UNIVERSITY OF ADELAIDE
21-5-02
WAITE CAMPUS LIBRARY

BIOLOGICAL CONTROL OF CROWN GALL DISEASE IN AUSTRALIAN GRAPEVINE NURSERIES

ALEXANDRA KEEGAN

B.Sc. (Hons.) Flinders University

Thesis submitted for the degree of

Doctor of Philosophy

in

The University of Adelaide

(Faculty of Agricultural and Natural Resources)

September, 2001.

TABLE OF CONTENTS

Acknowledgements		I
Declaration		II
Abbreviations		III
Summary		IV
CHAPTER 1	**LITERATURE REVIEW**	
1.1	Introduction	1
1.2	Causative organism of crown gall disease	1
1.3	Host-pathogen interactions	3
1.3.1	Bacterial attraction to wound sites	3
1.3.2	Ti plasmid genes	4
1.3.3	Virulence genes	5
1.3.4	Opine production	6
1.3.5	Host range of agrobacteria	7
1.4	T-DNA oncogenes	7
1.5	Symptoms of crown gall disease on grapevine	8
1.6	Survival of *A. vitis*	9
1.6.1	Survival of *A. vitis* in soil and plant tissue	9
1.6.2	Endophytic survival in grapevine	10
1.7	Importance of crown gall disease to the grapevine industry	11
1.8	Management of crown gall in vineyards	12
1.8.1	Current methods of crown gall control	13
1.8.2	Thermotherapy	13
1.8.3	Culture of pathogen free grapevines	14
1.9	Biological control of plant diseases	16
1.9.1	Factors affecting biocontrol activity	16
1.9.2	Antibiosis	17
1.9.3	Induced resistance	18
1.10	Biological control of crown gall on almond and stone fruits	18
1.11	Mechanisms of control for K84	19

1.12	Biocontrol strains for control of grapevine crown gall disease	20
1.13	The role of bacterial attachment in the control of crown gall disease	21
1.14	Aims of the study	23
CHAPTER 2	**GENERAL LABORATORY TECHNIQUES**	
2.1	Culture and storage of bacterial strains	24
2.1.1	Culture of bacterial strains	24
2.1.2	Storage of bacterial strains	24
2.1.3	Bacterial strains	24
2.1.4	Growth media	26
2.2	Bacterial growth rates	26
2.3	Isolation and enumeration of Agrobacteria from soil	27
2.4	Stonier's assay for detection of antagonism	27
2.5	Plasmid isolation and visualisation	28
2.6	Genetic modification of bacteria	28
2.6.1	Production of spontaneous rifampicin mutants	28
2.6.2	Insertion of the green fluorescent protein marker	28
2.6.3	Certification of the GFP transconjugants and rifampicin mutants	29
2.7	Growth of inoculum for field trials	32
2.7.1	Broth culture	32
2.7.2	Peat cultures	32
2.8	Pathogenicity testing on Tomato	32
2.9	Polymerase Chain Reaction (PCR) detection of *A. vitis*	33
2.9.1	DNA preparation	33
2.9.1.1	Cell lysate preparation method 1	33
2.9.1.2	Cell lysate preparation method 2	33
2.9.1.3	DNA purification from soil	34
2.9.2	PCR detection of *A. vitis*	34
2.9.3	Multiplex PCR	36
2.10	DNA fingerprinting with ERIC PCR	36

2.11	Immunological detection of *A. vitis*	37
2.12	Tissue culture of plantlets	38
2.12.1	Tissue culture of tomato seedlings	38
2.12.2	Tissue culture of *Arabidopsis thaliana*	38
2.13	Enumeration of nematodes from soil	38
2.14	Bacterial attachment assays	39
2.14.1	Basic attachment assay	39
2.14.2	Addition of excess bacteria	39
2.14.3	Interaction of biological control and pathogenic strains	40
2.14.4	Microwave preparation of soil	40
2.14.5	Root colonisation by *Agrobacterium* strains	40
2.14.5.1	Recovery of bacteria from roots	41
2.14.5.1.1	Loosely bound bacteria	41
2.14.5.1.2	Tightly bound bacteria	41
2.15	Grapevine material	42
2.15.1	Varieties, suppliers and dormancy	42
2.15.2	Storage of grapevines	42
2.15.3	Thermotherapy of grapevine cuttings	42
2.15.4	Callusing of grapevine cuttings	43
2.15.5	Inoculation f callused grapevine cuttings	44
2.15.6	Re-isolation of inoculated strains from callus material	44
2.15.7	Assessment of grapevine rootlings for disease	44
2.16	Isolation and quantification of Agrobacteria from galls	44
2.17	Extraction of vascular fluid from grapevine segments	45
2.18	Tissue culture of grapevine material	45
CHAPTER 3	**CHARACTERISATION AND DETECTION OF *A. vitis* AND BIOCONTROL STRAINS**	
3.1	Introduction	49
3.1.1	Detection of *A. vitis*	49
3.1.2	Characterisation of biocontrol isolates	51
3.2	Experimental Protocols	54
3.2.1	Characterisation of biocontrol isolates	54

3.2.1.1	Standardised bacterial levels	54
3.2.1.2	Selective media analysis	54
3.2.1.3	Pathogenicity testing on tomato	54
3.2.1.4	Plasmid screening of bacterial isolates	55
3.2.1.5	Antagonistic activity of bacterial isolates	55
3.2.1.6	DNA fingerprinting with ERIC-PCR	55
3.2.1.7	ERIC fingerprinting of K84 and plasmid cured derivatives	56
3.2.2	Detection of biocontrol and pathogenic *A. vitis* from grapevine	56
3.2.2.1	PCR detection of *A. vitis*	56
3.2.2.2	Multiplex polymerase chain reaction	57
3.2.2.3	Detection of *A. vitis* from vascular washing fluid	57
3.3	Results	58
3.3.1	Standardised bacterial numbers	58
3.3.2	Selective media analysis	58
3.3.3	Pathogenicity testing on tomato	59
3.3.4	Plasmid screening of bacterial isolates	62
3.3.5	Antagonistic activity of bacterial isolates	62
3.3.6	Pathogen selection for experimental use	65
3.3.7	PCR based detection of *A. vitis*	66
3.3.7.1	Individual primer set	66
3.3.7.2	Multiplex PCR	66
3.3.8	Detection of *A. vitis* from vascular washing fluid	69
3.3.9	DNA fingerprinting with ERIC-PCR	69
3.3.10	ERIC fingerprints of K84 and plasmid cured derivatives	69
3.4	Discussion	72
CHAPTER 4	**ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSERY: TRIAL 1 (1996-1997)**	78
4.1	Introduction	78
4.1.1	Field trial aims	81

4.2	Experiments	82
4.2.1	Field trial organisation	82
4.2.1.1	Grapevine variety and treatment	82
4.2.1.2	Field site	82
4.2.1.3	Field site organisation	83
4.2.2	Level of inoculum applied to grapevine cuttings	83
4.2.3	Colonisation of biocontrol agents on grapevine callus	83
4.2.4	Identification of *A. vitis* from callus material	84
4.2.5	Assessment of *A. vitis* populations in grapevine nursery soil	84
4.2.6	Efficacy of biocontrol agents in a grapevine nursery	86
4.2.7	Effect of thermotherapy on grapevines	86
4.2.8	Examination of field trial site for *Meloidogyne*	86
4.3	Results	88
4.3.1	Levels of inoculum applied to grapevines	88
4.3.2	Enumeration of levels of biological controls on grapevine callus	88
4.3.3	Assessment of *A. vitis* in nursery soil	89
4.3.4	Effect of thermotherapy on grapevine growth	90
4.3.5	Efficacy of biocontrol agents	90
4.3.6	Bacterial levels isolated from grapevine sap	90
4.3.7	Examination of field trial site for *Meloidogyne*	91
4.4	Discussion	95
CHAPTER 5	**ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSERY: TRIAL 2 (1997-1998)**	98
5.1	Introduction	98
5.1.1	Aims	99
5.2	Experiments	100
5.2.1	Field trial organisation	100
5.2.1.1	Vine variety selection and treatment	100
5.2.1.2	Inoculum preparation and application	100

5.2.1.3	Pathogen selection	101
5.2.1.4	Site selection	101
5.2.2	Inoculum levels in peat culture	101
5.2.3	Inoculum applied to callused grapevine cuttings	101
5.2.4	Field trial treatment groups and organisation	102
5.2.5	Bacterial colonisation of callused grapevine cuttings	102
5.2.6	Effect of biocontrol bacteria on gall frequency, position and size	104
5.2.7	Effect of thermotherapy and biocontrol application on vine growth	104
5.2.8	Effect of biocontrols, thermotherapy and variety on root development	104
5.2.9	Pathogenic colonisation of grapevine sap in the presence of biocontrol bacteria	104
5.3	Results	106
5.3.1	Inoculum levels in peat	106
5.3.2	Inoculum applied to callused grapevine cuttings	106
5.3.3	Bacterial colonisation of callused grapevine cuttings	107
5.3.4	Efficacy of biocontrol bacteria and thermotherapy on gall reduction	107
5.3.5	Effect of biocontrol agents on gall position and size	112
5.3.6	Effect of thermotherapy and biocontrol inoculation on vine growth	114
5.3.7	Pathogen colonisation of grapevine sap in the presence of biocontrol strains	118
5.4	Discussion	120
CHAPTER 6	**ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSEY:TRIAL 3 (1998-1999)**	**125**
6.1	Introduction	125
6.2	Experiments	
6.2.1	Field trial organisation	126

6.2.1.1	Vine variety selection and treatment	126
6.2.1.2	Inoculum preparation and application	126
6.2.1.3	Site selection and preparation	126
6.2.2	Inoculum levels in peat cultures	126
6.2.3	Inoculum applied to callused grapevine cuttings	126
6.2.4	Field trial treatment groups and organisation	127
6.2.5	Bacterial colonisation of callused grapevine cuttings	127
6.2.6	Efficacies of biocontrol bacteria on gall frequency, position and size	127
6.2.7	Effect of thermotherapy and biocontrol application on vine growth	129
6.2.8	Effect of biocontrol strains on canopy development	129
6.3	Results	130
6.3.1	Inoculum levels in peat	130
6.3.2	Inoculum applied to callused grapevine cuttings	130
6.3.3	Bacterial colonisation of callused grapevine cuttings	131
6.3.4	Efficacies of biocontrol bacteria on gall frequency, position and size	131
6.3.4.1	Non heat-treated vines	131
6.3.4.2	Heat-treated grapevines (10 minutes)	134
6.3.4.3	Heat-treated grapevines (20 minutes)	134
6.3.5	Assessment of biocontrol effect on gall position and size	135
6.3.5.1	Gall diameter	135
6.3.6	Effect of thermotherapy and biocontrol application on vine growth	137
6.3.7	Effect of biocontrol strains on canopy development	140
6.4	Discussion	142
CHAPTER 7	**THE ROLE OF BACTERIAL ATTACHMENT IN THE BIOLOGICAL CONTROL OF CROWN GALL ON GRAPEVINE**	145
7.1	Introduction	145
7.1.1	Aims	148

7.2	Experiments	149
7.2.1	Genetic modification of bacteria	149
7.2.2	Survival of bacteria in CAS medium	
7.2.3	Microscopic observation of bacterial attachment on plant root surfaces	150
7.2.4	Bacterial attachment on plantlet roots	150
7.2.5	Interaction of biocontrol and pathogenic bacteria	151
7.2.6	Microscopic observation of bacterial interaction on grapevine roots	152
7.2.7	Bacterial colonisation of *Vitis Vinifera* plantlets	152
7.3	Results	154
7.3.1	Fitness of genetically modified bacteria	154
7.3.2	Bacterial survival in CAS broth	155
7.3.3	Colonisation of root surfaces	159
7.3.4	Bacterial attachment on tissue cultured plant roots	159
7.3.5	Interaction between biocontrol agents and pathogens	160
7.3.5.1	Bacterial colonisation of tomato roots	161
7.3.5.2	Bacterial colonisation of grapevine roots	161
7.3.6	Microscopic observation of bacterial interactions on grapevine	163
7.3.7	Bacterial colonisation of *V. Vinifera* plantlets	167
7.3.7.1	Individual bacterial isolates on plantlets	167
7.3.7.2	Co inoculation of grapevines with biocontrol and pathogenic *A. vitis*	169
7.4	Discussion	171
CHAPTER 8	**GENERAL DISCUSSION**	177
	APPENDICES AND BIBLIOGRAPHY	187
	PUBLICATIONS AND PRESENTATIONS	206

ACKNOWLEDGEMENTS

I would like to thank my four PhD supervisors; although getting you all together in a room was difficult at times, we did manage. Firstly, Dr. Kathy Ophel Keller, for taking the time to introduce me to the finer points of molecular biology and useful discussion on crown gall disease. Next, Dr. Nick McClure, although further away from the action, provided support throughout the project with GFP marker systems and tissue cultured grapevines. Thanks to Dr. Peter Murphy for assistance in University matters and discussion groups which allowed many useful ideas to flow. Last but not least, Dr. Maarten Ryder, for allowing me to borrow everything in the lab and support for publication. Thanks to you all for your help and guidance throughout this project. To the staff and students in SARDI-Field Crops Pathology, thankyou for your time, assistance and friendship. A special thankyou to Professor Ann Matthysse, who took me into her lab at University of North Carolina for two months and taught me so much about bacterial attachment; interaction on plant surfaces and fluorescent microscopy. To Dr. Kerri Davies, thanks for your assistance with the nematode problems in the field trial. Thanks to Paul Wright and the staff at Orlando-Wyndham for thermotherapy and callusing on the final year's trial.

This project was supported by an Australian Postgraduate Award (Industry). I would like to thank the industry partners in this project Bio-Care Technology Pty. Ltd. and Temple Bruer Wines Pty. Ltd. Thankyou to both Gary Bullard and David Bruer for their undying enthusiasm for the project and new ideas.

Finally, I would like to thank my Mum, Joan Backhouse and my husband, Marc Keegan, both of whom have been extremely patient and understanding throughout this PhD with encouragement and support.

DECLARATION

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

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

SIGNED..

DATE.....10.9.01.................

ABBREVIATIONS

AB	AB minimal medium
ALS	Antibiotic like substance
bp	Base pairs
bv.	biovar
DNA	deoxyribonucleic acid
E26	*A. vitis* biocontrol strain
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERIC	Enterobacterial repetitive intergenic concensus
F2/5	*A. vitis* biocontrol strain F2/5
GFP	Green fluorescent protein
GTE	Glucose/tris/EDTA buffer
HLB2	*A. tumefaciens* biocontrol strain HLB2
K315	*P. fluorescens* biocontrol strain
K84	*A. rhizogenes* biocontrol strain
kbp	Kilo base pairs
Km	Kanamycin
LB	Luria Bertani (broth)
LHR	Limited host range
MQ	Milli Q water
NA	Nutrient agar
NB	Nutrient broth
OD_{600}	Optical density at 600nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pehA	Polygalacturonase A
Ri	Root inducing (plasmid)
Rif	Rifampicin
rRNA	ribosomal ribonucleic acid
RS	Roy and Sasser (medium)
SDDW	Sterile double distilled water
SDW	Sterile distilled water
ssDNA	Single stranded DNA
STE	Sucrose/Tris/EDTA (buffer)
TBE	Tris/borate/EDTA (buffer)
Tc	Tetracycline
T-DNA	Transfer DNA
TE	Tris/EDTA
Ti	Tumour inducing (plasmid)
TYA	Tryptone yeast agar
V	Volts
v/v	Volume per volume
*vir*A	Virulence
w/v	Weight per volume
WHR	Wide host range

SUMMARY

Crown gall disease on grapevine is common in most viticultural areas of the world. In the field the causal agent *Agrobacterium vitis* (formerly *A. tumefaciens* biovar 3) has been problematic for grapevine nurseries, vine improvement schemes and vineyards.

Agrobacterium vitis survives endophytically in the plant vascular system and in decaying grapevine matter. It has been easily disseminated via diseased propagating material, or infection from contaminated soils. In Australia, problems occur mainly in grapevine nurseries with galls developing at the crown and nodal sections below ground level and grafting unions.

Previously thermotherapy (hot water treatment) at 50°C for 20-30 minutes has been proven very effective at eliminating the pathogen from dormant grapevine cuttings. Although effective, when vines are planted into *A. vitis* contaminated soils, vines are readily reinfested over time.

This project has investigated the efficacy of four potential biological control bacteria to protect vines from reinfestation. The isolates F2/5 (*A. vitis*), E26 (*A. vitis*), HLB2 (*A. tumefaciens*) and K315 (*P. fluorescens*) were applied to heat-treated and non heat-treated dormant grapevine cuttings with pathogenic *A. vitis* and planted into field nurseries. Disease was assessed at nine months by the frequency of galling in 3 consecutive trials. Results have shown that F2/5 applied at 10^6 cfuml^{-1}, significantly reduced galling frequency by more than 50% when applied prior to the pathogen (10^6-cfu ml^{-1}) on callused cuttings. HLB2 and K315 (applied at 10^7 cfuml^{-1}) were also effective at controlling disease when applied at a higher rate than the pathogen.

Under field conditions E26 failed to protect grapevines from disease with no significant difference when compared to the pathogen alone treatment.

Diagnostic testing procedures were investigated in an attempt to allow faster detection of pathogenic *A. vitis* from grapevine samples. Previous tests using immunoblot and selective medium isolation were slow and laborious. Recent advances in PCR technology, allowed a multiplex PCR (modified from existing primers) to detect pathogenic and non-pathogenic *A. vitis* from a variety of sources, including soil, grapevine sap, callus and gall material using commercially available soil DNA purification kits. Together with quantification techniques, the test is faster and more sensitive for industry purposes.

The mechanism(s) of action of the biocontrol strains are currently unknown although previous research has indicated that agrocin production is not involved for F2/5. The role of bacterial competition for attachment was investigated using the roots of tissue culture grapevine plantlets. All strains were tested for attachment to the roots and showed positive binding. The use of green fluorescent protein (gfp) tagged pathogens in competition assays between biocontrol and agrocin resistant pathogens showed that pathogen was capable of colonising the root surface in the presence of a biocontrol agent. This was also observed in samples taken from the field.

F2/5 has been shown to be effective in controlling crown gall disease in glasshouse and field tests on grapevine. Through industry support, the development of this organism as a commercially available product would allow easy incorporation into current nursery management practices.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Crown gall disease of grapevine caused by *Agrobacterium vitis* (formerly *Agrobacterium tumefaciens* biovar 3) is common in most viticultural areas of the world (Panagopoulos and Psallidas, 1973: Sule 1978: Burr and Hurwitz, 1981). Crown gall causes high levels of damage to nursery stock and decreased productivity (Schroth *et al*, 1988) with galled rootlings weakened and discarded. In Australia, crown gall is primarily a problem for nurseries and vine improvement schemes, especially for some rootstock cultivars (Ophel *et al.*, 1988). *A. vitis* survives endophytically and in the plant rhizosphere. It can be disseminated via infected propagation material or clean material can become infested via diseased soils (Burr *et al.*, 1987a, Bishop *et al.*, 1988, Panagopoulos *et al.*, 1978, Burr and Katz, 1984).

1.2 Causative organism of crown gall disease

The causative organism of crown gall disease of grapevines is *Agrobacterium vitis* (Ophel and Kerr, 1990) formerly known as *Agrobacterium tumefaciens* biovar 3 (Kerr and Panagopoulos, 1977), belonging to the Family *Rhizobiaceae*. The species *A. vitis* are Gram negative, motile, non-sporing aerobic rods that are capable of invading plant tissue and living under reduced oxygen tension (Kersters and DeLey, 1984). All members of the *Agrobacterium* genus are capable of invading crown, roots and stems of a large range of dicotyledonous and some gymnospermous plants. Entry into the plant is via attraction to wound exudates and transformation of the plant cells is caused by incorporation of a segment of bacterial DNA into the plant nuclear genome. This results in autonomously proliferating tumour cells (Yanofsky *et al.*, 1985a).

Previously the delineation of *Agrobacterium* species was based on plasmid-borne characteristics and classified according to phytopathogenic characteristics. The genus was divided into three species, *Agrobacterium tumefaciens* (biovars 1, 2, and 3), *A. radiobacter* and *A. rubi* (Kersters and DeLey, 1984, Skerman, *et al.*, 1980). Reclassification of the genus was necessary as pathogenic and non-pathogenic isolates held high levels of genetic similarity using 16s rRNA fingerprinting (Sawada *et al.*, 1993) and *Agrobacterium* plasmids were found to be highly promiscuous (Holmes and Roberts, 1981). Comparisons of a short variable region of the 16S rRNA gene of type strains for agrobacterial species gave distinct differences between species (Sawada *et al.*, 1993). Pathogenic *A. vitis* have been shown to accept plasmids other than the Ti plasmids (tumour inducing) from the donor strain (Burr *et al.*, 1995). The genus is now divided into four species *A. tumefaciens* (formerly biovar 1), *A. rhizogenes* (formerly biovar 2), *A. vitis* (formerly biovar 3) and *A. rubi* being classified by chromosomal traits. The biovar 3 *A. tumefaciens* are sufficiently distinct from other agrobacteria to be recognised as a distinct species, *A. vitis* (Ophel and Kerr, 1990). *Agrobacterium vitis* was found to be most similar to *A. rubi* and most dissimilar to *A. tumefaciens* with 16s rRNA typing (Sawada *et al.*, 1992).

Agrobacterium vitis strains are capable of inhabiting only a limited number of dicotyledonous plants (Perry and Kado, 1982). In nature *A. vitis* infects only grapevines and not stone fruits, almond trees and roses which are infected by *A. tumefaciens* (Panagopoulos *et al.*, 1978). Although largely limited to grapevines, *A. vitis* has previously been isolated from *Chrysanthemum* (Bazzi and Rosciglione, 1982).

1.3 Host-pathogen interactions

Crown gall induced by tumorigenic *Agrobacterium* species is characterised by "autonomous outgrowths, which after initiation are independent of the bacterium and dependent of the host" (Lippincott and Lippincott, 1969). The infection and transformation of plants by agrobacteria is a complex process involving the transfer and incorporation of DNA from the bacteria into the plant nuclear genome.

