molecular and General Genetics* **181**, 36-43.
- Ellis J.G., Kerr A., Van Montagu M. & Schell J. (1979). *Agrobacterium*: genetic studies on agrocin 84 production and the biological control of crown gall. *Physiological Plant Pathology* **15**, 311-319.
- Engler G., Holsters M., Van Montagu M., Schell J., Hernalsteens J.P. & Schilperoort R. (1975). Agrocin 84 sensitivity: a plasmid determined property in *Agrobacterium tumefaciens*. *Molecular and General Genetics* **138**, 345-349.
- Farkas E. & Haas J.H. (1985). Biological control of crown gall in rose nursery stock. *Phytoparasitica* **13**, 121-127.

- Farkas G.L. & Király Z. (1962). Role of phenolic compounds in the physiology of plant diseases and disease resistance. *Phytopathologische Zeitschrift* **44**, 105-150.
- Farrand S.K., Slota J.E., Shim J.-S. & Kerr A. (1985). Tn5 insertions in the agrocin 84 plasmid: the conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. *Plasmid* **13**, 106-117.
- Farrand S.K., Wilson M., Lindow S.E. & Savka M.A. (1994). Modulating colonization by plant-associated microbes. In: *Improving Plant Productivity with Rhizosphere Bacteria*. (Eds M.H. Ryder, P.M. Stephens & G.D. Bowen) pp. 233-237. CSIRO Division of Soils, Adelaide.
- Figurski D.H. & Helinski D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function *in trans*. *Proceedings of the National Academy of Sciences USA* **76**, 1648-1652.
- Fry S.M. & Milholland R.D. (1990). Response of resistant, tolerant, and susceptible grapevine tissues to invasion by the Pierce's disease bacterium, *Xylella fastidiosa*. *Phytopathology* **80**, 66-69.
- Garfinkel D.J. & Nester E.W. (1980). *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. *Journal of Bacteriology* **144**, 732-743.
- Geiger W.B. (1946). The mechanism of the antibacterial action of quinones and hydroquinones. *Archives of Biochemistry* **11**, 23-32.
- Gietl C., Koukolíková-Nicola Z. & Hohn B. (1987). Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. *Proceedings of the National Academy of Sciences USA* **84**, 9006-9010.
- Goodman R.N. (1986). Transcellular and vascular transport. In: *The Biochemistry and Physiology of Plant Disease*. (Eds R.N. Goodman, Z. Király & K.R. Wood) pp. 287-317. University of Missouri Press, Columbia.

- Goodman R.N., Butrov D. & Tarbah F. (1987). The occurrence of *Agrobacterium tumefaciens* in grapevine-propagating material and a simplified indexing system. *American Journal of Enology and viticulture* **38**, 189-193.
- Goodman R.N., Grimm R. & Frank M. (1993). The influence of grape rootstocks on the crown gall infection process and on tumor development. *American Journal of Enology and Viticulture* **44**, 22-26.
- Graham P.H. (1964). Application of computer techniques to the taxonomy of root-nodule bacteria. *Journal of General Microbiology* **35**, 511-517.
- Grinsted J., de la Cruz F., Altenbuchner J. & Schmitt R. (1982). Complementation of transposition of *tnpA* mutants of Tn3, Tn21, Tn501, and Tn1721. *Plasmid* **8**, 276-286.
- Haas J.H., Zveibil A., Zutra D., Tanne B. & Manulis S. (1991). The presence of crown gall of grape incited by *Agrobacterium tumefaciens* biovar 3 in Israel. *Phytoparasitica* **19**, 311-318.
- Hammerschmidt R., Nuckles E.M. & Kúc J. (1982). Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiological Plant Pathology* **20**, 73-82.
- Hawes M.C., Smith L.Y. & Howarth A.J. (1988). *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Molecular Plant-Microbe Interactions* **1**, 182-186.
- Heberlain G.T., De Ley J. & Tijtgaat R. (1967). Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. *Journal of Bacteriology* **94**, 116-124.
- Hendson M., Askjaer L., Thomson J. & Van Montagu M. (1983). Broad-host-range agrocin of *Agrobacterium tumefaciens*. *Applied and Environmental Microbiology* **45**, 1526-1532.
- Hilderbrand D.C. & Schroth M.N. (1964). Arbutin-hydroquinone complex in pear as a factor in fire blight development. *Phytopathology* **54**, 59-63.