1.3.1 Bacterial attraction to wound sites

Bacteria are attracted to plant wound exudates, which initiates the infection cycle. The genes involved in chemotaxis are located on the chromosome (Sheng and Citovsky, 1996) while genes facilitating the copying and transfer of DNA into the plant nuclear chromosome are located on a large Ti (tumour inducing) plasmid present in pathogenic agrobacteria.

Early in the initiation process, agrobacteria adhere to the wounded plant cell wall to allow transformation to take place (Matthysse, 1987). The attachment process is necessary for the infection cycle to take place as cellulose minus (Cel^-) and attachment minus (Att^-) mutants are less pathogenic than wild-type agrobacteria and are unable to attach to the leaf surface (Matthysse, 1987). Although attachment to the plant surface is reduced, the disease cycle may still proceed (provided the bacteria are not removed from the plant surface). The non-attaching mutants have less ability to cause disease unless inoculated directly into a wound site.

The cell surface characteristics of the infecting bacterium may allow attachment of bacteria to specific sites on the plant cell surface (Lippincott and Lippincott, 1969).

The involvement of extracellular lipopolysaccharides, surface-active agents specific to agrobacteria has been suggested (Stachel *et al.*, 1985). After adherence to the host cell, fibrils are produced forming aggregates as agrobacteria multiply within the host cell. Cellulose-minus mutant agrobacteria were capable of attaching to the plant cell but aggregation could not occur, preventing colonisation of the plant cells (Matthysse, 1987). The attachment loci (*chvA, chvB, pscA (exoC)* and *att*) are all located on the chromosome and are essential for binding while genes involved in DNA transfer are located on the Ti (tumour inducing) plasmid (recently reviewed in Sheng and Citovsky, 1996).

1.3.2 Ti plasmid genes

Plasmids involved in the transfer of DNA from bacteria to the plant host are known as tumour inducing (Ti) or root inducing (Ri) plasmids (responsible for hairy root disease). Ti plasmids contain two important genetic components required for the initiation of tumours on plants:

1. T-DNA (transfer-DNA), the DNA transferred into the plant cell that encodes the synthesis of two growth regulators, auxin and cytokinin as well as opines (Nester *et al.*, 1991).
2. Virulence (*vir*) region: involved in the processing and transfer of T-DNA, but is not expressed when agrobacteria grow in the absence of plant cells.

A. vitis plasmids make up four diverse categories classified based on opine production and utilisation, number of T-DNA segments and size. The categories are:

1. Octopine/cucumopine type Ti plasmids with large TA-DNA regions

2. Octopine/cucumopine type Ti plasmids with small TA-DNA regions
3. Nopaline type Ti plasmids
4. Vitopine type Ti plasmids

TA-DNA refers to one of the two T- DNA's on the plasmids (Burr and Otten, 1999).

1.3.2 Virulence genes

Virulence genes are carried on the Ti plasmid and encode the known products that mediate T- DNA transfer from the Ti plasmid into the plant cells (recently reviewed in Sheng and Citovsky, 1996). These genes are not included in the segment of DNA transferred into the plant host. The *vir* region consists of seven complementation groups *virA*, *virB*, *virC*, *virD*, *virE*, *virG* and *virH* (Zambryski, 1992). *Vir* gene induction is via low molecular weight phenolics such as acetosyringone for *A. tumefaciens.* For *A. vitis* isolates, identification of inducer molecules showed that acetosyringone very weakly induced tumorigenesis while the methylated form (commonly found in vine wound exudates) induced virulence activity at a higher rate (Spencer *et al.*, 1990, Stachel *et al.*, 1985). *A. vitis* virulence genes differ greatly from the well studied *A. tumefaciens* genes. The vitopine producing strains lack DNA homology with known T-DNA genes under standard hybridisation conditions and only 50-70% homology between pTiS4 and pTiC58 (Gérard *et al.*, 1992).

Following induction of virulence genes a segment of Ti plasmid DNA is copied and integrated into the plant host genome. A copy of the T-DNA region is generated and is flanked by specific sequences (25 base pair imperfect direct repeats). This single stranded DNA (ss-DNA) is transferred with the help of *virD* and *virC* genes as a DNA/protein complex through the bacterial cell membrane, cell wall, plant cell wall and nuclear membranes to be integrated into the plant host genome (Sheng and Citovsky, 1996). Several loci located on the T-DNA border regions are important for pathogenicity and hormone production.

Both *virA* and *virG* are required for efficient induction of the remaining *vir* loci (Stachel and Zambryski, 1986). *Vir A* acts as an environmental sensor of plant derived inducer molecules secreted by wounded plant cells, acting in concert with *virG* to induce the other *vir* loci (Spencer *et al*, 1990). In the presence of a signal molecule *virA* undergoes autophosphorylation and transfers a phosphate group to the *virG* protein. This acts as a transcriptional regulator for the other *vir* genes (Sheng and Citovsky, 1996)

1.3.4 Opine production

Agrobacterial transformation of plant tissue causes the transformed cells to produce specific opines (Petit and Tempe, 1978, reviewed by Ream, 1989). Transformed plant cells synthesise and secrete particular opine(s) and the inducing bacteria carrying Ti-plasmid-borne genes are able to catabolise the synthesised opine. This may create a niche for agrobacteria to colonise the wound site as the produced opine is favourable for agrobacterial growth. Other bacteria are capable of opine catabolism (reviewed in Ream, 1989) which can result in competition for nutrients at the infection site. After primary infection has occurred the grapevine is more susceptible to secondary infection by other pathogens such as *Pseudomonas* species and fungi (Schroth *et al.*, 1988).

Agrobacteria respond to the expression of the T-DNA and opine synthesis with induced opine degradation (Kerr and Ellis, 1982; Klapwijk and Schilperoot, 1978; Tempe and Petit, 1982). The opines are specifically degraded by the bacteria acting as carbon and nitrogen sources, causing tumour formation and allowing bacteria to survive and proliferate within the gall (reviewed by Ream, 1989).

1.3.5 Host range of agrobacteria

It has been well documented that *A. vitis* shows strong host specificity to grapevines (Pangopoulos and Psallidis, 1973: Knauf *et al.*, 1983: Ma *et al.*, 1987). These strains are characterised as limited host range (LHR) and their Ti plasmids differ to wide host range (WHR) strains (Loper and Kado, 1979, Thomashow *et al.*, 1990) that are capable of colonisation of a wider range of plant varieties. Alterations in the *virA* gene product between *A. vitis* and other *Agrobacterium* species have been found to limit host range. The LHR has been transformed to WHR by the incorporation of an *A. vitis virG* gene. This increased the host range of a LHR plasmid and increased the tumorigenic potential on a wider range of plants (Jin *et al.*, 1987: Yanofsky *et al.*, 1985). The *virA* gene of LHR strains has only 45% homology to WHR *A. vitis* (reviewed in Burr and Otten, 1999).

1.4 T-DNA oncogenes

The main tumour inducing genes of octopine and nopaline strains of *A. tumefaciens* are *ipt* and *iaa*. These genes are responsible for the synthesis of cytokinins (*ipt*) and indoleacetic acid (*iaa*) in a two step process. For *A. vitis* isolates, the LHR plasmids do not have active *ipt* genes (normally found in WHR *A. tumefaciens*) on the T-DNA that is transferred into the plant host. The tumorous growth on grapevines does not require the presence of functional cytokinin genes, allowing the transfer of auxin genes into the plant cell. This is sufficient to induce cytokinin accumulation from the plant (Yanofsky *et al.*, 1985). Introduction of this gene into a LHR strain induced a hypersensitive response preventing gall induction (Buckholtz and Thomashow, 1984, Hoekema *et al.*, 1984).

A. vitis are specialised for existence within the grapevine because they use tartrate and malate as carbon sources (Szegedi *et al.*, 1992, Knauf *et al.*, 1983, Matsumoto *et al.*, 1992). High levels of tartrate within grapevine sap act as a selective carbon and nutrient source for *A. vitis* (Szegedi, 1985: Szegedi *et al.*, 1992, Ruffner, 1982), with many *A. vitis* strains containing tartrate utilising regions on plasmids (Salomone *et al.*, 1996: Gallie *et al.*, 1984). Specific differences in the virulence region on the Ti plasmid compared to other species of *Agrobacterium* resulted in poor tumour initiation for the attachment and transfer of T-DNA into the host when using plants other than grapevines (Yanofsky *et al.*, 1985a, Yanofsky *et al.*, 1985b).

1.5 Symptoms of crown gall disease on grapevine

Symptoms of *A. vitis* infection on grapevine can vary greatly (Schroth *et al.*, 1988). Infection can result in gall formation, reduced growth, stunting or no apparent symptoms. The growth and yield of vines infested with pathogenic *A. vitis* was assessed on the variety Zinfandel. The affects of crown gall disease on the grapevine was greatest when more than 50% of the trunk (circumference) was galled with significant decreases in grapevine growth (Schroth *et al.*, 1988). Infected vines can be asymptomatic or if the vine was infected in the first 2-3 years after transplantation the whole plant may die (Bazzi *et al.*, 1987).

In general, the infection of *A. vitis* develops at the base of cuttings, disbudding sites and graft unions (Bazzi *et al.*, 1987). Gall formation can also occur from wounds caused by mechanical damage during harvesting (Baldini and Interneri, 1982) and canopy management. Aerial galls occur in cold climate viticulture due to freeze fracturing of the vine (Baldini and Interneri, 1982; Tarbah and Goodman, 1986). The

bacteria attach to wounded plant tissue, which allows extensive colonisation of the site. Often the secondary invasion by other grapevine pathogens such as infestation of *Pseudomonas syringae* pv. *syringae*, or insect borers has a greater effect than crown gall on the vine after initial weakening of the vine from *A. vitis* infestation (Schroth *et al.*, 1988).

A grapevine specific necrosis has been observed on vines infected with *A. vitis* (Burr *et al.*, 1987b) and shown to be responsible for root specific decay via polygalacturonase activity (Burr *et al.*, 1987c). Both tumorigenic and non-tumorigenic *A. vitis* induce necrosis in 24-48 hours on all *Vitis* species, but not on other plants (Burr *et al.*, 1987b, Sule *et al.*, 1994).

1.6 Survival of *A. vitis*

1.6.1 Survival of *A. vitis* in soil and plant tissue

The survival of *A. vitis* in soil has been found to be closely associated with the presence of decaying grape debris (Burr *et al.*, 1988). The bacterium is limited to areas previously used as vineyards or grapevine nurseries (Burr and Katz, 1983, Bishop *et al.*, 1988). When soil (with no prior exposure to grapevines) was artificially inoculated with pathogenic *A. vitis* the pathogen was not detected (Bishop *et al.*, 1988). When the populations of *A. vitis* in living and cleared vineyards were measured there was no significant difference in the population of the pathogens between soils. This indicates that the bacteria are stably maintained within decaying grapevine material (Burr *et al.*, 1987a, Burr *et al.*, 1995). In glasshouse trials *A. vitis* was detected in soil for over twenty-three months (Burr *et al.*, 1995). Vineyard trials following the infestation of soil with *A. vitis* colonised roots and canes resulted in detection of the bacteria for at least two years (Burr *et al.*, 1995). Plasmids remained

intact in specific isolates with no galls induced in the grapevine material. This demonstrated that *A. vitis* were able to survive and stably maintain plasmids in decaying grapevine tissue. Research on other grapevine diseases has demonstrated that decaying root material remaining in the soil after vine harvest can harbour pathogenic organisms (Raskie *et al.*, 1983) highlighting the need for a method to protect of the vine from infestation by pathogenic *A. vitis*.

1.6.2 Endophytic survival in grapevine

In grapevine nurseries, cuttings are taken from symptomless mother vines that are assumed to be pathogen free for use as propagation material. However, *A. vitis* can survive asymptomatically within the vascular system (Lehoczky, 1978 Panagopoulos *et al.*, 1978; Burr and Katz, 1983) leading to transmission of the pathogen to uncontaminated areas (Gillings and Ophel-Keller, 1995; Burr and Katz, 1983). *A. vitis* has been detected in most *Vitis* species, cultivars and rootstocks in the United States (Goodman *et al.*, 1987). A similar result was found in Australia with varying levels of the pathogen being found in South Australia, Western Australia, Victoria and New South Wales (Gillings and Ophel-Keller, 1995).

In a survey of Australian grapevine material, 50-100% of cuttings were infected in vine nurseries while rootstocks and scions had 10-40% infection (Ophel *et al.*, 1988b). As the pathogen exists as an endophyte, crown gall can be transmitted to disease free areas. This has occurred in many vine nurseries in South Australia with 50-80% of vines infected in one season (Ophel *et al.*, 1988). The bacteria reside mainly in the xylem but also the phloem of the grapevine (Sule, 1986) and the layer of tissue underneath the bark on dormant cuttings (Jager *et al.*, 1989). Without prior

treatment of grapevine cuttings and material, *A. vitis* can be easily transferred to disease free areas.

1.7 Importance of crown gall disease to the grapevine industry

Crown gall disease of grapevine occurs worldwide and is a major concern for grapevine nurseries. Galled material cannot be sold and transport of infected material between states in Australia is controlled by inspection before sale (D. Haeusler, pers. comm.). In the late 1980's some nurseries reported 80% of some rootlings affected by the disease (Ophel Keller, pers. comm.). The restriction of movement also decreases the likelihood of other grapevine diseases such as phylloxera and grapevine yellows moving between grape-growing areas of Australia. For quarantine purposes the bacteria are usually detected by the presence of a gall on the stem of the cutting. As vines can be asymptomatic further testing is required to detect pathogenic *A. vitis* using vascular washing, semi-selective media, ELISA detection or PCR. In recent years through the use of alternative nursery management systems, reductions in gall frequencies have been observed. The bacterium is still present within the soil and can problematic which has resulted in galls occurring on thermotherapy treated grapevine rootlings (Haeusler, pers. comm.).

1.8 Management of crown gall in vineyards

Management practices affecting crown gall disease in vineyards include crop and canopy management, site and variety selection. In general, rootstocks of *Vitis riparia* and *V. rupestris* are more resistant to crown gall than *V. vinifera*. In South Australia, an increase in the incidence of crown gall disease was associated with the use of American rootstocks Ramsey and K51-40 from 1980 onwards. These rootstocks

confer the advantages of being salt and nematode tolerant and are ideal for some grape-growing districts (Riverland South Australia, Victoria and New South Wales) but are very susceptible crown gall infections often occurring at the graft union. *Vitis vinifera* cultivars are highly susceptible to crown gall but this varies between cultivars (Ferriera and van Zyl, 1986, Sule, 1994). Italian and French vineyards show particularly severe disease on *V. vinifera* that are more susceptible than intra specific hybrids and American cultivars such as Ramsey and Teleki currently used in Australia (Bazzi *et al.*, 1987). These varieties remain susceptible to the disease when wound sites and graft unions are exposed to the bacterium.

Currently at Temple Bruer Nursery, (Langhorne Creek, South Australia) soil used for grapevine propagation is rotated out of use for at least five years before replanting with grapevine (D. Bruer, pers. comm.). This process is costly as virgin soil is required each year and cannot be reused for an extended period.

Alternately, when grafted material is used, cuttings are planted into biodegradable paper pots with pasteurised soil and grown in glasshouses before transplant to the vineyard (P. Wright, Orlando-Wyndham). Grafting material is expensive and also susceptible to crown gall due to wounding that occurs at the graft union. When used in conjunction with the biodegradable pot system, frequency of galling events has been reduced to very low levels (5 /800,000 rootlings) when used in conjunction with thermotherapy (P. Wright, Orlando-Wyndham Vine Nursery, pers. comm.).

1.8.1 Current methods of crown gall control

A number of methods have been investigated for control of the crown gall pathogen that include methyl bromide application and the use of fumigants such as Vorlex®, with neither being effective (Goodman *et al.*, 1987, Pu and Goodman, 1993). Some bacterial and fungal antagonists tested against pathogenic *A. vitis* were also ineffective at disease control (Cooksey and Moore, 1980). Rotation of land out of use for at least five years and regular flooding of vineyards has been used as a land management practice for reducing the levels of root knot nematode (*Meloidogyne*) in the soil. This has the advantage of reducing levels of other pathogens within the soil such as *A. vitis* (D. Haeusler, Temple Bruer Wines, pers. comm.).

1.8.2 Thermotherapy

An alternative method of control of crown gall on grapevine is the use of thermotherapy, a heat treatment of 50°C for 30 minutes. Due to the systemic nature of *A. vitis* in grapevine, methods were sought to eliminate the pathogen from grapevine cuttings or rootlings by using hot water therapy on dormant vine cuttings (Burr *et al.*, 1989, Ophel *et al.*, 1990, Orffer, 1977). Hot water treatment showed great promise as a simple, economical and environmentally safe method for eradication of *A. vitis* (Burr *et al.*, 1989) reducing bacteria below detectable levels (Ophel *et al.*, 1990). However, low levels of bacteria can survive the treatment and manifestation of the disease can occur once planted into the vineyard. Potential sites of reinfestation include emergent roots and callus material. Reinfestation of the disease free grapevine can also occur on planting into vineyard with previous exposure to the pathogen.

Attempts to produce disease free grapevine material by increases in treatment time or temperature has been found to reduce bud burst or damage cuttings from various grapevine varieties (Orffer *et al.*, 1980, D. Haeusler, pers. comm., 1996, Burr *et al.*, 1989). This has also been associated with a reduction in callusing, delayed root initiation and inhibition of primary and secondary bud growth (Orffer *et al.*, 1979), although reports vary depending on geographical area and vine storage pre- and post-treatment (Wample, 1992).

Thermotherapy has been used on rootlings to eliminate soil and surface pathogens from vine cuttings and rootlings (D. Haeusler, pers. comm.). The removal of the residual soil from rootlings reduces numbers of other pathogens such as nematodes, fungal pathogens, insects and limits the spread of disease to other grape-growing areas (Orffer, 1977). However, once material has been treated it can be reinfected if it comes into contact with the pathogen (Goodman *et al.*, 1987). This requires that clean cuttings be planted into clean land without previous use for grapevine propagation.

1.8.3 Culture of pathogen free grapevines

Elimination of *A. vitis* using tissue culture has been investigated for control of crown gall disease (Goodman *et al.*, 1987). This was achieved by the use of tissue culture propagation using green shoots. Burr *et al.*, (1987c) found that early season green shoots were pathogen free if the mother vine was infested with *A. vitis*, however shoots produced later in the growing season were infected (Burr *et al.*, 1987c) allowing a window for collection of actively growing shoot tips that were pathogen free. Plants grown from green shoots were found to be pathogen free when planted

into potting soil and could maintain the pathogen free status for an extended period. When planted into pathogen infested soil, the vines were slowly infested with pathogenic *A. vitis* (Goodman *et al.*, 1987, Burr *et al.*, 1987c), indicating that although the vines were pathogen free initially, they were still susceptible to infection from the soil.

The reinfestation of clean grapevine material is the main factor affecting disease control in the vineyard. Disease free vines planted into vineyards often become infected. Biological control can complement the use of thermotherapy and tissue culture. Biological control strains for crown gall control on grapevines are capable of disease control in the glasshouse (Biggs, 1994). The introduction of a non-pathogenic biological control agent that will prevent reinfection of disease free vines would be a cost-effective means of disease control.

Prior to or co-inoculation of non-pathogenic agrobacterial cell wall preparations with pathogenic agrobacteria proved effective at inhibiting gall induction (Lippincott and Lippincott, 1969). This is indicative of the ability of non-pathogenic agrobacteria to occupy binding sites or prevent the attachment of pathogenic strains and inhibit the initiation of crown gall development.

1.9 Biological control of plant diseases

Biological control is the control of pests and weeds by other living organisms. Biological control has been investigated for its role in control of a variety of phytopathogens and restriction of diseases in plants. Many microorganisms have

been found to inhibit a range of plant pathogens using *in vitro* tests. These organisms include fungi, yeasts, and many species of soil bacteria including agrobacteria and *Pseudomonas* (Weller, 1988). Biological control mechanisms have been classified into three main areas that include antibiosis, competition and induced resistance (Cook and Baker, 1983).

1.9.1 Factors affecting biocontrol activity

These mechanisms can work independently but in general effective biocontrol has been shown to incorporate two or more modes of action. The addition of a biocontrol agent is a preventative measure and is not useful after the disease is present. A major problem for the use of biological control agents is performance in field trials. Testing isolates *in vitro* can be transferred to pot or glasshouse trials successfully, but transfer into field conditions can show inconsistencies (Cook, 1994).

The requirements for a useful and effective biocontrol agent include (a) plant host specificity and able to survive on plant tissue, (b) genetic stability, maintain biocontrol effectiveness, (c) low nutrient requirements (d) lack of pathogenicity on the host, (e) cost effectiveness, and (f) ease of incorporation into current management practices. The agent must tolerate the physical and chemical conditions within the plant host rhizosphere where it is introduced.

1.9.2 Antibiosis

Antibiosis is the production of antibiotics, volatile compounds and enzymes secreted by a biological control agent that are antagonistic towards a phytopathogen. The compounds produced can be toxic to pathogens resulting in cell death or other

metabolites that sequester nutrients from the soil such as manganese reductase, and siderophores (Leong, 1986). Antagonistic compounds may also have a role in induced resistance. Metabolites produced by the biocontrol organism stimulate the plant host defence system that reduces the ability of pathogens to infect the plant. This has been shown to be an important mechanism of biological control for *A. rhizogenes* K84 by the production of agrocins 84 (Kerr, 1980) and 434 (McClure *et al.*, 1994) along with good colonisation of the root surface (Shim *et al.*, 1987). *Pseudomonas fluorescens* strain 2-79 has been shown to use phenazine production and iron assimilation (Thomashow and Weller, 1988, Hamdan *et al.*, 1991). The production of other enzymes and compounds such as manganese reductase (Huber and McCay-Buis, 1993), cell wall lytic enzymes (Fridlender *et al.*, 1993), ammonia (Paulica *et al.*, 1978) and hydrogen cyanide (Astrom, 1991) have been involved in biological control activities.

Biological control agents may compete with pathogens for trace elements and nutrients to deplete the surrounding area of essential minerals and organic nutrients that are essential for growth and proliferation. Biocontrol agents may deprive the pathogen of essential elements for successful infection or lifecycle such as carbon, nitrogen and iron.

1.9.3 Induced resistance

Induced resistance in plants is the process of active resistance dependant on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (Kloepper *et al.*, 1992). The response of plants to the presence of an organism capable of aiding induced resistance includes the production of antimicrobial compounds such as

phytoalexins or synthesis of extra protective layers of lignin and cellulose (Elmer, 1995). Reviews by Tuzun and Kloepper, 1994 discuss the mechanisms in greater detail. Induced resistance has been observed for a variety of pathogens including viral, bacterial and fungal diseases on a wide range of plants (reviewed in Tuzan and Kloepper, 1994).

1.10 Biological control of crown gall on almond and stone fruits

In Australia and America, an agent for the biological control of crown gall on stone fruits, roses and nut trees is in commercial use (Kerr and Tate, 1984). *Agrobacterium radiobacter* strain K84 was developed as a biocontrol agent against *Agrobacterium tumefaciens* biovars 1 and 2 pathogens containing nopaline, agrocinopine A and octopine/agropine type plasmids, and has successfully controlled crown gall on stone-fruits, almonds and roses (Ellis and Murphy, 1981, Fermin and Fenwick, 1978). Biocontrol activity of strain K84 involves the production of agrocins (antibacterial compounds) one of which is actively transported into pathogens containing nopaline type Ti plasmids (agrocin 84) (Clare *et al.*, 1990). Control is achieved by dipping seedlings, seeds or cuttings in an inoculum containing K1026 (commercially available No Gall®) (Clare, 1990). The bacteria are capable of colonising the root system and are effective at reducing crown gall disease on various plants. The use of this agent has been shown to be more effective than use of commercially available antibiotic therapy and more cost effective (Schroth *et al.*, 1971, Moore, 1977). Disease control was 100 percent in naturally infested soils (Moore, 1977).

1.11 Mechanisms of control for K84

Two main mechanisms of biological control exist with *Agrobacterium radiobacter* K84. The first is the production of agrocins with agrocin 84 encoded on one of the three plasmids contained within the strain, plasmid pAgK84 (Kerr, 1989) and a second agrocin (agrocin 434), is encoded on a second larger plasmid (Donner *et al.*, 1993). Agrocin 84 appears to adversely effect DNA replication within a sensitive strain (Das *et al.*, 1978). These Agrocin 84 susceptible strains are the major disease causing bacteria in orchards and nurseries (Kerr and Tate, 1984) and are effectively controlled. The second mechanism involves the competition for and occupation of binding sites and nutrients within the host rhizosphere/ root system and the presence of high numbers of K84 around the roots for biocontrol (Lopez *et al.*, 1989). The biocontrol agent is able to survive in the soil and colonise root surfaces (Shim *et al.*, 1987, Macrae *et al.*, 1988). A third antibiotic like substance (ALS) has been proposed to be present with circumstantial evidence pointing to a role in competition (Peñalver *et al.*, 1994).

In field conditions the exchange of plasmids between *A. tumefaciens and A. radiobacter* could occur spontaneously and this created a genetic diversity of agrobacteria which may influence biocontrol efficiency of *A. radiobacter* K84 (Vicedo *et al.*, 1996). The rate of transfer from K84 to a recipient was 10^{-4} transconjugants per recipient, with the transconjugants retaining the plasmid pAgK84 for up to seven months in the field (Stockwell *et al.*, 1996). Within tumours the event is more common (Vicedo *et al.*, 1996). The uptake of this plasmid by a pathogenic strain would result in the breakdown of the biological control due to the presence of

an agrocin 84 producing pathogen. The pathogen is capable of eliminating all agrocin sensitive strains from the wound and can cause disease.

A transfer defective mutant of K84 was constructed (K1026) to eliminate the potential transfer of the plasmid encoding agrocin production to pathogenic strains (Farrand, 1985, Shim *et al.*, 1987). Transfer of agrocin production would render pathogens immune to the effects of agrocin 84. The genetically modified K84 strain (K1026) is in commercial use. This is the first example of a genetically modified strain being used as a bio-pesticide.

1.12 Biocontrol strains for control of grapevine crown gall disease

Due to the specific nature of the interaction between grapevine and *A. vitis*, the use of non-pathogenic *A. vitis* as a potential biocontrol strategy has been investigated in a number of laboratories (Burr and Reid, 1994, Liang *et al.*, 1990, Dickie and Bell, 1996, Staphorst *et al.*, 1985, Chen and Xiang, 1986, Biggs *et al.*, 1994). A similar action of non-pathogenic *A. vitis* or other non-pathogenic grapevine specific bacterium were sought to prevent pathogenic *A. vitis* from entering the grapevine when planted into pathogen infested soils similar to the activity of K84. Studies by Kerr and Panagopoulos (1977) showed that strain K84 was not effective in the biological control of *A. vitis* on grapevine and that *A. vitis* was not susceptible to the agrocins produced by K84 (Farrand, 1990, Donner *et al.*, 1993).

Four potential agents for the biocontrol of crown gall disease of grapevine are under investigation in Australia with similar studies being undertaken in America, Italy, Spain, China, South Africa and elsewhere. These strains are *A. vitis* F2/5 (from South

Africa, Staphorst *et al.*, 1985), *A. vitis* E26 (from Peoples Republic of China, Liang *et al.*, 1990), *A. radiobacter* biovar 1 HLB-2 (from Peoples Republic of China, Chen and Xiang, 1986) and *Pseudomonas fluorescens* K315 (Kerr, Australia). Isolates were obtained from vine galls or hops (HLB2) and were found to be effective at inhibiting *A. vitis* strains *in vitro* and *in planta* (Biggs, 1994, Biggs *et al.*, 1994).

1.13 The role of bacterial attachment in the control of crown gall disease

As previously discussed, attachment has many roles in the disease cycle of pathogenic *A. vitis* including tumour formation but may also be involved in the disease control strategy with biocontrol bacteria colonisation. When bacteria are unable to attach to the plant cell surface, virulence in significantly decreased (Matthysse and McMahan, 1998). Lippincott and Lippincott (1969) suggested the presence of specific sites of attachment for *Agrobacterium* strains and the ability of avirulent agrobacteria to inhibit the initiation of tumour formation by virulent strains, possible by blocking the available sites. The specificity of these 'receptors' was demonstrated by the failure of *Rhizobium meliloti* (closely related to *Agrobacterium*) and *Pseudomonas aeruginosa* to block virulent *A. tumefaciens* attachment. The introduction of bacterial biocontrol bacteria that are capable of crown gall disease control may target similar sites on the plant cell surface and therefore preclude the disease process.

Further observation of the *Agrobacterium*-plant cell interaction has shown attachment to be a two step process. The bacterium initially adheres to the plant surface as a single cell and then elaborates cellulose fibrils that act to entrap other bacteria into aggregates (Matthysse *et al.*, 1981). The bacteria are capable of binding very tightly at this phase. Cellulose minus mutants (unable to synthesise the cellulose fibrils)

were discussed in section 1.3.2. All genetic loci involved in the attachment process are chromosomally encoded and include *chvA, chvB, pscA and att* genes. Mutants that are unable to attach were shown to be avirulent when compared to the wild type pathogen (Matthysse, 1987) using mutations in the *att* loci. Phenotypic characterisation of the mutants showed differences in the outer membrane protein structure, but is currently unknown whether these are involved in binding of *A. tumefaciens* to the plant cell surface.

The presence of specific receptor molecules has been supported by the observation of a saturable levels of *A. tumefaciens* (Nett and Binns, 1985) and on grape root tip at 10^4 cfu per millimetre root using starting cultures at 10^7-10^8 cfu per millilitre (Pu and Goodman, 1993). The prevention of tumour formation by pre-inoculation with an avirulent but attaching bacterium was shown to be effective at swamping receptor sites on the plant (Lippincott and Lippincott, 1969) and preventing disease development when challenged with a pathogen.

The introduction of a biocontrol strain may reduce the ability of pathogens to attach by binding to specific receptor sites, prevent DNA transfer from bacterium to plant host or block attachment of the pathogen to plant cells. It is currently unknown how these biocontrol agents prevent disease development, as agrocin minus mutants are able to control disease (Burr *et al.*, 1997).

1.14 Aims of the Study

This project investigated the efficacy of the biocontrol agents under field conditions when tested against high levels of pathogenic *A. vitis* not normally encountered in the field. The aims of the project were planned to enable fast detection of the pathogen from grapevine samples, determine the interaction between biocontrol and pathogen and determine the efficacy of the four individual biocontrol strains previously tested under glasshouse conditions.

The aims are given below:

1. To determine the efficacy of four potential biological control strains in limiting crown gall disease of grapevine under field conditions.
2. To develop detection techniques using specific PCR primers for the detection and identification of pathogenic *Agrobacterium vitis* from grapevine and soil samples.
3. To investigate the mechanism of action of biocontrol agents with respect to attachment, competition and colonisation of grapevines.

CHAPTER 2

GENERAL LABORATORY TECHNIQUES

2.1 Culture and storage of bacterial strains

2.1.1 Culture of bacterial strains

Agrobacterium and *Pseudomonas* strains were grown on Nutrient Agar (NA, Oxoid) (Appendix 1), Tryptone Yeast Agar (TYA) (Appendix 1) or in nutrient/TY broth (shaking incubator at 200rpm) at 28°C for 2 days. *E. coli* strains were grown on Luria Bertani (LB) agar or broth at 37°C overnight. The origins of strains are listed in Table 1.

2.1.2 Storage of bacterial strains

Bacterial isolates were cultured in TY broth or Nutrient broth (NB) for 48 hours at 28°C with shaking (200rpm). Isolates were stored at –80°C in 15% (weight/volume) glycerol.

2.1.3 Bacterial Strains

Bacterial isolates used in this study are listed in Table 1.

Table 1: Bacterial isolates used in this study. (T) Indicates type strain.

Strain designation	Pathogen	Plant host	Origin	Other designation
A. vitis				
Ag57	yes	Grapevine	Panagopoulos, Greece	K252
K306	yes	Grapevine	Kerr, Australia	
K309 (T)	yes	Grapevine	Kerr, Australia	Type strain ICMP 10752
K377	yes	Grapevine	Kerr, Australia	
CG484	no	Grapevine	Burr, U.S.A	K1069, ICMP 10755
K1070	yes	Grapevine	Kerr, Australia	
K1072	yes	Grapevine	Kerr, Australia	
K1076	yes	Grapevine	Kerr, Australia	
673	no	Grapevine	Burr, U.S.A	K1265
675	yes	Grapevine	Burr, U.S.A	K1266
676	yes	Grapevine	Burr, U.S.A	K1267
680	yes	Grapevine	Burr, U.S.A	K1269
E26	no	Grapevine	Liang, P.R.C.	K1319
CG49	yes	Grapevine	Burr, U.S.A	K1332
F2/5	no	Grapevine	Staphorst, South Africa	K1334
A. rhizogenes				
K46	yes	Peach	Kerr, Australia	
K 66	yes	*Lippia canescens*	Kerr, Australia	R76
K84	no	Peach	New, Australia	
K434	no	Peach	Donner, Australia	derivative of K84
K1143	no	Peach	Donner, Australia	derivative of K84
K1347	no	Peach	McClure, Australia	derivative of K 1143
K1351	no	Peach	McClure, Australia	Cured K84
K1352	no	Peach	McClure, Australia	cured K84
K114	yes	Peach	Kerr, Australia	
A. *tumefaciens*				
K198	yes		Kerr, Australia	
A 892	yes	*Lippia canescens*	Moore, U.S.A	R72
HLB2	no	Hops	Chen, P.R.C.	K1051
K1073	no	Grapevine	Kerr, Australia	
K1074	no	Grapevine	Kerr, Australia	
K1075	no	Grapevine	Kerr, Australia	
P. fluorescens				
K315	no	Grapevine	Kerr, Australia	
B15132	no		Brisbane, Australia	derived from 2-79, smooth border
P. corrugata				
2140 0P1	no	wheat	Ryder, Australia	
Rhizobium				
SU303	no	Peas, beans	Gibson, Australia	R195, NA533
SU391	no	Peas	Gibson, Australia	R196
NA30	no	Clover	Gibson, Australia	R198
Bacillus subtilis				
R238	no		Wigmore, Australia	R238
E. coli *DH5* p519*ngfp*	no		McClure, Australia	pNJ5000, p519*ngfp*
A. vitis F2/5*ngfp*2	no	Km^r gfp	This study	p519*ngfp*
A. vitis E26*ngfp* 1	no	Km^r gfp	This study	p519*ngfp*
A. vitis K377*ngfp* 2	yes	Km^r gfp	This study	p519*ngfp*
A. vitis K1072*ngfp* 2	yes	Km^r gfp	This study	p519*ngfp*

2.1.4 Growth media

Growth media (described in Table 2) were used as broth or agar with antibiotic supplements as required. Media compositions are listed in Appendix 1. Cycloheximide ($250\mu g.ml^{-1}$) was incorporated (to limit fungal contamination) when isolations were made from field soil or vines.

Media	Selectivity	Antibiotic concentrations ($\mu g.ml^{-1}$)
Nutrient	Aerobic bacteria	rifampicin (100) tetracycline (10) kanamycin (25)
TY	Aerobic bacteria	
AB minimal	*Agrobacterium*	kanamycin (25)
RS	*A. vitis*	cycloheximide (250)
Biovar 1 medium	*A. tumefaciens*	cycloheximide (250)
Biovar 2 medium	*A. rhizogenes*	-
Biovar 3 medium	*A. vitis*	-
Kings B	*Pseudomonas* (fluorescent)	Cycloheximide (250)
PDA	Aerobic bacteria	Cycloheximide (250)

Table 2: Bacterial culture media and supplements of antibiotics or antifungal agents added as required.

2.2 Bacterial growth rates

Growth rates of bacterial isolates were determined in nutrient broth. Sterile broth was inoculated with a fresh plate culture and incubated for 2 days at 28°C in a shaking incubator at 200rpm. A standardised aliquot (100µl, OD600nm=1.0) was inoculated into fresh nutrient broths (3ml) in 5 ml Kimble tubes. Optical densities (600nm) were measured every 4 hours, up to 48 hours. All assays were done in triplicate with 5 tubes per treatment. Samples were taken at time intervals, diluted in PBS and spread on NA plates in 100µl aliquots. Colonies

were counted after incubation at 28°C for 3 days. Growth curves were created using colony forming units plotted against time for each strain.

2.3 Isolation and enumeration of agrobacteria from soil

Soil from field sites was collected from the top 20cm of soil, placed in plastic bags and stored at 4°C until required. Individual samples were thoroughly mixed and a 10g sub-sample taken. Soil samples were then inoculated into 100ml RS broth supplemented with 250μg.ml^{-1} cycloheximide and placed on a rotary shaker at 200rpm for 1 hour. Aliquots (100µl) were taken, diluted in PBS and plated onto RS agar with cycloheximide (250μg.ml^{-1}). Plates were incubated at 28°C for 5 days and putative *A. vitis* colonies counted.

2.4 Stonier's assay for detection of antagonism

Bacterial isolates to be tested for antagonism were grown in TY broth for 48 hours at 28°C. A drop (10µl) of culture (OD_{600} 0.5) was placed in the centre of a modified Stonier's agar plate (Biggs, 1994, Stonier, 1960) (Appendix 1), allowed to absorb into the agar and sealed in parafilm and incubated for 48 hours at 28°C. The culture was killed by inverting plates over filter paper soaked in chloroform for 10 minutes then aired for 20 minutes to allow any residual chloroform to evaporate. Overlay strains to be tested for sensitivity to bacteriocin(s) produced by antagonistic strains were grown in 5ml TY broth at 28°C, 200rpm. An aliquot (500µl) of culture was inoculated into 5ml of cooled (40°C) soft buffered agar overlay (Appendix 1) and poured aseptically onto the plate. Once the overlay was set, plates were incubated for at least 48 hours at 28°C and checked for zones of inhibition.

2.5 Plasmid isolation and visualisation

Plasmid isolation was done using the modified Birnboim and Doly method (Sambrook *et al.*, 1989). Cultures were grown in nutrient broth at 28°C for 2 days. The procedure was altered in the initial steps to remove excess exopolysaccharide by washing in STES solution (see Appendix 2) (Ophel, 1987). DNA was resuspended in 30µl sterile MQ water. Plasmid preparations were run on a 0.5% agarose gel in TBE at 4°C (Appendix 2) and visualised by ethidium bromide staining and UV visualisation. Photographs were taken using Polaroid 667 instant black and white film.

2.6 Genetic modification of bacteria

2.6.1 Production of spontaneous rifampicin mutants

Bacterial strains were grown in NB for 48 hours at 28°C. Undiluted cultures were spread onto NA containing 25µg.ml^{-1} rifampicin (Sigma). Plates were incubated at 28°C for 5 days. Putative rifampicin resistant mutants were then sub-cultured onto NA containing 50µg.ml^{-1} rifampicin and incubated at 28°C for 5 days. This procedure was repeated until isolates were capable of growth on NA containing rifampicin at 100µg.ml^{-1}. Isolates were maintained on this medium.

2.6.2 Insertion of the green fluorescent protein marker

Filter mating were used to introduce the green fluorescent protein (GFP) via plasmid p519*ngfp* (Figure 1) (Matthysse *et al*, 1996). Cultures of *E. coli* DH5α p519*ngfp*, pNJ5000, and *Agrobacterium* strains were grown in NB for 1 and 2 days at 37°C and 28°C respectively. An aliquot (1ml) of each culture was pelleted at 6000g for 5 minutes. The supernatant was decanted and the pellet was resuspended in 200µl PBS (Appendix 1). Aliquots (50µl) of each isolate were mixed on sterile 0.4µm filters and placed on NA.

Controls of each strain alone were performed. Plates were allowed to dry, sealed in parafilm and incubated at 28°C for 3 days.

To recover bacteria, filters were placed in 2ml PBS and vortexed for 1 minute or until bacterial growth was resuspended. Samples were plated onto AB minimal agar (see Appendix 1) containing $25\mu g.ml^{-1}$ kanamycin. This medium is selective for *Agrobacterium* transconjugants containing kanamycin resistance from plasmid p519*ngfp*. Plates were incubated for 7 days at 28°C and colonies were sub-cultured on AB minimal media (kanamycin $25\mu g.ml^{-1}$). Colonies were selected for further studies by expression of the green fluorescent protein using a light box with wavelength of 485nm. Isolates expressing GFP were maintained on selective media to ensure stability of the plasmid.

2.6.3 Certification of GFP transconjugants and rifampicin mutants

Genetically modified bacteria were assessed for growth rate, agrocin activity, stability and presence of the introduced plasmid and attachment on grapevine roots. Bacterial growth curves (Section 2.2) were used to determine bacterial growth rates of wild type and genetically modified bacteria. Production of antagonistic compounds by genetically altered strains was tested to ensure agrocin production had not been impaired. Stonier's assays (Section 2.4) were performed on all wild type and modified bacteria.

In order to check the stability of the plasmids in *Agrobacterium* transconjugants, strains were inoculated in NB and grown on a rotary shaker at 200rpm for 2 days. Samples of each culture were serially diluted in PBS and spread onto both NA and NA supplemented with $25\mu g.ml^{-1}$ kanamycin agar plates. Plates were incubated at 28°C for 3 days and colonies counted and analysed. A plasmid screen was performed on all transconjugants to check for

the presence of plasmid p519*ngfp* introduced via the plate mating technique. Plasmid preparations (Section 2.5) were separated on a 1.5 % agarose gel and visualised using ethidium bromide staining and UV detection. Attachment assays (Section 2.14.4) were performed on wild type and genetically modified bacteria to ensure attachment was not affected by the manipulations. All assays were performed on tissue cultured *V. vinifera* cv. Cabernet Sauvignon.

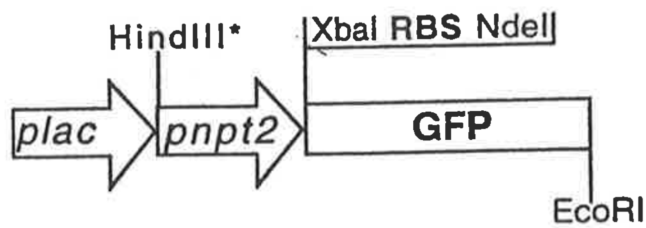


Figure 1: Plasmid construct p519*ngfp* used for genetic tagging of bacterial isolates. Plasmid contains kanamycin resistance, *lac* promoter and Mob^{+} function (Keen *et al.*, 1988, Matthysse *et al.*, 1996).

2.7 Growth of inoculum for field trial

2.7.1 Broth culture

A starter culture (5ml) was grown in TY broth for 48 hours at 28°C (200rpm). This culture was used to inoculate flasks of TY broth (50ml), that were grown at 28°C for 48 hours (200rpm). This culture was then inoculated into a 250ml NB flask and grown under the same conditions. The culture was then pelleted at 10,000g for 10 minutes and resuspended in 500ml PBS to an OD_{600}=1.0. Inoculum was used immediately after preparation. Estimates of bacterial numbers were determined by serial dilution and plating onto NA. Plates were incubated at 28°C for 4 days and colonies counted.

2.7.2 Peat cultures

Bio-Care Technology Pty. Ltd prepared peat cultures for field trials. Bags of 200g and 100g pasteurised peat were inoculated with bacteria grown in nutrient broth for 48 hours at 28°C (200 rpm). An aliquot (5ml) of inoculum was injected into the sterile peat bags with a sterile 29G needle and sealed to prevent contamination. Inoculated peat was incubated in the dark at room temperature for 2 weeks and then assessed for bacterial numbers and contaminants. A 10g sample was removed from individual bags, placed in 100ml SDW and placed on a rotary shaker at 200rpm for 30 minutes. Samples were taken, serially diluted in PBS and spread onto selective RS medium for *A. vitis* strains, Biovar 1 medium for *A. tumefaciens* and Kings B medium for *P. fluorescens*. Plates were incubated for 2-5 days at 28°C and colonies counted.