- Hille J., van Kan J. & Schilperoort R. (1984). *trans*-acting virulence functions of the octopine Ti plasmid from *Agrobacterium tumefaciens*. *Journal of Bacteriology* **158**, 754-756.
- Hirooka T. & Kado C.I. (1986). Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. *Journal of Bacteriology* **168**, 237-243.
- Hoekema A., de Pater B.S., Fellingner A.J., Hooykaas P.J.J. & Schilperoort R.A. (1984). The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. *The EMBO Journal* **3**, 3043-3047.
- Holmes B. & Roberts P. (1981). The classification, identification and nomenclature of agrobacteria. *Journal of Applied Bacteriology* **50**, 443-68.
- Hoos G. & Blaich R. (1990). Influence of resveratrol on germination of conidia and mycelial growth of *Botrytis cinerea* and *Phomopsis viticola*. *Journal of Phytopathology* **129**, 102-110.
- Hooykaas P.J.J., Hofker M., den Dulk-Ras H. & Schilperoort (1984). A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. *Plasmid* **11**, 195-205.
- Hooykaas P.J.J., Klapwijk P.M., Nuti M.P., Schilperoort R.A. & Rorsch A. (1977). Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to *Rhizobium ex planta*. *Journal of General Microbiology* **98**, 477-484.
- Hopkins D.L. (1985). Physiological and pathological characteristics of virulent and avirulent strains of the bacterium that causes Pierce's disease of grapevine. *Phytopathology* **75**, 713-717.

- Huang M.-L.W., Cangelosi G.A., Halperin W. & Nester E.W. (1990). A chromosomal *Agrobacterium tumefaciens* gene required for effective plant signal transduction. *Journal of Bacteriology* **172**, 1814-1822.
- Huss B., Tinland B., Paulus F., Walter B. & Otten L. (1990). Functional analysis of a complex oncogene arrangement in biotype III *Agrobacterium tumefaciens* strains. *Plant Molecular Biology* **14**, 173-186.
- Inze D., Follin A., Van Lijsebettens M., Simoens C., Gentello C., Van Montagu M. & Schell J. (1984). Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*; further evidence that two genes are involved in indole-3-acetic acid synthesis. *Molecular and General Genetics* **194**, 265-274.
- Jäger J., Frankmann K.T., Lorenz D.H. & Eichhorn K.W. (1989). The indexing of grapevine material latently infested by *Agrobacterium tumefaciens* biovar 3 - isolate identification by gas chromatography of whole cell fatty acids. *Wein-Wissenschaft, Wiesbaden* **44**, 177-182.
- Jäger J., Lorenz D., Plapp R. & Eichhorn K.W. (1990). Latent occurrence of *Agrobacterium tumefaciens* biovar 3 in grapevine, *Vitis vinifera* L. *Wein-Wissenschaft, Wiesbaden* **45**, 14-20.
- Jarchow E., Grimsley N.H. & Hohn B. (1991). *virF*, the host-range-determining virulence gene of *Agrobacterium tumefaciens*, affects T-DNA transfer to *Zea mays*. *Proceedings of the National Academy of Sciences* **88**, 10426-10430.
- Jones D.A. & Kerr A. (1989). *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant disease* **73**, 15-18.
- Jones D.A., Ryder M.H., Clare B.C., Farrand S.K. & Kerr A. (1988). Construction of a Tra- deletion mutant of pAgK84 to safeguard the biological control of crown gall. *Molecular and General Genetics* **212**, 207-214.