2.8 Pathogenicity testing on Tomato

Tomato plants were used to test pathogenicity of individual *Agrobacterium* and *Pseudomonas* strains. *Lycopersicon esculentum* (cv. 'Burnley Bounty') seedlings were

planted into fresh UC (University of California) soil mix and grown under glasshouse conditions. When stems were approximately 5mm in diameter, plants were inoculated. Inoculation was performed using a 29-gauge needle dipped in bacterial colonies (cultured for 48 hours). Plants were wounded several times by pricking the surface with the needlepoint at two positions on the stem, with two replicate plants per treatment. Control plants were wounded without inoculum. Plants were held upright with stakes and watered regularly. Assessment for the presence of galls, gall diameter and characteristics were completed after four weeks incubation.

2.9 Polymerase Chain Reaction (PCR) detection of *A. vitis*

2.9.1 DNA preparation

2.9.1.1 Cell lysate preparation method 1

DNA was isolated from reference strains using a rapid bacterial lysis method (Neilan *et al.*, 1993). A single colony from a 2 day old streak plate (NA) was resuspended in 20µl 0.1% (vol/vol) Triton X-100. The colony was lysed by the addition of 5µl of 0.4N NaOH followed by heating in a boiling water bath for one minute, then immediately chilled on ice. The solution was neutralised by the addition of 5µl 1M Tris-HCl, pH 7.6. DNA was stored at -20°C until required.

2.9.1.2 Cell lysate preparation method 2

Bacterial cell lysates were prepared using the method described by de Bruijn (1987). An overnight culture (5ml NB) was pelleted at 5000g for 10 minutes and was washed twice with 2ml SDW. Cells were resuspended in SDW to OD (600nm) = 1.5. To an aliquot (100µl) of cells, 100µl of 10-mM Tris-HCl pH8.0 and proteinase K ($5mg.ml^{-1}$) was added and

incubated overnight at 55°C. The lysate was heating to 100°C for 10 minutes to inactivate the proteinase K. Cell lysates were stored at -20°C.

2.9.1.3 DNA purification from soil

DNA was prepared using the Mo Bio Laboratories Inc. (California) Soil DNA Purification Kit Ultraclean ™. Grapevine sap, soil or callus material was suspended in 1ml of Bead solution with 0.6 grams small (0.5mm) and large glass (2mm) beads in 2ml Fast Prep® tube. Solution 1 (60µl) was added and shaken in the Fast Prep FP 120 (Savant Instruments, Holbrook, NY) for 30 seconds at speed 5.5. The lysate was pelleted for 1 minute at 10,000g and the supernatant removed to a fresh tube. Protein precipitating solution (Solution 2) was added (250µl) and chilled at 4°C for 5 minutes. Tubes were centrifuged at 10,000g for 1 minute and 500µl supernatant transferred to a fresh tube. A volume (900µl) of Solution 3 was added, thoroughly mixed and loaded onto a binding matrix with repeated centrifugation (10,000g for 1 minute) to load all sample. The matrix was washed with 300µl Solution 4, followed by centrifugation (10,000g for 1 minute). DNA was eluted by application of 50µl SDW to the matrix and centrifugation (10,000g for 1 minute). DNA was stored at -20°C until required.

2.9.2 PCR detection of *A. vitis*

Polymerase chain reaction (PCR) for the detection of pathogenic and non-pathogenic *A. vitis* was developed by Eastwell *et al.,* (1995). The primer set was selected to allow detection of both pathogenic and biocontrol *A. vitis* strains used in this study (vitopine strains are not detected with these primers). Primers *pehA* and *virA* (Figure 2) were obtained from Geneworks (formerly Bresatec, Thebarton, South Australia). The PCR reaction was optimised with the final cycling program given in Table 3. DNA was prepared by the cell lysate method (2.9.1.1) with 1µl used per 25 µl reaction.

Final concentrations of components in a 25µl reaction were 30nmole of each *vir*A primer/ 40nmole of each *peh*A primer, 1x Reaction buffer (GeneWorks, Thebarton, South Australia supplied with Taq polymerase as 10x buffer), 1mM magnesium chloride (supplied as 250mM, GeneWorks), 0.2 mM dNTP mix (from 10mM stock) and 0.25 units *Taq* DNA polymerase (Bresatec, South Australia). PCR reactions were cycled on the program given in Table 3 using an MJ Research PTC-225 Peltier Thermocycler DNA Engine Tetrad.

Aliquots of PCR product (10µl) were run on a 1.5 % agarose gel in TAE buffer at 80V for 1 hour with ethidium bromide staining and ultraviolet visualisation. Expected PCR product sizes were 199bp for *pehA* and 480bp for *virA*.

Temperature	Time	Number of Cycles
96°C	2 minutes	1
94°C	1 minutes	↑
54°C (*pehA*)/ 56°C (*virA*)	30 sec	40
72°C	30 sec	↓
72°C	5 minutes	1
20°C END	1 minute	1

Table 3: Cycling program for single primer set PCR reactions used on the Tetrad Thermal Cycler. Optimal annealing temperatures were determined for each primer set and found to be 54°C for *pehA* and 56°C for *virA*.

*pehA*1 5'CGA TGG CGG CGA GGA TTT3'

*pehA*2 5'ATC GGG CGT GAA ACA AGT3'

*virA*1 5'TTC AGT CGC GCA AGC AGTT3'

*virA*2 5'CGG CAA TTC GTA TCA CGGA3'

Figure 2: Oligonucleotide primer sequences used in detection of *A. vitis* pathogen and non-pathogenic strains (described by Eastwell *et al.*, 1995).

2.9.3 Multiplex PCR

Multiplex PCR combined the 2 sets of primers described in Figure 2 (originally used in single PCR reactions). The reaction was optimised with final concentrations in reaction mix for a 25µl reaction: 30nmole *virA* and 40nmole *pehA* primers, 1mM magnesium chloride, 0.2mM dNTP mix, 1x reaction buffer, and 0.026 units Taq DNA polymerase and 1ml template DNA (section 2.9.1.1). Reactions were cycled using the program shown in Table 4, on the Tetrad Thermal Cycler. Aliquots (10µl) of PCR products were analysed by agarose gel electrophoresis on a 1.5 % gel in TAE buffer with ethidium bromide staining followed by ultraviolet visualisation.

Temperature	Time	Number of Cycles
96°C	2 minutes	1
94°C	1 minutes	↑
56°C	30 sec	40
72°C	30 sec	↓
72°C	5 minutes	1
20°C END	1 minute	1

Table 4: Multiplex PCR cycling program modified from Eastwell *et al.*, 1995 to combine *virA* and *pehA* primers in a single reaction for the detection of pathogenic and non-pathogenic *A. vitis*.

2.10 DNA fingerprinting with ERIC PCR

PCR reactions were carried out in 200µl PCR tubes using REP-PCR buffer described by Versalovic *et al.*, (1991). A 10x stock buffer solution contained 166mM-ammonium sulphate, 67mM Tris-HCl pH 8.8, 67mM magnesium chloride, 100mM-ß-mercaptoethanol, 67µM EDTA, and 1.7mg.ml^{-1} bovine serum albumin (BSA). ERIC oligonucleotide primer sequences are given below.

ERIC1R 3′- CATCTAGGGGTCCTCGAATGTA -5′

ERIC2 5′- AAGTAAGTGACTGGGGTGAGC -3′

Individual PCR reactions consisted of the following per 25µl reaction with final concentrations: 50 pmol each primer, 0.5mM dNTP mix, 1x reaction buffer (freshly prepared) 10% DMSO, 0.5 units Taq DNA polymerase and 5µl cell lysate (section 2.9.1.2). The cycling procedure shown in Table 5 was used with the Corbett Research FTS-960 Thermal sequencer.

Temperature	Time	Number of Cycles
94°C	1 minute	↑
52°C	1 minute	34
65°C	8 minutes	↓
65°C	16 minutes	1
20°C END	1 minute	1

Table 5: ERIC PCR thermocycling program for DNA fingerprinting of pure bacterial isolates.

Aliquots (15µl) of PCR products were run on 1.5 % agarose gel in TBE (Appendix 2) at 50V for 4 hours. Gels were stained with ethidium bromide and destained in MQ® water for 1 hour to increase clarity of bands. Photographs were taken using Polaroid 667 black and white film.

2.11 Immunological detection of *A. vitis*

A monoclonal antibody developed by Burr *et al.*, (1988a) was used in an ELISA (Enzyme Linked Immuno-Sorbent Assay) for the detection of *A. vitis* isolates recovered from soil and grapevine vascular fluid samples. Putative *A. vitis* isolates were aseptically removed from RS agar, resuspended in sterile water and tested in a modified immunoblot assay on nitrocellulose membrane for binding to the specific monoclonal antibody (as described by Leach *et al.*, (1987)). Colorimetric detection with nitro-blue-tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP, Boehringer-Mannheim) substrate

and anti-mouse IgG alkaline phosphatase conjugate (Sigma) was used for the positive identification of detection of *A. vitis* isolates by visual inspection.

2.12 Tissue culture of plantlets

2.12.1 Tissue culture of tomato seedlings

Tomato seeds (*Lycopersicon esculentum* cv. Floridade) from S & G Seeds, (Novartis, NSW) were surface sterilised in 3% hypochlorite solution (bleach) for 20 minutes, then rinsed clean of bleach solution with 5 successive washes in SDW until bubbles were removed. Seeds were placed on water agar (1.5 % Difco Bacto agar, Appendix 1) and incubated in the dark at 25°C for 5 days until roots developed. Contamination was detected on seedlings by the presence of bacterial or fungal growth on the water agar. Contaminated seedlings were discarded.

2.12.2 Tissue culture of *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Landsburg erecta seeds were surface sterilised in 1.6% sodium hypochlorite, stored at 4°C for 3 days and planted onto petri-dishes containing MS salts, Gamborg's B5 Vitamin mixture and 2% sucrose in 0.8% Phytagar (all obtained from Gibco Laboratories). Seeds were incubated in a 14-hour light, 10-hour dark cycle for up to 6 weeks (Matthysse and McMahan, 1998).

2.13 Enumeration of nematodes from soil

Soil samples (100g) were placed onto nematode separating trays covered with tissue in a catchment tray (Dr. K. Davies, Dept. Applied and Molecular Ecology, University of Adelaide, South Australia, pers. comm.). Trays were then filled with water until the tissue became wet and were left at room temperature for 7 days to allow the nematodes to encyst

and move into the water phase. Nematodes were concentrated in a Büchner funnel, then counted.

2.14 Bacterial attachment assays

2.14.1 Basic attachment assay

Bacterial isolates were cultured in NB (5ml) for 2 days at 28°C (200rpm). Cultures were diluted to 10^5 $cfu.ml^{-1}$ in PBS immediately before use. Tissue cultured *V. vinifera* (cv. Cabernet Sauvignon) plantlets were removed from culture medium and roots were excised under sterile conditions. Roots were washed in SDW and placed in 2ml calcium sucrose broth (0.4M Ca, 0.1% sucrose, Matthysse, 1998) in 36mm petri dishes (2ml).

An aliquot (100µl) of diluted bacterial cells was added to the root assay system, gently mixed and incubated at room temperature for 1 hour. Samples were taken at 0 and 60 minutes, diluted in PBS and spread on the appropriate media and incubated at 28°C for 3 days. All plating was done in triplicate. Each treatment was replicated at least 5 times.

Alternative assays performed utilised tomato and *Arabidopsis* roots instead of *V. vinifera*. Where optimisation of bacteria survival in the assay was performed, no roots were added to the system.

2.14.2 Addition of excess bacteria

Where excess bacteria were used, a drop of 2 day old bacterial culture was added to calcium sucrose broth and plant roots. Roots were incubated for 24, 48 and 72 hours for microscopic observation.

2.14.3 Interaction of biological control and pathogenic strains

Interaction between pathogenic and biocontrol isolates was determined on cut roots in the assay system. Pathogenic *A. vitis* strains were selected for agrocin resistance to individual biocontrol strains. Detection of individual isolates was completed using green fluorescent protein (GFP) tagging of one of the bacteria in the competition assay. Competition assays were as per Section 2.14.1 and interactions were observed at 24 and 48 hours using Nomarski optics or fluorescent microscopy (Olympus, Vanox). Photographs were taken using Tmax 400 film in colour and black and white.

2.14.4 Microwave preparation of soil

Soil was pasteurised for use in root colonisation assay experiments using a microwave treatment. UC soil (University of California Mix) was bagged (4kg) in microwave safe polyethylene bags. Moisture was added to the soil at a rate of 50 grams per 500g soil and microwave treated for 7.5 minutes. Soil was stored for at least 1 month in the dark prior to use (Ferris, 1984).

2.14.5 Root colonisation by *Agrobacterium* strains

Rifampicin resistant isolates were diluted in PBS (10^5-10^6 bacteria per ml). Tissue culture plants (Section 2.12) were removed from culture medium and washed in SDW. Intact plantlets were soaked in bacteria for 1 minute then planted into sterile 50 ml Falconer tubes (with 3 drainage holes in the base) in microwave sterilised UC soil (Section 2.14.4). Assay tubes were covered in parafilm and incubated in a 12/12-hour day/night cycle growth cabinet at 15°C. Plantlets were allowed to grow for 0, 2, 5 and 10 days then removed and assayed to determine levels of bacteria that were loosely, tightly or irreversibly bound to the grapevine roots using a series of washing steps.

2.14.5.1 Recovery of bacteria from roots

2.14.5.1.1 Loosely bound bacteria

Roots were excised from plantlets and suspended in PBS (5ml) and washed by gentle inversion (10 times). The supernatant was decanted and the step repeated. The combined fluid was serially diluted and plated on selective media (NA rifampicin $100\mu g.ml^{-1}$) as loosely bound bacteria.

2.14.5.1.2 Tightly bound bacteria

To estimate tightly bound bacterial levels, roots were sonicated (Branson B220, 125 Watts) in PBS (5ml) for 90 seconds. Treatment of pure cultures for 0, 10, 20, 30, 45, 60 and 90 seconds sonication time was used to assess the time required for bacterial release from the plant root surface and limit bacterial cell death. As no difference was observed between bacterial populations at 0 and 90 seconds, 90-seconds was used. Samples were serially diluted, plated on NA rifampicin ($100\mu g.ml^{-1}$) and incubated at 28°C for 2 days.

Estimation of irreversibly bound bacteria on plant root surfaces was assessed by spreading root material onto NA rifampicin plates ($100\mu g.ml^{-1}$) and covering in 0.7% water agar overlay. Plates were incubated at 28°C for 2 days and bacterial colonies counted. All samples were plated in triplicate and all assays had at least 5 replicates per treatment.

2.15 Grapevine material

2.15.1 Varieties, suppliers and dormancy

Grapevine varieties used in these studies are given in Table 6 along with varietal names and disease susceptibility.

Variety	Resistance/tolerance	Suppliers
Vitis vinifera cv Shiraz	*A. vitis* susceptible	Temple Bruer Vine Nursery, Langhorne Creek, South Australia
Vitis champinii cv. Ramsey	*Meloidogyne* (nematode) and salt tolerant *A. vitis* highly susceptible	RVIA- Riverland Vine Improvement Association, Monash, South Australia

Table 6: Grapevine varieties used in these studies including resistance, tolerance of varieties and suppliers used in Australia.

2.15.2 Storage of grapevine cuttings

Dormant grapevine cuttings were stored as bundles of 100 in sealed plastic bags at 2°C until required.

2.15.3 Thermotherapy of grapevine cuttings

Two methods of thermotherapy of grapevines were utilised. The first involved dormant grapevine cuttings that were removed from cold storage and completely immersed in tap water to allow rehydration of the vines for at least 24 hours. Rehydrated vines were submerged into a hot water bath at 50°C for 20 minutes. Settings on the bath were checked and a 2°C buffer was used to compensate for the decreased temperature effects of vine addition. After treatment vines were plunged into tap water to cool. Bacterial inoculum was added at a final concentration of 10^6 cfu/ml to allow infusion of the bacteria into the vascular system. Vines were placed into callusing bins (Section 2.15.4.1).

The second thermotherapy method was completed at Temple Bruer Wines Pty. Ltd. that used a specially modified water bath consisting of a recirculating 10,000L-water bath maintained at 50°C using a controlled temperature system. Vines were placed into the thermotherapy unit for 30 minutes and then removed and plunged into cold water for 2 hours to allow vines to return to ambient temperature. Vines were placed into callusing beds as described in Section 2.15.4.2.

2.15.4 Callusing of grapevine cuttings

Three methods of callusing were used in the field trials. Heat-treated and untreated vines were placed into 80-L plastic bins with drainage holes in the base. A 10cm bed of washed river sand was placed on the base of the bins. Vines were placed into vertical sand beds in an inverted position leaving the basal end upward to be callused. Individual vine callusing beds were packed with sand covering the entire length of the cane with a 5cm cover of sand placed over the callusing ends. Callusing beds were placed in the sun and watered regularly to prevent dehydration of the cuttings. Vines were incubated for 2 months until callus could be seen developing on the basal section.

The 1997-98 field trial callusing was completed at Temple Bruer Wines Pty. Ltd. After thermotherapy, vines were placed basal end up into callusing beds using washed river sand. Vines were completely covered in sand and callused for 8 weeks until callus was developing with regular watering to prevent dehydration and encourage callus development.

The 1998-99 field trial callusing was completed after thermotherapy and rehydration treatment. Vines were packed into polystyrene boxes with pasteurised vermiculite and incubated in a controlled temperature room at 27-28°C for 2 weeks (Orlando-Wyndham Vine

Nursery, Rowland Flat, South Australia). Vine temperature was checked daily to maintain optimal callusing conditions (G. Pearson, Orlando-Wyndham vine Nursery).

2.15.5 Inoculation of callused grapevine cuttings

Callused material was removed from callusing beds and was rinsed with tap water to remove excess sand. Cuttings were then placed into buckets of peat inoculum and allowed to stand in individual biocontrol strains for a 1-hour period. Vines were then transferred to a second inoculum of pathogen peat mix for 15 minutes. Vines were immediately planted into field sites as required.

2.15.6 Re-isolation of inoculated strains from callus material

Samples of inoculated callus material were aseptically removed from the vine cutting and placed in 400μl sterile PBS. Callus material was crushed and plated onto selective agar with cycloheximide (250$\mu g.ml^{-1}$). Plates were incubated at 28°C for 5 days and counted.

2.15.7 Assessment of grapevine rootlings for disease

After removal from the field, vines were washed in water and stored at 2°C. Vines were individually assessed for the presence of galls by observation. Galls were recorded for each treatment group for size, position and frequency.

2.16 Isolation and quantification of agrobacteria from galls

Gall samples were removed from grapevine cuttings by initially surface sterilising the surface with 70% ethanol. Cuttings were dried and using a sterile scalpel, samples cut from the gall and placed into a pre-weighed tube. Sample weights were recorded and gall material

was crushed (400µl PBS) using a sterile sharp-ended glass rod. Crushed material was then spread plated onto RS agar (cycloheximide, 250µg.ml^{-1}) and incubated at 28°C for 5 days.

2.17 Extraction of vascular fluid from grapevine segments

Isolation of *A. vitis* and other saprophytic bacteria from grapevine material was completed on dormant cuttings using a sap extraction procedure. Sterile plastic tubing was attached to both ends of grapevine cuttings (Figure 4). The segment was then attached to a Venturi pump via directed flow conical flask and 0.5 ml PBS was drawn through using the vacuum system (Bazzi *et al.*, 1987). Sap was collected in a sterile 10ml tube and stored at 4°C until use and also preserved in 30% glycerol/NB at -80°C for future use. Samples were serially diluted and plated on selective media (cycloheximide 250µg ml^{-1}) as required (RS agar for *A. vitis*, Biovar 1 agar for *A. tumefaciens* and Kings B agar for *P. fluorescens*). Plates were incubated at 28°C for 5 days and colonies counted.

Putative *A. vitis* isolates were selected from RS medium and were inoculated into NB in 96 well microtitre trays. Isolates were cultured at 28°C for 2 days until cultures reached an optical density (OD_{600}) of 1.0, 30% glycerol in NB was added and stored at -80°C for future use.

2.18 Tissue culture of grapevine material

Vitis vinifera cv. Riesling was cultured from actively growing shoots 6-8 nodes in length. Shoots were cut into nodal segments and surface sterilised in 70% ethanol for 1 minute. Segments were then transferred to 1.5 % hypochlorite solution for 20 minutes with regular vigorous shaking to remove air bubbles from the vine surface. Segments were rinsed in SDW a total of 4 times. Individual segments were placed in sterile culture tubes containing

rooting media (Appendix 1) and incubated in a growth cabinet with 14-hour light/10 hour dark cycle. Vines were checked regularly for the presence of contamination. Any contaminated stock was discarded. Plantlets were incubated for up to 3 weeks before use.

Vitis vinifera cv. Cabernet Sauvignon plantlets were obtained from Dr. B. Stummer (University of Adelaide, Department of Applied and Molecular Ecology) and were maintained on root inducing media (Appendix 1) with 14/10 hour light/dark cycle at 28°C. Plantlets were regularly sub-cultured on rooting media.