- Jones D.A., Ryder M.H., Clare B.G., Farrand S.K. & Kerr A. (1991). Biological control of crown gall using *Agrobacterium* strains K84 and K1026. In: The Biological Control of Plant Diseases, FFTC Book Series No. 42. (Ed. J. Bay-Peterson) pp. 161-170. Food and Fertilizer Technology Center, Taipei, Taiwan.
- Jones J.B. & Raju B.C. (1988). Systemic movement of *Agrobacterium tumefaciens* in symptomless stem tissue of *Chrysanthemum morifolium*. Plant Disease **72**, 51-54.
- Kan-Rice P. (1988). Heavy crown gall infestation follows Zinfandel grafting. California Grape Grower **19**, 21-23.
- Katz B.H. & Burr T.J. (1983). Recovery of *Agrobacterium tumefaciens* biovar 3 from grapevine cuttings. Phytopathology **73**, 368.
- Keane P.J., Kerr A. & New P.B. (1970). Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. Australian Journal of Biological Science **23**, 585-595.
- Kearney B., Ronald P.C., Dahlbeck D. & Staskawicz B.J. (1988). Molecular basis for evasion of plant host defence in bacterial spot disease of pepper. Nature **332**, 541-543.
- Kerr A. & Htay K. (1974). Biological control of crown gall through bacteriocin production. Physiological Plant Pathology **4**, 37-44.
- Kerr A. & Panagopoulos C.G. (1977). Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopathologische Zeitschrift **90**, 172-179.
- Kerr A. & Roberts W.P. (1976). *Agrobacterium*: correlations between transfer of pathogenicity, octopine and nopaline metabolism and bacteriocin 84 sensitivity. Physiological Plant Pathology **9**, 205-211.
- Kerr A. & Tate M.E. (1984). Agrocins and the biological control of crown gall. Microbiological Sciences **1**, 1-4.

- Kerr A. (1969). Transfer of virulence between isolates of *Agrobacterium*. *Nature* **223**, 1175-1176.
- Kerr A. (1972). Biological control of crown gall: seed inoculation. *Journal of Applied Bacteriology* **35**, 493-497.
- Kerr A. (1989). Commercial release of a genetically engineered bacterium for the control of crown gall. *Agricultural Science* **2**, 41-44.
- Kerr A. (1992). The genus *Agrobacterium*. In: *The Prokaryotes*, 2nd edition, Vol. 3. (Eds A. Balows, H.G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer) pp. 2214-2235. Springer-Verlag, New York.
- Kerr A., Young J.M. & Panagopoulos C.G. (1978). Genus II. *Agrobacterium* Conn 1942. *New Zealand Journal of Agricultural Research* **21**, 155-156.
- Kerstens K. & De Ley J. (1984). Genus III. *Agrobacterium*. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 1. (Eds N.R. Krieg & J.G. Holt) pp. 244-254. Williams & Wilkins Co., Baltimore.
- Kerstens K., De Ley J., Sneath P.H.A. & Sackin M. (1973). Numerical taxonomic analysis of *Agrobacterium*. *Journal of General Microbiology* **78**, 227-239.
- Klee H.J., Gordon M.P. & Nester E.W. (1982). Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid mutations affecting oncogenicity. *Journal of Bacteriology* **150**, 327-331.
- Knauf V., Yanofsky M., Montoya A. & Nester E. (1984). Physical and functional map of an *Agrobacterium tumefaciens* tumor-inducing plasmid that confers a narrow host range. *Journal of Bacteriology* **160**, 564-568.
- Knauf V.C. & Nester E.W. (1982). Wide host range cloning vectors: A cosmid clone bank of an *Agrobacterium* Ti-plasmid. *Plasmid* **8**, 45-54.

- Knauf V.C., Panagopoulos C.G. & Nester E.W. (1982). Genetic factors controlling the host range of *Agrobacterium tumefaciens*. *Phytopathology* **72**, 1545-1549.
- Köhn S. & Beiderbeck R. (1982). Octopinverwertung durch *Pseudomonas* spp. *Phytopathologische Zeitschrift* **105**, 305-310.
- Lam S.T., Gaffney T.D., Frazelle R.A., Gates K., Torkewitz N., Hill S., Goodwin S., Ligon J. & Kempf H.-J. (1994). Genetic regulation of biocontrol factors in *Pseudomonas fluorescens*. In: Improving Plant Productivity with Rhizosphere Bacteria. (Eds M.H. Ryder, P.M. Stephens & G.D. Bowen) pp. 97-99. CSIRO Division of Soils, Adelaide.
- Langcake P. & Pryce R.J. (1977). A new class of phytoalexins from grapevines. *Experientia* **33**, 151.
- Langcake P., Cornford C.A. & Pryce R.J. (1979). Identification of pterostilbene as a phytoalexin from *Vitis vinifera* leaves. *Phytochemistry* **18**, 1025-1027.
- Larson R.A. (1988). The antioxidants of higher plants. *Phytochemistry* **27**, 969-978.
- Leach J.E., Ramundo B.A., Pearson D.L. & Claflin L.E. (1987). Dot-immunobinding assay for detecting *Xanthomonas campestris* pv. *holcicola* in sorghum. *Plant Disease* **71**, 31-33.
- Leatham G.F., King V. & Stahmann M.A. (1980). *In vitro* protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. *Phytopathology* **70**, 1134-1140.
- Lehoczky J. (1968). Spread of *Agrobacterium tumefaciens* in the vessels of grapevine, after natural infection. *Phytopathologische Zeitschrift* **63**, 239-246.
- Lehoczky J. (1971). Further evidences concerning the systemic spreading of *Agrobacterium tumefaciens* in the vascular system of grapevines. *Vitis* **10**, 215-221.