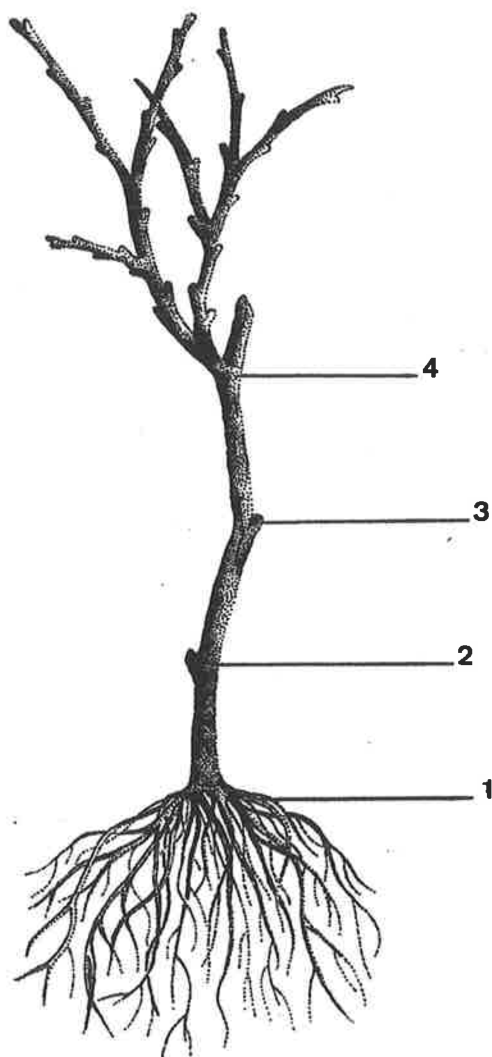


Figure 3: Position of galls on grapevine rootlings after 9 months growth. Vines were assessed for the position and size of galls present at the base (position 1), first node (position 2), second node (position 3) and third node (position 4) (Robinson, 1994).

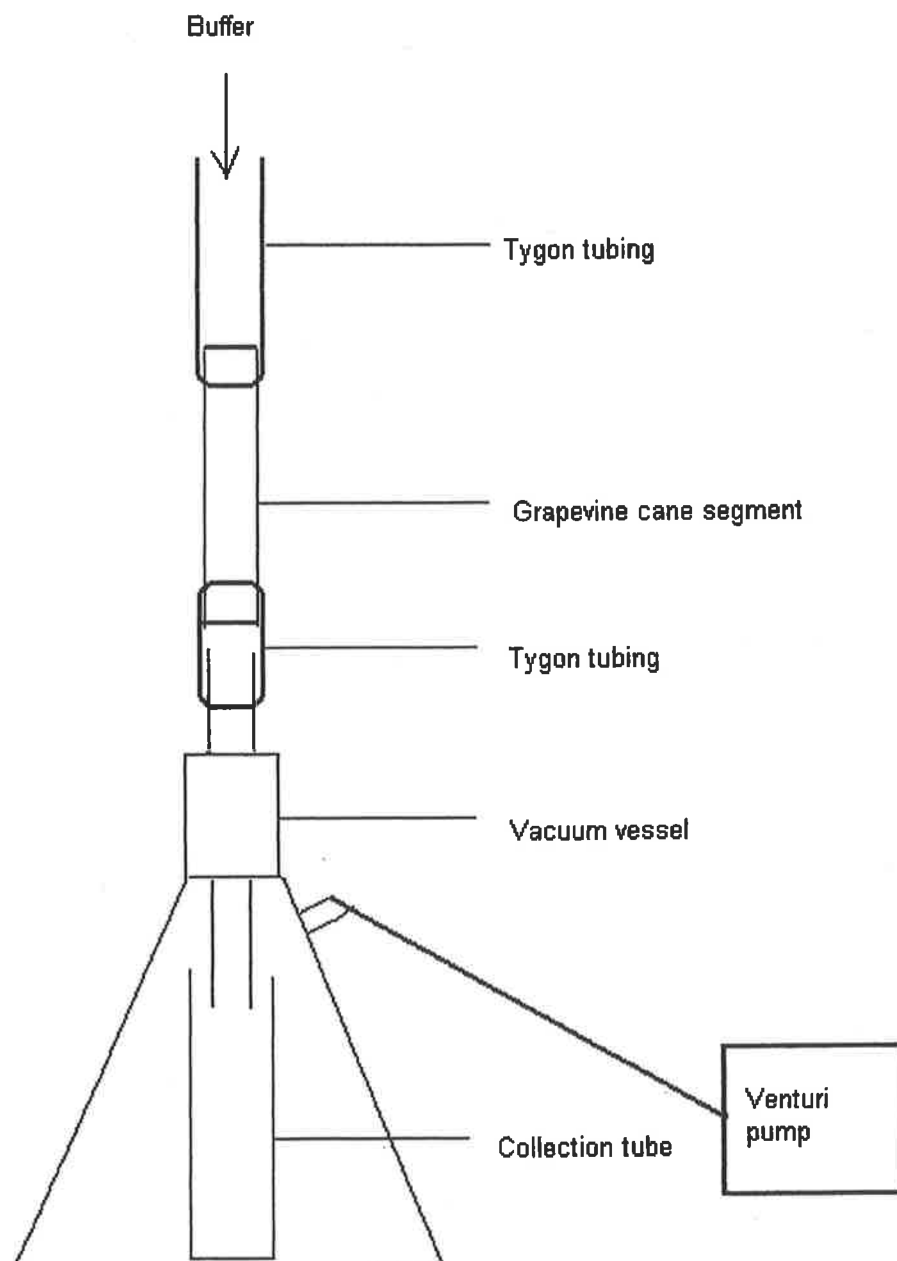


Figure 4: Design of Venturi pump used for the extraction of vascular fluid from grapevine segments.

CHAPTER 3

CHARACTERISATION AND DETECTION OF *A. VITIS* AND BIOCONTROL STRAINS

3.1 INTRODUCTION

3.1.1 Detection of *A. vitis*

Methods for the rapid detection and characterisation of *A. vitis* have been developed during this study. Previously, methods were developed to allow isolation and detection of *A. vitis* but these are slow. Two *A. vitis* semi-selective media used were based on carbon-source utilisation (RS medium, Roy and Sasser, (1983) and Biovar 3 medium, Brisbane and Kerr, (1983)). Bacteria associated with grapevines are capable of utilising adonitol (from RS agar), tartaric acid or and D-glutamic acids (in Biovar 3 agar) as carbon sources which are present in grapevine sap along with other compounds such as tartaric and malic acids used in the selection media (Süle, 1978). These compounds comprise 70-90% of the organic fraction of grapevine sap (Ruffner, 1982). As other species of *Agrobacterium* can utilise these compounds in the vine, they can grow on selective media making identification of *A. vitis* difficult.

To allow positive identification of putative *A. vitis* isolates from grapevine sap, a method was developed using an *A. vitis* specific monoclonal antibody (Burr *et al.*, 1988) which can be used in an ELISA or immunoblot protocol (Leach *et al.*, 1987). This method cannot distinguish pathogenic strains and requires isolation of individual colonies on semi-selective medium with an incubation period of 5 days. Tumour induction on tomato stems or DNA hybridisation to Ti plasmid sequences (Burr *et al.*, 1990) can be used to assess pathogenicity.

This procedure is time consuming, expensive and shown to seriously retard the rate at which diagnosis can be confirmed (Eastwell *et al.*, 1995).

PCR techniques for the amplification of specific DNA sequences have been successfully utilised for the detection of *A. vitis* from agar plate cultures and also from grapevine sap extracted using the vacuum extraction method (Eastwell *et al.*, 1995). Primers specific for a polygalacturonase gene (*pehA*) which encodes a pectin enzyme hydrolase produced by all *A. vitis* responsible for root decay and pectinolytic activity were used. The primers allow amplification of a 199bp fragment from the coding region of *A. vitis* strain CG49 (Eastwell *et al*, 1995). Detection of virulent *A. vitis* was accomplished with *virA* primers which were derived from a limited host range Ti plasmid pTiAg162 and wide host range plasmid pTiA6 (Leroux *et al.*, 1987). The primers amplify a 480bp fragment from the coding region of *virA* (Eastwell *et al.*, 1995).

Initially grapevine vascular washing fluid was plated onto selective agar medium and incubated for 5 days. Colonies were then lysed and used as PCR template. To reduce the assay time and increase accuracy of detection, bacterial isolation from the grapevine by *in situ* lysis followed by DNA purification with ion exchange chromatography was used (Eastwell *et al.*, 1995). This resulted in a reduction in inhibition of the PCR due to effective removal of polyphenolics and carbohydrate polymers from the template DNA (Rezaian and Krake, 1987).

PCR allows rapid detection of DNA of interest and can be assessed using agarose gel electrophoresis. The utilisation of PCR techniques will advance testing procedures for *Agrobacterium* detection for the grape and wine industry, in Australia and overseas. These

methods will enhance efforts to propagate nursery stock free from *A. vitis* and reduce losses from galled vines. Currently testing of mother vines is not completed before sale, leading to the potential transfer of diseased material to new areas. Standardised testing for all vines before sale would aid in decreasing disease transfer between vineyards, across state borders and countries.

3.1.2 Characterisation of biocontrol strains

Bacterial isolates used in this study were characterised to determine growth characteristics, pathogenicity, and antagonism of the individual strains. Four biocontrol strains were investigated. F2/5 is a non-pathogenic *A. vitis*, that produces agents that are inhibitory to a range of pathogenic *A. vitis* (Staphorst *et al.*, 1985) and was found to be inhibitory to both tumorigenic and non-tumorigenic *A. vitis in vitro* and under glasshouse conditions (Burr and Reid, 1994). It was also capable of reducing gall formation when inoculated at equal concentration to the pathogen, either with or before the pathogens. In glasshouse trials, F2/5 reduced galling on *Vitis vinifera* cv. French Columbard from 48% to 0% (Biggs *et al.*, 1994). The mode of action of this strain is not fully understood but may reduce T-DNA transfer from the pathogen into the plant host (Burr *et al.*, 1997). Burr also showed that F2/5 did not bind to the probe pTHE17, specific for Ti plasmids indicating that F2/5 does not harbour a Ti plasmid or a deletion mutant (Burr and Reid, 1994).

Strain E26 (*A. vitis)* was isolated from a grapevine gall and has proven to be inhibitory to a broad spectrum of isolates including *A. tumefaciens, A. rhizogenes* and *A. vitis* (Liang *et al.*, 1990). The bacterium is capable of production of an inhibitory compound(s) and was able to survive for over one month on wounded grapevine material (Liang *et al.*, 1990). In

glasshouse trials, E26 reduced galling frequency on *V. vinifera*, but was not as effective as F2/5 (Biggs *et al.*, 1994).

HLB2 is a non-pathogenic *A. tumefaciens* (biovar 1) isolated from hops (*Humulus lupulus)* in China (Chen and Xiang, 1986). It produces antagonistic compound(s) inhibitory to a range of *A. vitis* strains. When co-inoculated with a pathogen on grapevine shoots it was capable of significantly reducing gall formation (Chen and Xiang, 1986). A study in Australia showed HLB-2 decreased the level of pathogen and was not as effective as the other potential biocontrol agents used in glasshouse trial (Biggs *et al.*, 1994).

The fourth strain investigated, as a potential biocontrol agent was isolated in South Australia, *Pseudomonas fluorescens* isolate K315 from the Kerr Collection (Waite Research Institute, South Australia). Galling was reduced to less than five percent when applied to rootlings with a decrease in levels of *A. vitis* survival in xylem fluids (Biggs *et al*, 1994).

Of the strains trialed under glass house conditions in South Australia, F2/5 and E26 and HLB2 were shown to reduce galling, and F2/5 was capable of lowering rhizosphere and systemic *A. vitis* pathogen densities (Biggs *et al.*, 1994). The mechanism of action of F2/5 is not agrocin production. This was demonstrated through the use of agrocin minus mutants (Burr *et al.*, 1997). Therefore development of resistance to the agrocin produced would not inhibit disease control.

The aim of the work outlined in this chapter was to characterise *Agrobacterium* and *Pseudomonas* strains from grapevine and other plant species and to improve the specificity

and speed of detection procedures. This investigation was carried out on a wide range of *A. vitis*, *A. tumefaciens* and other vine and soil isolates.

3.2 EXPERIMENTAL PROTOCOLS

3.2.1 Characterisation of biocontrol isolates

3.2.1.1 Standardised bacterial levels

Bacterial counts for isolates were determined to allow estimation of bacterial numbers using spectrophotometry. Standardised bacterial cultures (OD600nm =1.0) were serially diluted in PBS. Optical density for each dilution was recorded and samples plated onto NA for estimations of bacterial counts. All plates were incubated at 28°C for 4 days and colonies were counted. All samples were plated in triplicate and repeated 5 times.

3.2.1.2 Selective media analysis

Bacterial strains from the Kerr collection (Department of Applied and Molecular Ecology (formerly Crop Protection), Waite Campus, University of Adelaide, South Australia) given in Table 1 were initially plated onto nutrient agar for recovery from lyophilised cultures, with incubation at 28°C. Isolates were characterised by plating onto selective media including RS agar (Roy and Sasser, 1983, Appendix 1), Biovar 1 agar, Biovar 2 agar and Biovar 3 agar (Brisbane and Kerr, 1983, Appendix 1).

3.2.1.3 Pathogenicity testing on tomato

Pathogenicity of *Agrobacterium* isolates was conducted on tomato plants (*Lycopersicon esculentum* cv. Burnley Bounty), as tomato is highly susceptible to infection by *A. vitis* and other agrobacteria. Each plant was inoculated with a single bacterial strain at 2 sites on the stem with 2 replicate plants per treatment. Control plants were wounded but not inoculated. Plants were maintained in a glasshouse and assessed after 4 weeks for the presence of galls and gall diameter.

3.2.1.4 Plasmid screening of bacterial isolates

Plasmid DNA was prepared from bacterial isolates following a modified Birnboim and Doly method (Section 2.5). All preparations were performed using wide bore tips (5mm-tip section removed) to reduce shearing of plasmid DNA due to the large size of *Agrobacterium* plasmids (20-400kbp). DNA was run on 0.5% agarose gel at 4°C in TBE buffer (section 2.5). Plasmids of *A. rhizogenes* strain K84 were used as molecular weight markers. Plasmid sizes are 47kb, 195kb and 300-400kbp (Szegedi *et al.*, 1992). Plasmid profiles were compared to previous reports for plasmid content (Burr and Reid, 1994, Szegedi *et al.*, 1992, Merlo and Nester, 1977).

3.2.1.5 Antagonistic activity of bacterial isolates

Six bacterial isolates were screened for antagonistic activity towards a wide range of agrobacteria and other rhizosphere bacteria using the overlay technique (Biggs, 1994, modified from Stonier, 1960, Section 2.4). The defined medium allows testing for the production of bacteriocins and the susceptibility of overlay strains to compounds produced by potential antagonists. Susceptibility to an antagonistic strain is determined by the production of a zone of inhibition around the site of application of the test strain. Each assay was performed in triplicate.

3.2.1.6 DNA fingerprinting with ERIC-PCR

ERIC (Enterobacterial Repetitive Intergenic Consensus) fingerprinting was investigated for its ability to distinguish between bacterial strains from field samples in the laboratory. The ERIC PCR protocol is based on the presence of intergenic repeat units, which are present in Gram negative bacterial genomes (de Bruijn, 1992). Individual bacterial strains have unique banding patterns when separated with agarose gel electrophoresis ranging from 100-1500bp.

Fingerprinting of the bacterial chromosome can be used to distinguish between closely related *Agrobacterium*, *Pseudomonas* and *Rhizobium* strains. PCR was used to obtain individual fingerprints for biocontrol and pathogenic strains used in this study (Section 2.10).

3.2.1.7 ERIC fingerprinting of K84 and plasmid cured derivatives

Agrobacterium plasmids have been shown to be promiscuous and there is evidence for plasmid loss in the course of a 9-month field trial (Stockwell *et al.*, 1993, Stockwell *et al.*, 1996). The effect of plasmid loss on DNA fingerprinting was investigated due to the presence of a number of large plasmids within biocontrol strains. This was investigated using strain K84 and its derivatives containing a range of plasmids (Table 7). Strains were prepared and cycled through the ERIC PCR procedure (section 2.10). PCR products were run on 1.5 % agarose in TAE buffer (Appendix 2).

Strain	Plasmid content	Agrocin	Description/Reference
K84	pAgK84, pAtK84b and pAgK434	84, 434	Biological control strain (Kerr and Htay, 1974)
K434	pAgK434 and pAtK84b	434	Cured derivative of K84 (Donner *et al.*, 1993)
K1143	pAgK434	434	Cured derivative of K84 (Donner *et al.*, 1993)
K1347	None	None	Cured derivative of K1143 (McClure *et al.*, 1994)

Table 7: *A. radiobacter* strain K84 and plasmid cured derivative strains from the wild type strain. Agrocin(s) produced by the strains are included.

3.2.2 Detection of biocontrol and pathogenic *A. vitis* from grapevine

3.2.2.1 PCR detection of *A. vitis*

Due to the extended time that is required for immunoblot detection of *A. vitis* strains (up to 7 days) an alternative method was preferable. PCR (polymerase chain reaction) was used to

allow faster and simpler detection of *A. vitis* isolates from soil and grapevine materials. Primers for the detection of *A. vitis* (*pehA*) and *A. vitis* pathogens (*virA*) were developed by Eastwell *et al*, (1995). These primers allow the detection of all *A. vitis* strains used in this study.

PCR reactions were optimised using single primer sets with a range of pathogenic and non-pathogenic *A. vitis, A. rhizogenes, A. tumefaciens* and *P. fluorescens* strains to determine primer specificity. Template DNA was prepared as cell lysates from pure cultures on agar plates (section 2.9.1.1). Reactions were run and visualised using the protocol described in section 2.9.2.

3.2.2.2 Multiplex polymerase chain reaction

Reactions were modified to perform multiplex PCR including both *virA* and *pehA* primer sets in a single reaction (Section 2.9.3). Conditions were optimised for the combined primer reactions using the same test isolates produced by the cell lysate method (Section 2.9.1.1).

3.2.2.3 Detection of *A. vitis* from vascular washing fluid

For the detection of *A. vitis* from vascular washing fluid, DNA preparation was completed using the Ultraclean™ Soil DNA purification Kit (Section 2.9.1.3). Grapevine vascular fluid was recovered from vine segments (from field trial vines) and stored at -80°C in 15% glycerol in nutrient broth (Section 2.17). An aliquot (100µl) was inoculated into 5ml RS broth supplemented with cycloheximide (0.2g.L^{-1}) and incubated overnight at 28°C (200 rpm). Total DNA was extracted from 2ml of broth culture (Section 2.9.1.3) and resuspended in a final volume of 25µl SDDW. PCR was performed using individual and multiplex PCR (Sections 2.9.2 and 2.9.3).

RESULTS

3.3.1 Standardised bacterial numbers for inoculum

Standardised bacterial numbers were determined allowing estimates of bacterial numbers using optical density. No significant differences were observed in growth for K1072 (*A. vitis* pathogen) and F2/5 (*A. vitis* biocontrol)(Figure 5). K1072 was capable of growing to a higher optical density over a two-day period but bacterial numbers were unaffected which allowed application of equivalent levels of bacteria to field trials.

3.3.2 Selective media analysis

Bacterial strains were inoculated onto selective media and incubated for 5 days at 28°C. Bacterial growth of pure cultures was assessed on each medium to determine the degree of selectivity of the agar media prior to isolation from field samples. The results show that the growth media are only semi-selective allowing a wide range of isolates to grow (Table 8). Although RS agar is the selective media for *A. vitis*, growth of strain F2/5 was generally slow with small colonies resulting.

Strain	Growth on selective media			
	Biovar 1 agar	Biovar 2 agar	Biovar 3 agar	RS agar
A. tumefaciens HLB2 (1 isolate) Biovar 1	+	Sc	+	+
A. rhizogenes K84 (1 isolate) Biovar 2	+	+	sc	sc
A. vitis (15 isolates) Biovar 3	-	-	+	+
P. fluorescens K315 (1 isolate)	+	+	sc	sc

Table 8: Summary of species growth on media selective for *A. tumefaciens* (Biovar1 medium), *A. rhizogenes* (Biovar 2 medium), and *A. vitis* (Biovar 3 medium and RS medium). Biovar media described by Brisbane and Kerr (1983), RS media by Roy and Sasser, (1983)). Plates were inoculated with pure cultures and incubated for 5 days at 28°C. (+) Growth of bacterial strain to typical species morphology, (-) no growth of isolate on selective media, (sc) small colony reduced growth of isolate on media, not conforming to typical species morphology. *A. vitis* isolates used in this experiment are listed in Section 2.1.3.

Results indicate that growth of bacteria on species specific media is not limited to the species for which the media was designed. Growth of *A. tumefaciens*, *A. rhizogenes* and *P. fluorescens* was observed on RS and Biovar 3 media designed to isolate *A. vitis* (Table 8). Colony morphology can be used as a distinguishing feature when selecting colonies from pure culture but this is difficult from a field sample. All P. fluorescens isolates appear orange in colour with a diffuse/irregular border. All agrobacteria appeared white in colour, circular, raised and glossy. Differences between agrobacteria species were variable.

3.3.3 Pathogenicity testing on tomato

After 4 weeks growth under glasshouse conditions tomato plants were assessed for galls, gall diameter and characteristics. Galling occurred for all pathogenic strains while no galls were observed for all non-pathogenic and biocontrol strains. Figure 6 shows the galling observed on tomato stems at 4 weeks post-inoculation. Galls varied in size between the bacterial strains. Results showed that gall size varied according to pathogenicity and host range of the inoculated strain (Figure 7). Galls produced by K252 (pathogenic *A. vitis*) were significantly smaller than those produced by all other pathogenic *A. vitis* isolates ($p<0.001$). K252 is a limited host range strain of *A. vitis*.

Characteristics of the galling varied with the strain. No galling or necrosis was visible for all biocontrol and non-pathogenic strains on the tomato stem (isolate details in section 2.1.3). After inoculation with pathogenic *A. vitis* 680 tissue necrosis was visible, while root induction was visible after inoculation with K306 and K1072. Necrosis of plant material has been frequently recorded and associated with the presence of pectinolytic enzymes in *A. vitis* when applied at high levels to grapevine roots (Burr *et al.*, 1987).

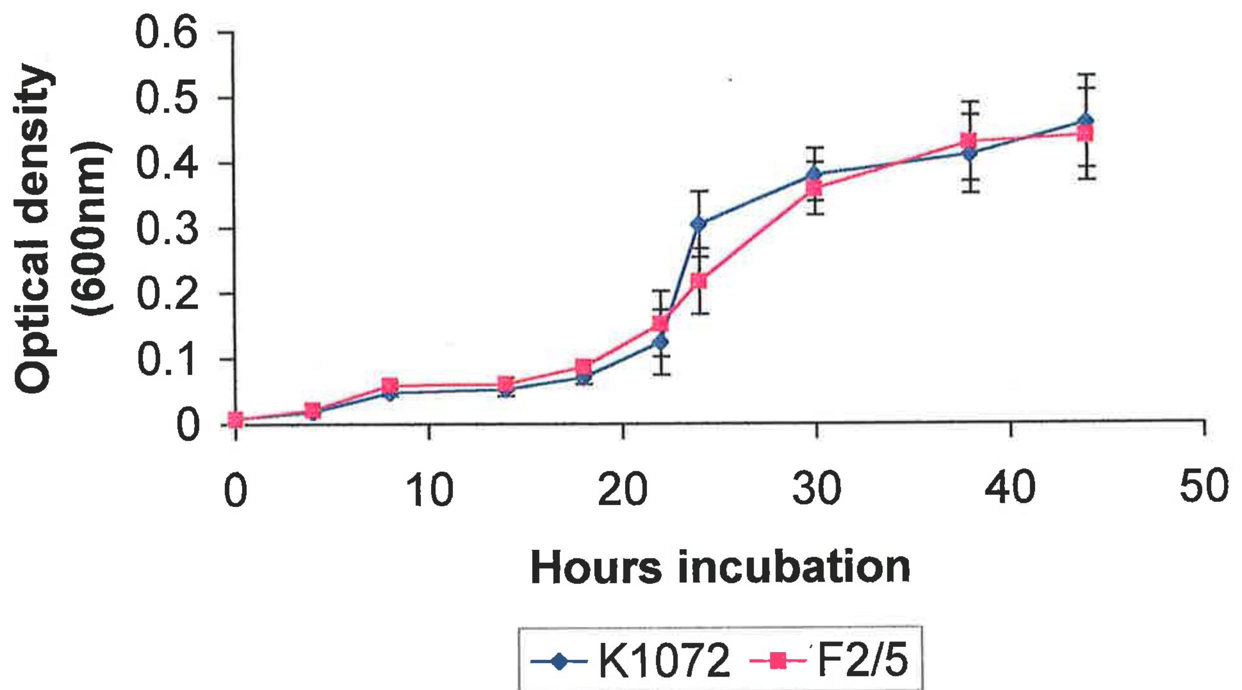


Figure 5: Bacterial growth rates of K1072 (pathogenic *A. vitis*) and F2/5 (biocontrol *A. vitis*) isolates in nutrient broth over 48 hours. Growth curves were fitted and assessed for differences between the isolates.

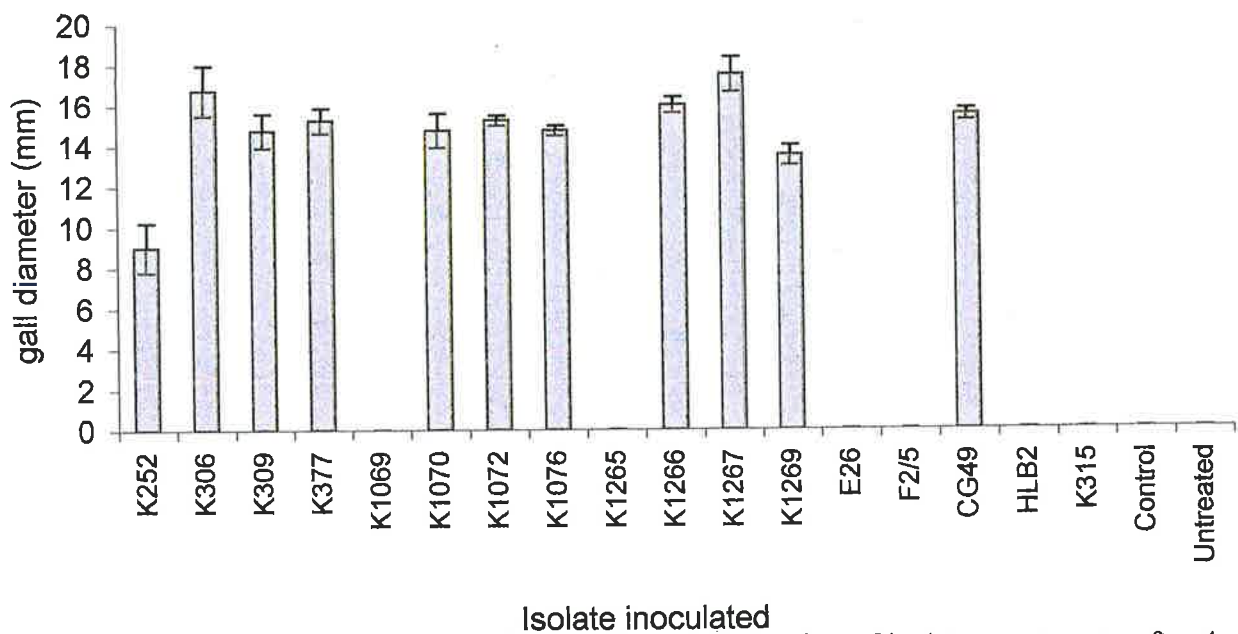


Figure 6: Assessment of gall diameter from pathogenicity testing of isolates on tomato after 4 weeks. Gall diameter (mm) was measured at the widest section of each gall. Analysis of gall diameter showed galls induced by K252 were significantly smaller than all other pathogens. A range of isolates was used to determine pathogenicity. Isolate details are in Section 2.1.3.

3.3.4 Plasmid screening of bacterial isolates

Plasmid isolation showed a range in the number and size of plasmids in the strains. Plasmid profiles of biocontrol and pathogenic strains are shown in Figure 8, and are consistent with previous reports (Burr and Reid, 1994, Merlo and Nester, 1977, Szegedi *et al.*, 1999) with Ti sized plasmids present for all pathogenic strains and absent for non-pathogenic biocontrol strains. F2/5 (*A. vitis* biocontrol) contained 3 plasmids as previously reported (Burr and Reid, 1994) including a tartrate utilising plasmid (Szegedi *et al.*, 1999).

3.3.5 Antagonistic activity of bacterial isolates

Production of antagonistic compounds by biocontrol and pathogenic strains was determined using a modified Stonier's overlay technique (Section 2.4). Zones of inhibition were measured after 3 and 5 days incubation. A selection of grapevine and rhizosphere isolates was included to determine any effects of introducing a biological control agent into the field. The production of inhibitory compounds by agrobacteria has been well documented but little is known of the effects of these compounds in microbial communities.

Antagonistic compounds produced by the biocontrol strain F2/5 were inhibitory to 9 of 15 *A. vitis* isolates tested; E26 inhibited 10, K315 inhibited 13 and HLB2 inhibited 14 of the isolates. *In vitro* agrocin production has often been utilised to detect potential biocontrol agents from mixed populations in field samples. Table 9 shows the diameter of the zones of inhibition observed for all strains tested with 15 pathogenic *A. vitis* and a range of other strains.

Figure 7: Plasmid profiles of bacterial isolates used in this study. Plasmid DNA was isolated and run on a 0.5% TBE agarose gel at 50V for 4 hours using K84 plasmid DNA as a standard marker (size measured in kbp). Lane1 K84 (non-pathogenic biocontrol strain), 2. K315 (non-pathogenic biocontrol strain), 3. K377 (pathogenic), 4. HLB2 (non-pathogenic biocontrol strain), 5. K1265 (non-pathogenic biocontrol strain), 6. K1269 (pathogenic), 7. E26 (non-pathogenic biocontrol strain), 8. F2/5 (non-pathogenic biocontrol strain), 9. K1345 (pathogenic), 10. K84 (non-pathogenic biocontrol strain).

		Strains tested for Agrocin production					
Isolate tested for Susceptibility	Pathogen +/-	Mean diameter of zone of clearing (mm) ± standard deviation					
		K309	K315	HLB2	E26	F2/5	K1069
A. tumefaciens							
R72	+	+++	0	+++	++++	0	0
K198	+	0	0	0	+++	0	0
HLB2	-	0	0	-	+++	0	0
A. rhizogenes							
R70	+	0	+	0	0	0	0
R76	+	0	++	+++	0	0	0
K84	-	0	0	+	0	0	0
A. vitis							
Ag57	+	++	+	+	++	0	0
K306	+	+	++	++++	0	+	0
K309	+	0	++	+++	+	++	0
K377	+	++	++	0	++	0	0
K1069	-	++	0	++	++	++	-
K1070	+	0	++	+++	0	+	++
K1072	+	0	++	+++	++	0	0
K1076	+	0	+	+++	++	+	0
K1265	-	0	++	+++	0	0	0
K1266	+	0	++	++	++	++	0
K1267	+	0	+++	++	+	++	0
K1269	+	0	+	+++	+++	+++	0
E26	-	0	0	++	-	0	0
CG49	+	0	++++	+	0	+	0
F2/5	-	++	+	+++	++	-	0
P. corrugata							
2140 OP1	-	0	++	0	0	0	0
B15132	-	0	+	0	0	0	0
R. meliloti							
SU303	-	ng	ng	ng	ng	ng	ng
SU391	-	ng	ng	ng	ng	ng	ng
NA30	-	ng	ng	ng	ng	ng	ng
B. subtilis							
R238	-	0	0	0	0	0	0
B908	-	0	+	0	0	0	0

Table 9: Screening for the production of antagonistic compounds from biocontrol strains (*P. fluorescens* K315, *A. tumefaciens* HLB2, *A. vitis* E26 and *A. vitis* F2/5), pathogenic strain (K309) and non-pathogenic strain (K1069) against a range of *A. tumefaciens, A. rhizogenes, A. vitis* and other rhizosphere bacteria on Stonier's medium (Stonier, 1960). ng = no growth of overlay strain on Stonier's medium. Isolate details are given in Table 1. *R. meliloti* isolates were tested but failed to grow on the medium. 0 =0mm, += 1-15mm, ++= 16-30mm, +++= 31-45mm, ++++= >45mm inhibition zone. - = not tested against self.

3.3.6 Pathogen selection for experimental use

Antagonistic activities of compounds produced by the biocontrol strains were effective against a wide variety of isolates tested using the modified Stonier's assay (1960). Sizes of inhibition zones varied (Table 9) and a number of resistant and susceptible strains were present. Resistance to agrocins was determined by the absence of an inhibition zone on the agar plate. Pathogens were selected for use in field trials and competition experiments, to be used in conjunction with the *A. vitis* biocontrol strains F2/5 and E26. Pathogens resistant to antagonistic compounds produced by these two isolates were favoured as previous glasshouse trials indicated these were the most successful at controlling crown gall disease (Biggs, 1994).

Strains selected for use in these experiments are listed in Table 10. The isolates selected for field trials were K306 and K1072 as this combined the effects of an agrocin resistant and susceptible pathogenic *A. vitis* for field trials. For competition assays (Chapter 7) individual pathogens were selected for each biocontrol strain. Combinations of biocontrol and pathogen are given: E26 (biocontrol) and K306 (pathogen), F2/5(biocontrol) and K1072, HLB2 (biocontrol) and K377 (pathogen) and K315 (biocontrol) and K1072 (pathogen).

	Susceptibility/Resistance of biocontrol strain to agrocin produced by pathogen	
Biocontrol strain	K306	K1072
E26	Resistant	Susceptible
F2/5	Susceptible	Resistant
HLB2	Susceptible	Susceptible
K315	Susceptible	Susceptible

Table 10: Susceptibility/resistance of pathogens selected for use in field trial to antagonistic compounds produced by biocontrol strains *in vitro* using the Stonier's antagonistic activity assay (Section 2.4).

3.3.7 PCR based detection of *A. vitis*

3.3.7.1 Individual primer set:

PCR using individual primer sets with cell lysates were effective at detecting all *A. vitis* strains with *pehA* primers and pathogenic *A. vitis* strains with *virA* primers (Figure 8). There was a clear band after both *virA* (480bp) and *pehA* (199bp) PCR amplification from *A. vitis* and no bands for non-*A. vitis* DNA. Control reactions were utilised to detect contamination within individual reaction sets using water (negative control) and a known *A. vitis* isolate (positive control). Different annealing temperatures are required for the two primer sets due to the differences in melting temperature (Tm) of the primers (56°C for *virA* and 54°C for *pehA* primers) when a single set of primers were used in the reaction.

3.3.7.2 Multiplex PCR

The multiplex PCR was optimised using the same cycling protocol as used for *virA* individual set PCR (Section 2.9.3). The annealing temperature was set at 56°C. In each reaction 30nmol *virA* primers and 40nmol *pehA* primers were used. Reactions were optimised using this reaction mix to ensure good amplification of both products without the appearance of other random bands in a single reaction. When pathogenic *A. vitis* were tested two bands were observed at 199bp and 480bp while non-pathogenic strains resulted in a single band at 199bp (Figure 9). No amplification was observed for non-*A. vitis* DNA tested.

Figure 8: PCR detection of non-pathogenic and pathogenic *A. vitis* with *pehA* and *virA* primer sets in individual PCR reactions. PCR product was run on 1.5% agarose gel in TAE buffer.

Lane

1. Molecular weight markers100bp ladder	11. K1069 (*A. vitis* non pathogen) *virA*
2. F2/5 (*A. vitis* non pathogen) *pehA*	12.K1072 (*A. vitis* pathogen) *pehA*
3. F2/5 (*A. vitis* non pathogen) *virA*	13. K1072 (*A. vitis* pathogen) *virA*
4. K306 (*A. vitis* pathogen) *pehA*	14. E26 (*A. vitis* non pathogen) *pehA*
5. K306 (*A. vitis* pathogen) *virA*	15. E26 (*A. vitis* non pathogen) *virA*
6. K309 (*A. vitis* pathogen) *pehA*	16.K1070 (*A. vitis* pathogen) *pehA*
7. K309 (*A. vitis* pathogen) *virA*	17. K1070 (*A. vitis* pathogen) *virA*
8. K377 (*A. vitis* pathogen) *pehA*	18. *A. vitis* 675 (*A. vitis* non pathogen) *pehA*
9. K377 (*A. vitis* pathogen) *virA*	19. *A. vitis* 675 (*A. vitis* non pathogen) *virA*
10. K1069 (*A. vitis* non pathogen) *pehA*	-

Figure 9: Multiplex PCR for the detection of non-pathogenic and pathogenic *A. vitis* using *pehA* and *virA* primer sets in a single reaction with annealing temperature at 54°C. Reactions tested a wide range of isolated from grapevine and other sources. PCR product (10µl) was run on a 1.5% agarose gel in TAE buffer for 1 hour at 80Volts. Gels were stained in ethidium bromide and visualised by UV. 480bp= *virA*, 199bp= *pehA*

Lane	Sample	Pathogen on grapevine	Lane	Sample	Pathogen on grapevine
1	100bp ladder (Promega)	-	14	*A. vitis* K377	+
2	*P. fluorescens* K315	-	15	*A. vitis* K1053	+
3	*A. tumefaciens* K2	+	16	*A. vitis* K1070	+
4	*A. tumefaciens* K6	+	17	*A. vitis* K1072	+
5	*A. tumefaciens* K8	-	18	*A. vitis* K1076	+
6	*A. tumefaciens* K30	+	19	*A. vitis* K1265	-
7	*A. tumefaciens* K41	+	20	*A. vitis* K1266	+
8	*A. tumefaciens* HLB2	-	21	*A. vitis* K1267	+
9	*A. rhizogenes* K46	+	22	*A. vitis* K1269	+
10	*A. rhizogenes* K84	-	23	*A. vitis* E26	-
11	*A. rhizogenes* K114	+	24	*A. vitis* CG49	+
12	*A. vitis* K252	+	25	*A. vitis* F2/5	-
13	*A. vitis* K306	+	26	Water control	-

3.3.8 Detection of *A. vitis* from vascular washing fluid

PCR amplification of DNA prepared from vascular washing fluid (using the Ultraclean™ DNA Extraction kit) was effective for *virA* and *pehA* primer sets, with no inhibition or contamination occurring. Vascular fluid was inoculated into RS broth supplemented with cycloheximide to prevent fungal growth. Incubation of the fluid in RS broth allows for an increase in bacterial numbers of *A. vitis* that allowed detection of lower levels by PCR within the grapevine sap. There was no need to increase bacterial numbers when isolating from callus material.

3.3.9 DNA fingerprinting with ERIC-PCR

Individual banding patterns were obtained for biocontrol and pathogenic strains used in field trials to allow identification of strains after recovery from vines. Strain specific banding patterns indicate some common bands within *A. vitis* as well as differences between individual strains within the species (Figure 10). Strain specific bands were observed in the higher molecular weight bands. This allows identification of individual isolates when reisolating bacteria from field trials with strain specific bands.

3.3.10 ERIC fingerprints of K84 and plasmid cured derivatives

Analysis of DNA fingerprints of strain K84 and its derivatives containing a variety of plasmids indicate that loss of plasmid(s) affected the ERIC DNA fingerprint. The reduction in plasmid content of strain K84is reflected in a decrease in the amplification of lower molecular weight bands was observed (Figure 11). The size of higher molecular weight bands remained constant.

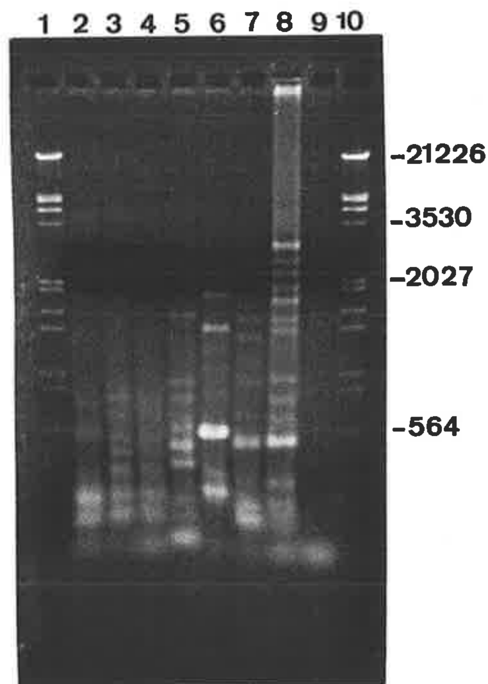


Figure 10: DNA fingerprinting of bacterial isolates using ERIC-PCR (section 2.10). PCR products (15μl) were run on 1.5-% agarose gel in TAE buffer at 50 Volts for 4 hours.

Lane

1. Marker III (Boehringer Mannheim)
2. K252 (*A. vitis*, pathogen)
3. K306 (*A. vitis*, pathogen)
4. K309 (*A. vitis* pathogen)
5. K315 (*P. fluorescens* biocontrol)
6. HLB2 (*A. tumefaciens* biocontrol)
7. E26 (*A. vitis* biocontrol)
8. F2/5 (*A. vitis* biocontrol)
9. Water control (negative)
10. Marker III (Boehringer Mannheim)

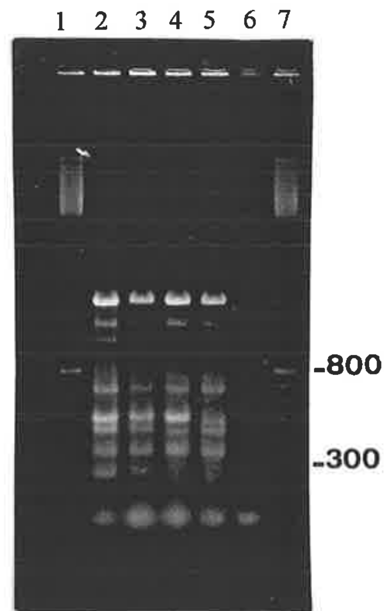


Figure 11: ERIC PCR fingerprints for strain K84 and cured derivatives K434, K1143 and K1347 (section 2.10). An aliquot (15µl) of PCR product was run on 1.5% agarose in TAE buffer, 50V for 4 hours.

Lane

1. Promega 100bp ladder
2. K84 (plasmids pAgK84, pAtK84b and pAgK434)
3. K434 (plasmids pAtK84b and pAgK434)
4. K1143 (plasmid pAgK434)
5. K1347 (plasmid free)
6. Water control (negative)
7. Promega 100bp ladder

3.4 DISCUSSION

The aim of this chapter was to characterise the bacterial isolates of interest and develop faster detection procedures for the isolation, detection and enumeration of pathogenic *A. vitis* and the biocontrol strains used in the field trials (Chapters 4, 5, and 6). Bacterial isolates were typed for a range of characteristics including growth on selective media, pathogenicity, plasmid content, DNA fingerprinting and screened for antagonistic activity. Methods were developed to allow bacterial isolation from field soil samples, callus and vascular washing with the identification of strains of interest. Diagnostic testing procedures were developed to obtain a testing procedure that is useful to the grape and wine industry for the identification of infected mother-vines. This will give the industry the potential to reduce the spread of crown gall disease by propagation of clean cuttings.

Species specific primers (developed by Eastwell *et al.*, 1995) were used for the detection of a virulence gene (*virA*) on the Ti plasmid (pathogenic strains of *A. vitis*) and pectin enzyme hydrolase (*pehA*) gene on the chromosome of all *A. vitis*. PCR using *virA* and *pehA* primers can detect both pathogenic and non-pathogenic *A. vitis* isolates from Australia and from around the world (in Table 1). However, the primers are unable to detect *A. vitis* isolates that contain the vitopine type Ti plasmid, strains which produce vitopine (Eastwell *et al.*, 1995).