- Leroux B., Yanofsky M.F., Winans S.C., Ward J.E., Ziegler S.F. & Nester E.W. (1987). Characterization of the *virA* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. *The EMBO Journal* **6**, 849-856.
- Liang Y.J., Di Y.B., Zhao J.Y. & Ma D.Q. (1990). Strain E26 of *Agrobacterium radiobacter* efficiently inhibits crown gall formation on grapevines. *Chinese Science Bulletin* **35**, 1055-1056.
- Lippincott B.B. & Lippincott J.A. (1969). Bacterial attachment to a specific wound site as an essential stage in tumour initiation by *Agrobacterium tumefaciens*. *Journal of Bacteriology* **97**, 620-628.
- Lippincott B.B., Whatley M.H. & Lippincott J.A. (1977). Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. *Plant Physiology* **59**, 388-390.
- Lippincott J.A., Beiderbeck R. & Lippincott B.B. (1973). Utilisation of octopine and nopaline by *Agrobacterium*. *Journal of Bacteriology* **116**, 378-383.
- Loper J.E. & Buyer J.S. (1991). Siderophores in microbial interactions on plant surfaces. *Molecular Plant-Microbe Interactions* **4**, 5-13.
- Loper J.E. & Kado C.I. (1979). Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **139**, 591-596.
- Loper J.E. (1988). Role of fluorescent siderophore production in biological control of *Pythium ultimum* by a *Pseudomonas fluorescens* strain. *Phytopathology* **78**, 166-172.
- López M.M., Gorris M.T., Temprano F.J. & Orive R.J. (1987). Results of seven years of biological control of *Agrobacterium tumefaciens* in Spain. *EPPO Bulletin* **17**, 273-279.
- Loubser J.T. (1978). Identification of *Agrobacterium tumefaciens* biotype 3 on grapevine in South Africa. *Plant Disease Reporter* **62**, 730-731.

- Lovrekovich L., Lovrekovich H. & Stahmann M.A. (1967). Inhibition of phenol oxidation by *Erwinia carotovora* in potato tuber tissue and its significance in disease resistance. *Phytopathology* **57**, 737-742.
- Lyr H. (1962). Enzymatische detoxification der kernholztoxine. *Flora* **152**, 570.
- Ma D., Yanofsky M.F., Gordon M.P. & Nester E.W. (1987). Characterisation of *Agrobacterium tumefaciens* strains isolated from grapevine tumors in China. *Applied Environmental Microbiology* **53**, 1338-1343.
- Marbach I., Harel E. & Mayer A.M. (1984). Molecular properties of extracellular *Botrytis cinerea* laccase. *Phytochemistry* **23**, 2713-2717.
- Matthews D.E. & Van Etten H.D. (1983). Detoxification of the phytoalexin pisatin by a fungal cytochrome P-450. *Archives of Biochemistry and Biophysics* **224**, 494-505.
- Matthysse A.G., Holmes K.V. & Gurlitz R.H.G. (1981). Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. *Journal of Bacteriology* **145**, 583-595.
- Mayer A.M. (1987). Polyphenol oxidases in plants - recent progress. *Phytochemistry* **26**, 11-20.
- McGuire R.G., Rodriguez-Palenzuela P., Collmer A. & Burr T.J. (1991). Polygalacturonase production by *Agrobacterium tumefaciens* biovar 3. *Applied and Environmental Microbiology* **57**, 660-664.
- Miller H.N. (1975). Leaf, stem, crown, and root galls induced in chrysanthemum by *Agrobacterium tumefaciens*. *Phytopathology* **65**, 805-811.
- Miller J.H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Moffett M.L. & Colwell R.R. (1968). Adansonian analysis of the Rhizobiaceae. *Journal of General Microbiology* **51**, 245-266.

- Mollenhauer H.H. & Hopkins D.L. (1976). Xylem morphology of Pierce's disease-infected grapevines with different levels of tolerance. *Physiological Plant Pathology* **9**, 95-100.
- Montoya A.L., Chilton M.-D., Gordon M.P., Sciaky D. & Nester E.W. (1977). Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumour cells: role of plasmid genes. *Journal of Bacteriology* **129**, 101-107.
- Montoya A.L., Moore L.W., Gordon M.P. & Nester E.W. (1978). Multiple genes coding for octopine-degrading enzymes in *Agrobacterium*. *Journal of Bacteriology* **136**, 909-915.
- Moustafa (Darweish) F.A. & Whittenbury R. (1970). Properties which appear to allow phytopathogenic pseudomonads to counteract plant defence mechanisms. *Phytopathologische Zeitschrift* **67**, 214-224
- Murphy P.J. & Roberts W.P. (1979). A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. *Journal of General Microbiology* **114**, 207-213.
- Nebert D.W. & Gonzalez F.J. (1987). P450 genes: structure, evolution, and regulation. *Annual Review of Biochemistry* **56**, 945-993.
- New P.B. & Kerr A. (1972). Biological control of crown gall: field measurements and glasshouse experiments. *Journal of Applied Bacteriology* **35**, 279-287.
- Nothnagel E.A., McNeil M., Albersheim P. & Dell A. (1983). Host-pathogen interactions XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiology* **71**, 916-926.
- Ophel K. & Kerr A. (1987). Ecology of *Agrobacterium*: plasmids and biovars. In: *Molecular Genetics of Plant-Microbe Interactions*. (Eds D. P. S. Verma & N. Brisson) pp. 3-5. Martinus Nijhoff, Boston.
- Ophel K. & Kerr A. (1990). *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systemic Bacteriology* **40**, 236-241.

- Ophel K. (1987). *Agrobacterium*: plasmids and biovars. Thesis, University of Adelaide.
- Ophel K., Burr T.J., Magarey P.A. & Kerr A. (1988). Detection of *Agrobacterium tumefaciens* biovar 3 in South Australian grapevine propagation material. *Australasian Plant Pathology* **17**, 61-66.
- Ophel K., Nicholas P.R., Magarey P.A. & Bass A.W. (1990a). Hot water treatment of dormant grape cuttings reduces crown gall incidence in a field nursery. *American Journal of Enology and Viticulture* **41**, 325-329.
- Ophel K.M., Jones D.A. & Kerr A. (1990b). Molecular cloning of cell wall degrading enzymes from *Agrobacterium tumefaciens* biovar 3. (Abstract) *Phytopathology* **80**, 892.
- Panagopoulos C.G. & Psallidas P.G. (1973). Characteristics of Greek isolates of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. *Journal of Applied Bacteriology* **36**, 233-240.
- Panagopoulos C.G., Psallidas P.G. & Alivizatos A.S. (1978). Studies on biotype 3 of *Agrobacterium tumefaciens* var. *tumefaciens*. In: *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria*. (Ed. M. Ride) pp. 221-228 INRA, Angers.
- Panagopoulos C.G., Psallidas P.G. & Alivizatos A.S. (1979). Evidence of a breakdown in the effectiveness of biological control of crown gall. In: *Soil-borne Plant Pathogens*. (Eds B. Schippers & W. Gams) pp. 569-578. Academic Press, London.
- Parke D., Ornston L.N. & Nester E.W. (1987). Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **169**, 5336-5338.
- Perry K.L. & Kado C.I. (1981). *Agrobacterium tumefaciens* biotypes 2 and 3 from *Rubus* and grape in California. (Abstract) *Phytopathology* **71**, 249.