In Australia, the major disease causing *A. vitis* pathogens are octopine, nopaline and cucumopine opine producing isolates with no vitopine isolates detected to date (Ophel, 1990). The use of this PCR technique has overcome the need for secondary testing of individual isolates as previously required with the immunoblot. New primers are now available that detect all *A. vitis* isolates including those containing vitopine type Ti plasmids (Momol *et al.*, 1998). These primers were based on the *virE2* primer that amplifies a

characteristic product with vitopine Ti plasmids. To date there has been no isolation of the vitopine type Ti plasmids from pathogenic *A. vitis* in Australia. Further surveys of grapevine material within Australia may reveal the presence of pathogenic vitopine *A. vitis*. Limits of detection of pathogenic *A. vitis* could be improved by including a step that concentrated the *A. vitis* bacteria and removes inhibitory compounds such as that developed by Kaufman *et al*., 1996) or the use of Dynal beads (Dynal, Oslo, Norway). This allows the sample to be thoroughly washed to remove the majority of compounds in grapevine sap that may inhibit PCR. The purified *A. vitis* bacteria can then be tested for pathogenicity for simple grapevine indexing. PCR allows a result to be obtained quickly when combined with direct detection using vascular washing. The elimination of the plating step allows testing to be performed on grapevine sap without inhibition or contamination.

The development of this test is important for the grape and wine industry and grapevine nurseries. Mother vines can be tested prior to cuttings being taken to determine whether vines are disease free. The elimination of *A. vitis* from nursery stocks would aid in decreasing the transfer of diseased material and increase the viability of grapevines worldwide (Eastwell *et al*., 1995). The development of the multiplex PCR allows two primer sets to be combined into a single PCR reaction. This will detect pathogenic and non-pathogenic *A. vitis* from grapevine vascular washing samples. For samples where pathogenic *A. vitis* are detected, the second primer set coding for polygalacturonase gives a control reaction.

Characterisation of biocontrol and pathogenic strains was used to determine the differences between the individual bacteria. A series of *in vitro* tests were used to ensure that the

biocontrol strains selected were active against pathogenic *A. vitis* confirming results with previous studies.

Plasmid profiles of the isolates showed that all *A. vitis* pathogens contained a large Ti sized plasmids. Strain F2/5 has a band corresponding to the size of a Ti plasmid but has previously been shown not to contain a Ti plasmid through DNA hybridisation to a Ti DNA probe pTHE17 (Burr and Reid, 1994). Strain HLB2 did hybridise to the Ti probe but is non-pathogenic on grapevine and all plant varieties tested (this study, Pu and Goodman, 1993, Biggs *et al.*, 1994, Burr and Reid, 1994, Chen and Xiang, 1986). This isolate may potentially contain a Ti plasmid with deletions in the virulence region. Further investigation would be required if this isolate were used commercially as a biological control agent.

Antagonistic activity through the production of antagonistic compounds by both pathogenic and non-pathogenic isolates was observed if disease control was due solely to production of antagonistic compounds. The resistance of some pathogens to inhibitory agents produced by a biocontrol strain such as F2/5 would be problematic. Burr *et al.*, (1997) has shown using agrocin minus mutants of F2/5 that control is still possible without production of inhibitory compounds. The strains that are resistant to F2/5 contain mainly octopine type plasmids. The resistance is not constant across biocontrol strains, as not all octopine-producing strains are resistant.

F2/5 has been demonstrated to be good at controlling disease in glasshouse (Burr and Reid, 1994, Biggs, 1994) and field trials (this study). Other potential biocontrol strains were very effective when tested for antagonism *in vitro*. For HLB2 fourteen of fifteen *A. vitis* isolates were inhibited in the Stonier's assay while K315 inhibited thirteen of fifteen tested. The

mechanism of action of these isolates may be antagonism through production of antagonistic compounds but requires further investigation. Sensitivity of pathogenic *A. vitis* to these compounds can be dependent on the medium used in the overlay medium (Fajardo *et al.*, 1994) which may be related to the changes seen *in vitro* and *in planta.*

The mechanism of action for crown gall disease control on almonds and stone fruits was demonstrated to be two-fold for *A. radiobacter* K84. Production of agrocins 84 and 434 is effective *in vitro* and *in planta,* but biocontrol was still effective with a plasmid free mutant K1347 (McClure *et al.*, 1994) indicating that disease control is not entirely agrocin driven. When used in leaf disc and stem tumorigenesis assays strain K1347 (plasmid free) reduced gall formation by a pathogen using a high concentration of K1347. This strain may produce other inhibitory compounds that are chromosomally encoded although it seems likely that control is due to competition for infection sites, nutrients or more efficient root colonisation (McClure *et al.*, 1994).

The use of ERIC fingerprinting for the identification of specific strains has been investigated and has shown that all *A. vitis* isolates tested have a high degree of similarity in lower molecular weight banding patterns. For identification of specific strains, the higher molecular weight bands vary considerably between strains and individual strain specific bands were identified. This allows the identification of inoculated biocontrol and pathogenic strains from grapevine material.

Although ERIC fingerprinting is generated by amplification of intergenic repeats on the chromosome, the loss of plasmids from the bacterial cell can also affect DNA fingerprints. Fingerprints from K84 and its cured derivatives (K434, K1143 and K1347) demonstrated

that loss of the plasmid pAtK84b resulted in the absence of a lower molecular weight band. This has been observed previously with *A. tumefaciens* strain C58 (pTi) and C58C1 (lacking pTi)(de Bruijn *et al.*, 1992). In this study, the loss of plasmid pAtK84b from the bacterial cell resulted in the loss of the smallest molecular weight band from the DNA profile. This demonstrates that the ERIC primers are binding to sequences on the Ti plasmid and loss of a Ti plasmid may result in changes to the DNA profile. As the biocontrol strains E26, HLB2 and F2/5 contain large plasmids, similar results may be seen with plasmid loss. F2/5 has been shown to be free of any Ti type plasmid using DNA hybridisation to the pTHE17 probe (Burr *et al.*, 1990). K315 contains no detectable plasmids while E26 has a plasmid of approximately 200kb that correspond to the size of Ti plasmids. Hybridisation of E26 DNA with pTHE17 has not been tested but all biocontrol strains are non-pathogenic on a wide range of plants tested (Burr and Reid, 1994, Pu and Goodman, 1993a, Ma *et al.*, 1987 and this study). It is important that biocontrol strains not contain Ti plasmid sequences as this may allow transformation of the plant host.

Although ERIC PCR is a useful tool for the identification of bacterial strains, it was not used further in this study. This was due to the development of alternative techniques that do not rely on plating on selective media and selection of putative *A. vitis* colonies for testing.

A range of techniques were used to characterise the bacterial isolates used in this study. The biocontrol strains produce inhibitory products *in vitro*, which were active against a range of pathogens. Rapid methods for strain identification were developed to allow population dynamics to be investigated while detection of pathogenic *A. vitis* was possible using a quick and relatively simple PCR test. This overcomes limitations encountered with selective media and ELISA. Analysis of cultured organisms on selective agar media for the detection

of crown gall disease on grapevine reduces the detection limits (Eastwell *et al.*, 1995) and hinders diagnosis, indicating the need for alternative reliable techniques. Diagnostic tests have been used for the assessment of field trials and population dynamics on grapevine samples (this study).

CHAPTER 4

ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSERY: TRIAL 1 (1996-1997)

4.1. INTRODUCTION

Field trials were designed to assess the efficacy of four potential biocontrol strains in the control of crown gall disease on grapevine. Biocontrol strains had previously been tested in glasshouse studies with promising results (Biggs, 1994). The application of biocontrol strains E26, F2/5 and HLB2 prior to planting into pathogenic *A. vitis* infested soil resulted in a significant reduction in gall formation (from > 57% to 0%) (Biggs, 1994).

Field trials are necessary to demonstrate biocontrol efficacy at reducing gall frequency in field conditions before use in the commercial market (Pu and Goodman, 1993). Variations in field conditions may be due to a number of factors including soil moisture, temperature, pH and presence or absence of competition.

The field trial was also designed to determine the effects of thermotherapy on grapevine cuttings. Thermotherapy (or heat treatment) was developed as a method of eliminating indigenous bacteria within grapevine cuttings (Burr *et al.*, 1989, Ophel *et al.*, 1990). Cuttings were heat-treated for 20 to 30 minutes at 50°C to allow the internal sections of the cane to reach the set temperature resulting in death of the majority of bacteria present within the vascular system (Lehoczky, 1968, Burr *et al.*, 1987, Burr *et al.*, 1989). This is now an

accepted grapevine nursery practice and thermotherapy treated grapevines are considered *A. vitis* free as levels after thermotherapy are below detection (Ophel *et al.*, 1990). *A. vitis* are capable of surviving the heat-treatment but take at least 41 months to grow to large enough numbers to affect the grapevine on wounding (Biggs, 1994).

The effects of thermotherapy on grapevine growth (shoot development, callus size and canopy development) have varied with grapevine variety, treatment time and post-thermotherapy treatment of the grapevines (Orffer *et al.*, 1977, Orffer *et al.*, 1979). In previous trials on fully dormant grapevines, thermotherapy was shown to eliminate indigenous *A. vitis* populations effectively from the grapevines to below detectable levels (Ophel *et al.*, 1989). In vineyard nurseries the effects of thermotherapy have been variable. Grapevine growth can be limited due to excess heat-treatment or shorter treatment periods at higher temperatures. Total control of the disease is not possible using this method as not all bacteria are killed (Goodman *et al.*, 1989). This is due to variations in dormancy of the cuttings, period of treatment and treatment of the vines before and after hot water dipping and callusing (Orffer *et al.*, 1977, Orffer *et al.*, 1979).

Current practice for callusing and propagation of cuttings varies between grapevine nurseries with two methods currently in use in South Australia. Grapevines are inverted and placed into callusing bins and covered in sand to callus in the sun with regular watering for up to 2 months (D. Haeusler, pers. comm.). The other method involves callusing for three weeks with grapevines horizontally packed into polystyrene boxes filled with vermiculite and incubated at 27°C (P. Wright pers. comm.). Callused cuttings are then planted into the field or into biodegradable paper pots and kept in a glasshouse. There is minimal risk if the biodegradable pot system is used as soil is pasteurised and sourced from areas with no

previous exposure to grapevines. The potential risk of exposure to grapevine pathogens is increased when planting into contaminated field sites. Increased levels of grapevine pathogens can remain in the soil through re-use of propagation sites (Burr *et al.*, 1995). These pathogens pose a serious risk to grapevine nurseries. Virgin land (with no previous history of grapevine propagation) or the use of pasteurised soil is ideal for planting grapevine nurseries. These options are particularly costly, as suitable land is often limited and expensive.

Previous research has focused on obtaining disease-free grapevines using shoot tip culture and tissue culture of grapevines (Burr *et al.*, 1987). These methods have proved successful in eliminating pathogenic *A. vitis* from the vascular system to below detectable levels. However, on planting into a glasshouse or the field, grapevines become reinfested (Goodman *et al.*, 1993).

The potential use of a biological control agent to reduce the risk of crown gall infection on grapevine cuttings and rootlings was the main aim of this project. Grapevines free of *A. vitis* may become reinfested over a long period of time. The application of a biological control strain that is antagonistic towards pathogenic *A. vitis* after thermotherapy should allow good colonisation of the vascular system with little competition from other *A. vitis* strains. Under glasshouse conditions, all biocontrol strains E26 and F2/5 (*A. vitis*), HLB2 (*A. tumefaciens*) and K315 (*P. fluorescens*) were capable of colonising the vascular system of the grapevine (Biggs *et al.*, 1994).

This study has focused on the use of these four potential biocontrol strains to protect grapevine cuttings from reinfestation by indigenous *A. vitis* in a field site at Langhorne

Creek, South Australia. The site was used previously as a grapevine nursery which produced galling on Shiraz and Merlot grapevine varieties ensuring an indigenous *A. vitis* population within the soil. Biological control strains were applied to grapevine cuttings in conjunction with thermotherapy treatments and callused in sterile river sand to allow colonisation of the vascular system to occur. The effect of individual biocontrol strains were assessed under field conditions to determine if a reduction in galling frequency was achieved.

4.1.1 Field trial aims

1. To determine the efficacy of four potential biological control agents to control crown gall disease in naturally infested soil and cuttings.

2. To determine whether biocontrol agents are capable of decreasing disease as measured by gall diameter and frequency of galling compared to uninoculated grapevines.

3. To determine the effect of thermotherapy on dormant grapevine cuttings and its interaction with biocontrol agents.

4.2 EXPERIMENTS

4.2.1 Field trial organisation

4.2.1.1 Grapevine variety and treatment

Temple Bruer and Bay View Wines Pty. Ltd. grapevine nursery, (Langhorne Creek, South Australia) supplied grapevine cuttings of *Vitis vinifera* Shiraz clone BVRC12. Heat-treated and non-heat treated cuttings (section 2.15.3.1) were prepared and plunged into tap water with bacterial inoculum added ($10^{5\text{-}6}$ cfu/ml) to aid in inoculation and rehydration of grapevines. Addition of inoculants was performed in this way to conform to current grapevine nursery practices.

Grapevines were inoculated with one of four biocontrol strains (E26, F2/5, HLB2 or K315) and a Control grapevines received no inoculum. Grapevines were placed into callusing bins and stored outdoors to allow warmth from the sun to aid in grapevine callusing (Section 2.15.4.1) (approximately 8 weeks). Grapevines were watered weekly to prevent sand and grapevines from dehydrating. Individual bins were used for different treatments to prevent cross contamination.

4.2.1.2 Field site

The 1996-97 field site at Temple Bruer Winery (Langhorne Creek, South Australia) was selected as a potential site with high levels of *A. vitis* as it had been used as a field nursery in the 1995/96 growing season. Disease had been detected on several rootlings removed from the area on Shiraz and Merlot grapevines. Areas were pegged off to allow planting where disease had been observed (D. Haeusler, pers. comm.). The soil at the site was sandy/loam that is favourable for crown gall disease (Dr K. Ophel Keller, pers. comm.).

4.2.1.3 Field site organisation

The field site was arranged into a randomised block design with 5 replicate plots for each treatment (2m per plot and 25 grapevines) (Figure 14). The trial consisted of non-heat treated and heat-treated grapevine cuttings inoculated with one of four bacterial inoculants and control grapevines (no inoculant applied) in separate plots. Before planting, soil was hoed and the block pegged out. Vines were planted in late October and remained in the soil until August of the following year. Watering and fertilising of the grapevines was performed using the standard nursery practice at Temple Bruer. Grapevines remained in the ground until reaching full dormancy and were then harvested using a disc hoe to allow grapevines to be lifted with minimal damage to the root system. Grapevines were bagged, sealed and returned to the laboratory for thorough washing and analysis of gall numbers. All grapevines were stored at 2°C until assessed.

4.2.2 Level of inoculum applied to callused grapevine cuttings

Biocontrol strains were prepared for use and diluted in 10 litres of tap water before inoculation onto cuttings. This allowed re-hydration of the grapevines after thermotherapy (Section 2.15.3.1). Aliquots of the bacterial inoculum were taken and assessed for bacterial numbers to determine bacterial levels. Serial dilutions were performed, plated onto selective media and incubated at 28°C for 5 days. Selective media was used as follows: RS agar for *A. vitis*, Biovar 1 media for *A. tumefaciens*, Kings B media for *P. fluorescens*.

4.2.3 Colonisation of biocontrol agents on grapevine callus

Biocontrol strains were applied to grapevine cuttings prior to the callusing period and were re-isolated from grapevine callus samples. A sample from each treatment group was tested for the presence of biocontrol strains after the callusing period. Callus and root material was

removed, macerated in PBS and serially diluted before plating onto selective media (Section 2.15.6) supplemented with cycloheximide and incubated at 28°C for 5 days. Remaining samples were stored in 15% glycerol at –80°C.

It was assumed that contamination with *A. tumefaciens* was unlikely for all heat-treated vines but this species may have been present on non heat-treated vines. Detection of the biocontrol strain HLB2 (*A. tumefaciens*) on heat-treated vines was assessed to determine the colony morphology expected for HLB2. Plates detecting the same strain from non heat-treated vines were assessed in comparison with the heat-treated vines. An immunoblot (Section 2.11) was performed to determine the level of *A. vitis* present in the callus of non-heat treated vines. Kings B and Biovar 1 agars were found to be selective in isolating fluorescent Pseudomonads and *A. tumefaciens,* respectively and no additional confirmatory testing of these isolates was performed.

4.2.4 Identification of *A. vitis* from callus material

Due to the non-specific nature of the selective media available for isolation of pathogenic *A. vitis* it was necessary to confirm the identity of the isolates on RS media. An immunoblot asssay (Section 2.11) was used to correctly identify putative *A. vitis* isolates. Colonies from selective media were replated on selective media then tested with the immunoblot (Section 2.11). A total of four colonies per vine were checked with two replicates for each treatment and 5 vines per treatment.

4.2.5 Assessment of *A. vitis* populations in grapevine nursery soil

Pathogen levels at the field site were determined by plating out samples (Section 2.3) of the soil by serial dilution in PBS and plating onto RS agar. Plates were incubated at 28°C for

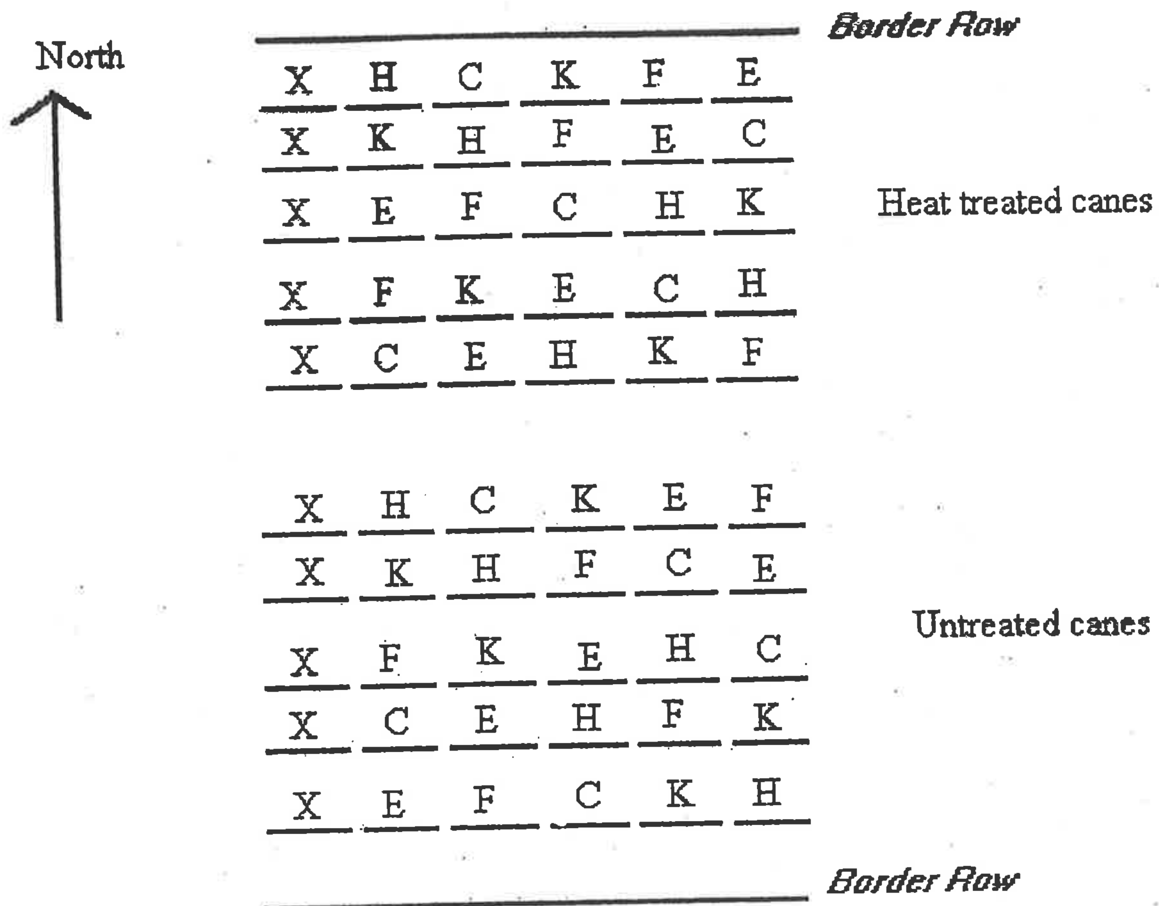


Figure 14: Field site organisation of plots for the 1996-97 field trial. Plots were organised in a random block design at Temple Bruer Winery, Langhorne Creek, South Australia on the previous years nursery site.

Legend:

E = E26 inoculum applied

F= F2/5 inoculum applied

H= HLB2 inoculum applied

K= K315 inoculum applied

C= control (no inoculum)

X= no inoculum applied, border row to protect vines

5 days. Seven samples were taken in total with a control sample taken from an area without vine propagation.

4.2.6 Efficacy of biocontrol agents in a grapevine nursery

Vines were washed and assessed individually for the site, size and position of galls and sap extractions performed on a sample from each treatment group (Section 2.15.7). Bacterial numbers were recorded after 5 days incubation at 28°C. Galling was assessed on all rootlings, but due to the excessive nematode damage no galls were observed.

4.2.7 Effect of thermotherapy on grapevines

Vines were assessed after the growing season to determine the total number of treated and untreated vines that grew. Assessment including observations for root and canopy development. Analysis was completed using 1-way ANOVA for individual bacterial treatments and statistically compared to the control groups.

4.2.8 Examination of field trial site for *Meloidogyne* (root knot nematode)

Throughout the growing season it was noted that the vines had poor canopy development. Additional foliar fertiliser was applied to grapevines in an attempt to increase nutrient uptake, however, this was ineffective. Further investigation revealed that root mass on individual vines was greatly reduced and stunted compared to normal vine development seen in the adjoining commercial nursery. After closer observation it was determined the field site had a major infestation of root knot nematode (*Meloidogyne spp.)* which prevents normal root development (J. Nicol, Dept. Crop Protection, pers. comm.).