- Perry K.L. & Kado C.I. (1982). Characteristics of Ti plasmids from broad-host-range and ecologically specific biotype 2 and 3 strains of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **151**, 343-350.
- Petit A. & Tempé J. (1978). Isolation of *Agrobacterium* Ti-plasmid regulatory mutants. *Molecular and General Genetics* **167**, 147-155.
- Petit A., Dessaux Y. & Tempé J. (1978a). The biological significance of opines: I. - A study of opine catabolism by *Agrobacterium tumefaciens*. In: *Proceedings of the Fourth International Conference on Plant Pathogenic Bacteria*. (Ed. M. Ride) pp. 143-152 INRA, Angers.
- Petit A., Tempé J., Kerr A., Holsters M., Van Montagu M. & Schell J. (1978b). Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti-plasmids. *Nature* **271**, 570-571.
- Pu X.-A. & Goodman R.N. (1993a). Effects of fumigation and biological control on infection of indexed crown gall free grape plants. *American Journal of Enology and Viticulture* **44**, 241-248.
- Pu X.-A. & Goodman R.N. (1993b). Tumor formation by *Agrobacterium tumefaciens* is suppressed by *Agrobacterium radiobacter* HLB-2 on grape plants. *American Journal of Enology and Viticulture* **44**, 249-254.
- Ried J.L. & Collmer A. (1985). An activity stain for the rapid characterisation of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate polyacrylamide gels. *Applied and Environmental Microbiology* **50**, 615-622.
- Riker A.J. (1923). Some morphological responses of the host tissue to the crown gall organism. *Journal of Agricultural Research* **26**, 425-435.
- Roberts W.P., Tate M.E. & Kerr A. (1977). Agrocin 84 is a 6-*N*-phosphoramidate of an adenine nucleotide analogue. *Nature* **265**, 379-380.
- Rodriguez-Palenzuela P., Burr T.J. & Collmer A. (1991). Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar 3. *Journal of Bacteriology* **173**, 6547-6552.

- Rothamsted Experimental Station (1977). GENSTAT - a general statistical program. The Statistics Department, Rothamsted Experimental Station.
- Roubelakis-Angelakis K.A. & Kliwer W.M. (1979). The composition of bleeding sap from Thompson Seedless grapevines as affected by nitrogen fertilization. *American Journal of Enology and Viticulture* **30**, 14-18.
- Roy M.A. & Sasser M. (1983). A medium selective for *Agrobacterium tumefaciens* biotype 3. (Abstract) *Phytopathology* **73**, 810.
- Sánchez-Serrano J.J., Villarroel R., Maenhaut R., Serrada J. & Beltrá R. (1985). Characterization of *Agrobacterium tumefaciens* strain ATV isolated from a grapevine tumor. *Zentralblatt fuer Mikrobiologie* **140**, 59-72.
- Schröder G., Waffenschmidt S., Weiler E.W. & Schröder J. (1984). The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *European Journal of Biochemistry* **138**, 387-391.
- Schroth M.N., McCain A.H., Foott J.H. & Huisman O.C. (1988). Reduction in yield and vigor of grapevine caused by crown gall disease. *Plant Disease* **72**, 241-246.
- Shim J.-S., Farrand S.K. & Kerr A. (1987). Biological control of crown gall: construction and testing of new biocontrol agents. *Phytopathology* **77**, 463-466.
- Shirasu K., Morel P. & Kado C.I. (1990). Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. *Molecular Microbiology* **4**, 1153-1163.
- Simon R. (1984). High frequency mobilization of gram-negative bacterial replicons by the *in vitro* constructed Tn5-Mob transposon. *Molecular and General Genetics* **196**, 413-420.

- Simon R., Priefer U. & Pühler A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology* **1**, 784-791.
- Slota J.E. & Farrand S.K. (1982). Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. *Plasmid* **8**, 175-186.
- Smith D.A. (1982). Toxicity of phytoalexins. In: *Phytoalexins*. (Eds J.A. Bailey & J.W. Mansfield) pp. 218-252. Blackie, Glasgow.
- Smith E.F. & Townsend C.O. (1907). A plant tumour of bacterial origin. *Science*, New York **25**, 201-226.
- Smith E.F., Brown N.A. & McCulloch L. (1912). The structure and development of crown gall, a plant cancer. U.S.D.A., B.P.I. Bulletin **225**, 60.
- Stachel S.E. & Nester E.W. (1986). The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *The Embo Journal* **5**, 1445-1454.
- Stachel S.E. & Zambryski P.C. (1986). *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* **46**, 325-333.
- Stachel S.E., Messens E., Van Montagu M. & Zambryski P. (1985b). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* **318**, 624-629.
- Stachel S.E., Nester E.W. & Zambryski P.C. (1986a). A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences* **83**, 379-383.
- Stachel S.E., Timmerman B. & Zambryski P. (1986b). Generation of single-stranded T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* **322**, 706-712.

- Staphorst J.L., van Zyl F.G.H., Strijdom B.W. & Groenewold Z.E. (1985). Agrocin-producing pathogenic and non-pathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. *Current Microbiology* **12**, 45-52.
- Stapp C. (1936). Der Pflanzenkrebs und sein Erreger *Pseudomonas tumefaciens*. IV. Mitt. Eine neue Wirtspflanze (*Dahlia variabilis* Desf.) mit hochvirulentem Erreger. *Zentralblatt fuer Bakteriologie* **97**, 273-283.
- Stefani E. & Bazzi C. (1989). The detection *in situ* of *Agrobacterium tumefaciens* biovar 3 in grapevine. In: *Proceedings of the Seventh International Conference on Plant Pathogenic Bacteria*. (Ed. Z. Klement) pp. 851-856. Budapest, Hungary.
- Stewart F.M. & Levin B.R. (1973). Partitioning of resources and the outcome of interspecific competition: a model and some general considerations. *The American Naturalist* **107**, 171-198.
- Stonier T. (1960). *Agrobacterium tumefaciens* Conn II. Production of an antibiotic substance. *Journal of Bacteriology* **79**, 889-898.
- Szegedi, E. (1985). Host range and specific L(+)-tartrate-utilization of biotype 3 of *Agrobacterium tumefaciens*. *Acta Phytopathologica Academiae Scientiarum Hungaricae* **20**, 17-22.
- Tarbah F.A. & Goodman R.N. (1986). Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. *Plant Disease* **70**, 566-568.
- Tarbah F.A. & Goodman R.N. (1987). Systemic spread of *Agrobacterium tumefaciens* biovar 3 in the vascular system of grapes. *Phytopathology* **77**, 915-920.
- Tarbah F.A. & Goodman R.N. (1988a). Anatomy of tumor development in grape stem tissue inoculated with *Agrobacterium tumefaciens* biovar 3, strain AG63. *Physiological and Molecular Plant Pathology* **32**, 455-466.

- Tate M.E., Murphy P.J., Roberts W.P. & Kerr A. (1979). Adenine N6-substituent of agrocin 84 determines its bacteriocin-like specificity. *Nature* **280**, 697-699.
- Tempé J. & Goldmann A. (1982). Occurrence and biosynthesis of opines. In: *Molecular Biology of Plant Tumors*. (Eds. G. Kahl & J.S. Schell) pp. 427-449. Academic Press, New York.
- Tempé J., Petit A., Holsters M., Van Montagu M. & Schell J. (1977). Thermosensitive step associated with transfer of the Ti plasmid during conjugation: possible relation to transformation in crown gall. *Proceedings of the National Academy of Sciences USA* **74**, 2848-2849.
- Tepfer D., Goldman A., Pamboukdjian N., Maille M., Lepingle A., Chevalier D., Dénarié J. & Rosenberg C. (1988). A plasmid of *Rhizobium meliloti* 41 encodes catabolism of two compounds from root exudates of *Calystegium sepium*. *Journal of Bacteriology* **170**, 1153-1161.
- Thies K.L., Griffin D.E., Graves C.H. & Hegwood C.P. Jr. (1991). Characterization of *Agrobacterium* isolates from muscadine grape. *Plant Disease* **75**, 634-637.
- Thomashow L.S., Reeves S. & Thomashow M.F. (1984). Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proceedings of the National Academy of Sciences* **81**, 5071-5075.
- Thomashow M.F., Karlinsey J.E., Marks J.R. & Hurlbert R.E. (1987). Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *Journal of Bacteriology* **169**, 3209-3216.
- Thomashow M.F., Panagopoulos C.G., Gordon M.P. & Nester E.W. (1980). Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. *Nature* **283**, 794-796.

- Toro N., Datta A., Yanofsky M. & Nester E. (1988). Role of the overdrive sequence in T-DNA border cleavage in *Agrobacterium*. Proceedings of the National Academy of Sciences **85**, 8558-8562.
- Tukey J.W. (1953). The problem of multiple comparison. Mimeographed publication of limited circulation.
- Turk S.C.H.J., Nester E.W. & Hooykaas J.J. (1993). The *virA* promoter is a host-range determinant in *Agrobacterium tumefaciens*. Molecular Microbiology **7**, 719-724.
- Van Etten H.D., Matthews D.E. & Matthews P.S. (1989). Phytoalexin detoxification: importance for pathogenicity and practical implications. Annual review of Phytopathology **27**, 143-164.
- Van Larebeke N., Genetello C., Schell J., Schilperoort R.A., Hermans A.K., Hernalsteens J.P. & Van Montagu M. (1975). Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature **255**, 742-743.
- Vance C.P., Kirk T.K. & Sherwood R.T. (1980). Lignification as a mechanism of disease resistance. Annual Review of Phytopathology **18**, 259-288.
- Voisard C., Keel C., Haas D. & Défago G. (1989). Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. European Molecular Biology Organization Journal **8**, 351-358.
- Volk W. (1969). Applied Statistics for Engineers, 2nd Edition. McGraw-Hill Book Company, New York.
- Wample R.L., Barry A. & Burr T.J. (1991). Heat tolerance of dormant *Vitis vinifera* cuttings. American Journal of Enology and Viticulture **41**, 67-72.
- Wang K., Herrera-Estrella L., Van Montagu M. & Zambryski P. (1984). Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell **38**, 455-462.

- Watson B., Currier T.C., Gordon M.P., Chilton M. & Nester E.W. (1975). Plasmid required for virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **123**, 255-264.
- Webster J., dos Santos M.D. & Thomson J.A. (1986). Agrocine-producing *Agrobacterium tumefaciens* strain active against grapevine isolates. *Applied and Environmental Microbiology* **52**, 217-219.
- White F.F. & Nester E.W. (1980). Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. *Journal of Bacteriology* **141**, 1134-1141.
- White L.O. (1972). The taxonomy of the crown-gall organism *Agrobacterium tumefaciens* and its relationship to Rhizobia and other Agrobacteria. *Journal of General Microbiology* **72**, 565-574.
- Willmitzer L., De Beuckeleer M., Lemmers M., Van Montagu M. & Schell J. (1980). DNA from Ti plasmid present in nucleus and absent from plastids of crown gall tumours. *Nature* **287**, 359-361.
- Wilson E.E. & Magie A.R. (1964). Systemic invasion of the host plant by the tumor-inducing bacterium, *Pseudomonas savastanoi*. *Phytopathology* **54**, 576-579.
- Winans S.C. (1992). Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiological Reviews* **56**, 12-31.
- Winans S.C., Ebert P.R., Stachel S.E., Gordon M.P. & Nester E.W. (1986). A gene essential for *Agrobacterium* virulence is homologous to a family of positive regulatory loci. *Proceedings of the National Academy of Sciences USA* **83**, 8278-8282.
- Winans S.C., Kerstetter R.A. & Nester E.W. (1988). Transcriptional regulation of the *virA* and *virG* genes of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **170**, 4047-4054.
- Yadav N.S., Vanderleyden J., Bennett D.R., Barnes W.M. & Chilton M.-D. (1982). Short direct repeats flank the T-DNA on a nopaline Ti plasmid. *Proceedings of the National Academy of Sciences USA* **79**, 6322-6326.

- Yanofsky M. & Nester E.W. (1986). Molecular characterization of a host-range-determining locus from *Agrobacterium tumefaciens*. *Journal of Bacteriology* **168**, 244-250.
- Yanofsky M., Lowe B., Montoya A., Rubin R., Krul W., Gordon M. & Nester E. (1985a). Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. *Molecular and General Genetics* **201**, 237-246.
- Yanofsky M., Montoya A., Knauf V., Lowe B., Gordon N. & Nester E. (1985b). Limited-host-range plasmid of *Agrobacterium tumefaciens*: molecular and genetic analysis of transferred DNA. *Journal of Bacteriology* **163**, 341 -348.
- Yanofsky M.F., Porter S.G., Young C., Albright L.M., Gordon M.P. & Nester E.W. (1986). The *virD* operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell* **47**, 471-477.
- Zambryski P.C. (1992). Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 465-490.
- Zaprometov M.N. (1992). On the functional role of phenolic compounds in plants. *Soviet Plant Physiology* **39**, 802-809.