Roots with cysts were incubated overnight in sterile tap water and observed under a dissecting microscope. Due to the high numbers of nematodes and cysts found on the roots,

the nursery soil was then tested for nematode levels (Section 2.13). Nematodes were identified by Dr. K. Davies and counts were completed on soil samples taken from around the roots of mildly and badly affected grapevine rootlings. Mildly affected vines were classified as having greater than 20cm of roots with canopy. Badly affected vines were classed as having limited stunted roots with a greatly reduced canopy. None of the vines from the field trial were as vigorous as grapevines in the commercial nursery showing less than 50 percent of normal vine canopy development.

4.3 RESULTS

4.3.1 Level of inoculum applied to grapevines

Bacterial numbers were estimated from serial dilution of pure cultures. All inocula contained approximately 10^7 cfu/ml of bacteria - *A. vitis* E26 (1.05×10^7 cfu.ml^{-1}) and F2/5 (1.35×10^7 cfu.ml^{-1}), *A. tumefaciens* HLB2 (3.05×10^7 cfu.ml^{-1}) and *P. fluorescens* K315 (2.55×10^7 cfu.ml^{-1}). There was no significant difference between the levels of bacteria in each inoculum (p level =0.05).

4.3.2 Enumeration of levels of biological controls on grapevine callus

Biocontrol bacteria were reisolated from the vine callus and roots after the callusing period in sand (Section 2.15.6). Samples were plated on selective media and a comparison between non-heat treated and heat-treated cuttings was made to determine if heat-treated vines were colonised more easily due to the lack of alternative antagonistic bacteria in the vascular fluids.

Further identification of the bacteria isolated on RS agar (treatments F2/5 and E26) was required using the immunoblot. All colonies isolated on Biovar 1 agar (HLB2) and Kings B agar (K315) did not require further identification due to the selective nature of the medium. Direct comparisons were made between plates from non heat-treated and heat-treated samples. No observable difference was found in the levels of HLB2 and K315 (data not shown).

Results from the immunoblot (Section 2.11) showed that many of the putative *A. vitis* colonies from selective RS media for treatments F2/5 and E26 were not *A. vitis*. For all vine treatments 50% to 75% of colonies isolated with RS media were later identified as strains

other than *A. vitis*. Bacterial populations from the control group showed low levels of *A. vitis*, while F2/5 treated vines had between 62.5% (non heat-treated) and 75% (heat-treated) *A. vitis*. Table 12 summarises the results from immunoblot tests to detect *A. vitis* isolates after recovery from callus and root material.

Biocontrol treatment	% *A. vitis* NHT vines	Calculated cfu/ml *A. vitis* NHT vines	% *A. vitis* HT vines	Calculated cfu/ml *A. vitis* HT vines
E26	62.5 (12.5)	3.76×10^6	62.5 (12.5)	2.2×10^6
F2/5	75 (25)	2.1×10^6	62.5 (12.5)	9.5×10^5
Control	0 (0)	0	25 (12.5)	5.2×10^2

Table 12: Immunoblot testing of putative *A. vitis* isolated from callus/root samples after callusing in the 1996-97 field trial using the antibody F21-ID3G7C8 (Leach *et al.*, 1987). Standard error given in (). Calculated: based on % *A. vitis* x cfu/ml detected.

Levels of *A. vitis* present on E26 and F2/5 treated vines were significantly higher than on un-inoculated control vines ($p < 0.05$) (data not shown). Levels of pathogenic *A. vitis* present on HLB2 and K315 treated vines were not significantly different to those seen on the uninoculated vines. There were no significant differences between biocontrol treatments with approximately 10^6cfu.ml^{-1} present on all treatments. On application of the biocontrol strains, they were all capable of colonising the callus, root and vascular system of the grapevine.

4.3.3 Assessment of *A. vitis* in nursery soil

Prior to planting the level of *A. vitis* present in the soil was assessed. The level of *A. vitis* detected in the field samples including the control sample (site 4) showed that site 1 (previous exposure to grapevine material) had a significantly higher level of *A. vitis* present

($5.0x10^7$ cfu.gram^{-1} soil) compared to the control with no previous exposure to grapevine ($2.1x10^5$ cfu.gram^{-1} soil). For all other sites there was no significant difference in the levels of *A. vitis* present at 2.0- $2.5x10^5$ *A. vitis*.gram^{-1} soil.

4.3.4 Effect of thermotherapy on grapevine growth

Establishment of grapevines was assessed and no significant differences were observed for all treatments when comparing non heat-treated and heat-treated vines to the control (no inoculum applied) except for F2/5 heat-treated vines (Figure 12). The proportion of vines which grew after heat-treatment combined with F2/5 application was greatly decreased (64%), when compared to non heat-treated vines (93%) ($p= 0.007$) and to control vines ($p= 0.035$). As the non heat-treated vines were unaffected by the inoculation with F2/5, the decrease in vine growth may be due to treatment during and post-thermotherapy.

4.3.5 Efficacy of biocontrol agents

Due to the lack of normal root development on the vines, no galling was observed. This was directly linked to the nematode infestation.

4.3.6 Bacterial levels isolated from grapevine sap

On recovery of vines from the field site after 9 months growth, sap extractions were performed to determine whether the biocontrol strains were present in the grapevine vascular system. Samples were serially diluted and plated on selective media as required for the isolation of *A. vitis*, *A. tumefaciens* and *P. fluorescens*. The recovered levels of bacteria are given in Figure 13. No significant difference was observed between all non heat-treated and heat-treated groups.

4.3.7 Examination of field site for *Meloidogyne* (root knot nematode)

Nematode levels were calculated from soil from the rhizosphere of healthy and unhealthy grapevine rootlings (Figure 14). Levels above 50 nematodes per 100g soil are deleterious to plant growth (S. Taylor, pers. comm.). Due to the patchiness of the nematode infestation across the site vine health varied, but the root and canopy development of most vines were badly affected by the nematode infestation when compared to the commercial nursery. Significant differences existed between mild and badly affected sites examined within the field site ($p=0.01$).

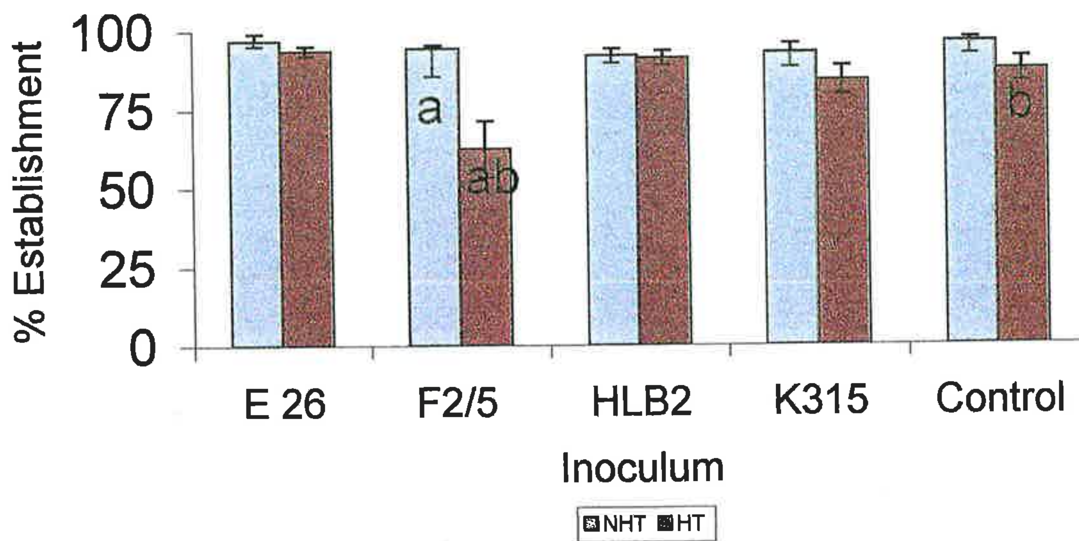


Figure 12: Establishment of vines in the 1996-97 field trial. Vines from each treatment group were assessed individually and statistical comparisons made between non heat-treated and heat treated vines for each inoculum applied and to the control (no inoculum applied) using ANOVA (1-way) analysis.

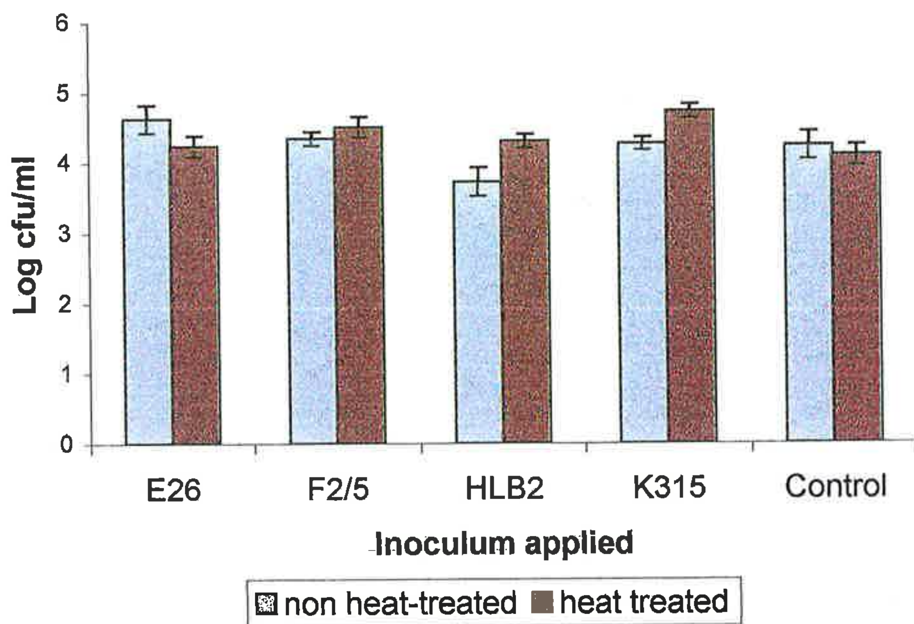


Figure 13: Bacterial isolates recovered from sap extractions of 1996-97 field trial Shiraz BVRC12 vines post harvest on selective media (RS agar for *A. vitis* E26, F2/5 and control vines, Biovar 1 agar for HLB2 and Kings B agar for K315). (+/- standard error of the mean)

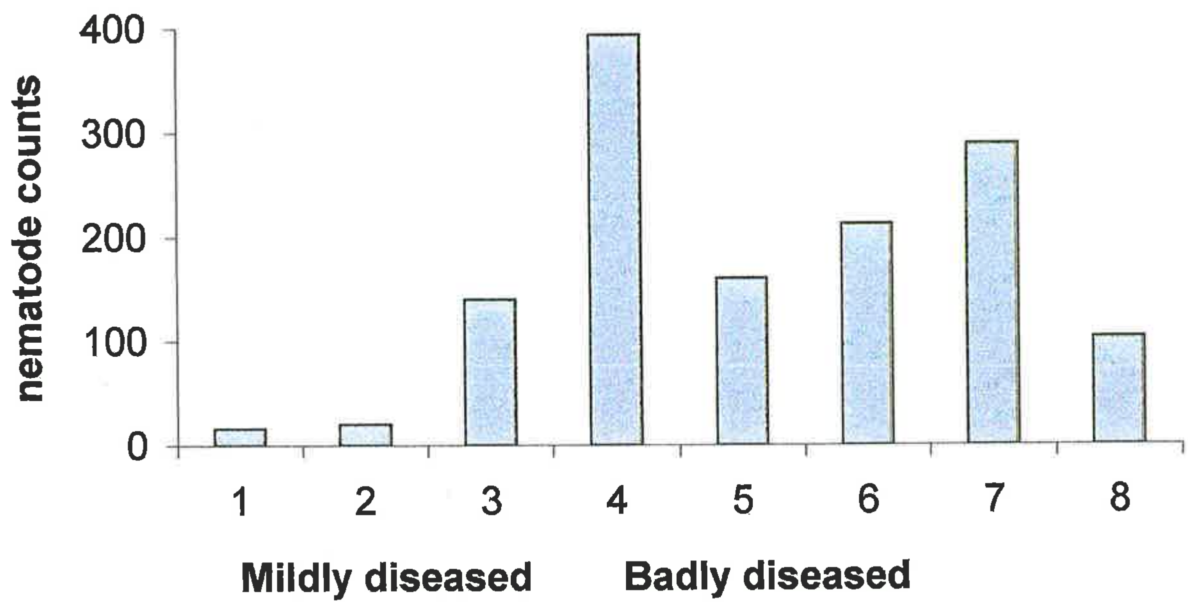


Figure 14: Levels of root knot nematodes recovered from soil samples taken from the rhizosphere surrounding grapevine roots from the 1996-97 field site. Sites 1, 2 and 3 contained mildly affected vines and sites 4, 5, 6, 7 and 8 contained badly affected grapevines.

4.4 DISCUSSION

A field trial was established to determine the efficacy of 4 potential biocontrol strains to control crown gall disease on grapevine nursery stock. Biocontrol strains were applied to heat-treated and untreated grapevine cuttings before callusing to allow colonisation of the vascular system.

The biocontrol strains were capable of colonising grapevine material after thermotherapy. On re-isolation from the callus and root material, biocontrol strains HLB2 and K315 were present at approximately 10^7 cfu.vine^{-1} for each treatment. The isolation of *A. vitis* strains E26 and F2/5 was confirmed with the immunoblot procedure. Both strains were present at 10^6 cfu.ml^{-1}. Overall colonisation by biological control agents on the callus material was not improved with the use of thermotherapy, but it was successful in decreasing pathogen numbers in the vascular system. Control vines (no inoculum applied) had significantly fewer bacteria present in the sap post callusing when compared to the other treatment groups. The bacteria are also capable of colonising the cork layer underneath the bark as the entire vine was immersed into the bacterial inoculum before callusing.

The indigenous levels of *A. vitis* present in the field soil were well below levels expected from a site on which vines were grown in the previous season. Information from nursery managers indicated that *A. vitis* levels would be very high due to observed galling on the site. On isolation with the semi-selective RS agar it appeared that *A. vitis* levels were approximately 10^5 cfu.g^{-1} soil. However, due to the non-selective nature of the medium, actual *A. vitis* levels were estimated at 10^4 cfu.g^{-1} soil or lower. The field trial was conducted prior to the development of the PCR based detection system for *A. vitis*. This level of *A. vitis* should be capable of inducing disease, but due to the lack of vine root development galls did not occur.

Major problems were encountered in the field trial due to nematode infestation. The trial site had been used recently for grapevine propagation. Although this was done to obtain a higher level of indigenous *A. vitis* (levels typically seen in the field soil), it resulted in the enhancement of other grapevine diseases including *Meloidogyne* (root knot nematode). The presence of nematodes in soil and nematode colonisation of immature root systems drastically reduced normal root development and affected plant growth. The nematode levels varied across the site with some samples recording very high levels (greater than 300 nematodes per 100g soil). A severe infestation was observed with all vines affected to some degree. No galling was observed on the grapevines because of poor root development caused by the nematode infestation.

Thermotherapy is not capable of killing all bacteria present within the sap. Bacteria in control vines were present in crushed callus material (10^3-10^4 cfu.ml^{-1}). Lower levels of *A. vitis* were present in the heat-treated vines indicating that less than ten percent of the bacteria survived the treatment. Re-colonisation of the vascular system can occur if the bacteria present in the sap are resistant to the heat-treatment. This eliminated the presence of competition and allows surviving bacteria to flourish. When F2/5 (biocontrol agent) was applied to heat- treated cuttings it was able to colonise callus and root system at a higher rate than untreated vines (Table 12). Similar results were seen for E26 treated vines with an increase in the levels of *A. vitis* detected from grapevine callus and root material.

The effect of thermotherapy on vines is variable but can inhibit bud development and decrease vine fitness if done incorrectly (Orffer *et al.*, 1977, Orffer *et al.*, 1979). In this trial, callusing was slower for heat-treated cuttings. All vines were planted at the same time and the degree of callusing for heat-treated vines was less than for non heat-treated cuttings.

Under current vineyard management practices, large volumes of vines are heat-treated and callused at one time so longer callusing time could be easily be integrated into vineyard management practices. In this field trial heat-treated grapevines should have been allowed to callus for a longer period.

There was no observable effect of thermotherapy on vine establishment. Only the F2/5 heat-treated group was significantly affected by the heat treatment. A significant decrease in vine growth is most likely due to thermotherapy at a temperature above 50°C. This can occur when the heated water bath is too hot for the duration of the treatment (Orffer *et al.*, 1977). Any deleterious effects caused by the bacterium would be seen on non heat-treated rootlings also. Potentially the combination of heat-treatment and F2/5 application may have an effect on vine establishment, when a single treatment is used this may be overcome. Assessment of the effects of the biocontrol isolates and thermotherapy on treated vines was not possible from this trial. Previous thermotherapy studies by Bazzi *et al.*, (1991) demonstrated that in most cases the treatments and times did not affect bud survival and increased the level of callus formed at the base of cuttings on many varieties of grapevine.

This initial trial allowed an assessment of the experimental design. Planting into a sandy/loam site previously used for grapevine propagation was not effective for determining the efficacy of the biocontrol strains against a natural population of *A. vitis*. To overcome this, application of the pathogen to vines and planting into a pathogen free soil was considered necessary for the next trial. Other modifications to the field trial design were made to ensure a successful trial in the 1997-98 period. This included (a) heavier clay based soil to reduce nematode infestations; (b) application of pathogen directly to callused grapevine cuttings and (c) use of peat cultures for inoculation.

CHAPTER 5

ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSERY: TRIAL 2

(1997-1998)

5.1 INTRODUCTION

The second field trial was performed to overcome the limitations observed in the initial trial (Chapter 4). The changes in experimentation included production of inoculum in peat, use of virgin soil, inoculation of the trial with pathogenic bacteria, as well as modifications to the callusing methods.

Due to the absence of galling in the first field trial, a pathogenic *A. vitis* mixed inoculum was applied to callused cuttings, after inoculation with biocontrol agents. This would test the efficacy of the individual biocontrol strains in the presence of an extremely high level (above those normally encountered in vineyards) of two pathogenic *A. vitis* strains. The trial was also designed to determine if any of the four potential biocontrol strains were capable of disease control or reducing gall size.

Burr *et al.*, (1995) investigated bacterial biocontrol and pathogen application for grapevine. Timing of application of a biocontrol strain was critical. The biocontrol agent should be applied before or at the same time as the pathogen otherwise disease control is greatly reduced. Co-inoculation of biocontrol and pathogen provided effective disease control.

The application protocol was based on that of Stockwell *et al.*, (1993) with the biocontrol strains applied for one hour followed by pathogen application for fifteen minutes immediately before planting. This allows maximum exposure of the fresh callus to

biocontrol inoculum enabling the biocontrol strains to occupy many of the binding sites on the callus, prior to exposure to the pathogen.

Peat cultures (used commercially by Bio-Care Technology) were sourced as a more reliable method of culturing bacteria and inoculating vines as used in commercial application. The less active metabolic state of the bacteria would allow more successful colonisation and survival. Peat cultures were also used for pathogen application. The *A. vitis* pathogen was applied directly to the vines and planted into virgin soil.

A new trial site was selected to decrease the potential for the presence of indigenous pathogenic *A. vitis* and damage due to nematodes. Heavy clay soil of the proposed site would limit the survival of nematodes, as they prefer sandy soils (J. Nicol, pers. comm.) and the site chosen had no previous grapevine propagation occurring on the site. The site was flooded in the previous year to ensure minimisation of pathogen levels within the soil.

5.1.1 Aims

1. To determine the efficacy of four potential biological control bacteria for control of crown gall disease in a grapevine nursery
2. To determine the effect of thermotherapy on vine growth and establishment
3. To determine the levels of bacterial colonisation of pathogen in vines treated with biocontrol strains

5.2 EXPERIMENTS

5.2.1 Field trial organisation

5.2.1.1 Vine variety selection and treatment

Two grapevine varieties (Shiraz BVRC12 and Ramsey) were chosen to allow a comparison of disease control. All Ramsey cuttings were heat-treated (Section 2.15.3.2) due to fungal growth occurring during transport from the supplier as vines were not maintained at 2°C (Riverland Vine Improvement Association (RVIA), South Australia). The vines were stored at temperatures above 2°C on arrival until use. Shiraz vines (supplied by Temple Bruer Wines Pty. Ltd) were heat-treated or untreated for the experiments as described and callused in sand (section 2.15.4).

5.2.1.2 Inoculum preparation and application

Inoculum was prepared in peat by Bio-Care Technology Pty. Ltd. (Section 2.7.2). Biocontrol inoculum (125g wet weight peat) was resuspended in 10L of fresh tap water (to a final concentration of approximately 10^6 cfu.ml^{-1} bacteria) at the Langhorne Creek vineyard (Section 2.15.5). Pathogen inoculum was prepared by resuspending 100g peat combined of *A. vitis* pathogens K306 and K1072 in 10L of fresh tap water. Callused grapevine cuttings were inoculated for 1 hour in a biocontrol agent and immediately followed by pathogen inoculation for fifteen minutes. A fifteen-minute treatment was performed when only pathogen was applied. All vines were treated immediately before planting. Fresh pathogen mix was used for each treatment to prevent cross contamination and to allow equal exposure to the pathogen.

5.2.1.3 Pathogen selection

A mixture of 2 pathogens K1072 and K306 (described in Section 3.3.6) were used to inoculate the grapevines. To maintain homogeneity across the field trial the same pathogens were applied to all treatments.

5.2.1.4 Site selection

The 1997-98 field site at Temple Bruer Wines Pty. Ltd., Langhorne Creek (South Australia) was selected with the following criteria

1. Heavy clay based soil
2. Low background of *A. vitis* pathogen
3. Reduced risk of other grapevine diseases including bacteria, fungi and nematodes

5.2.2 Inoculum levels in peat cultures

Biocontrol and pathogen levels in peat inoculants were estimated using serial dilution and plating onto selective media (Section 2.7.2). Bacterial colonies were counted after 5 days incubation at 25°C. Bio-Care Technology Pty. Ltd carried out quality control. Contamination levels were determined by estimation of non-*Agrobacterium* and non-*Pseudomonas* bacteria and fungi present on semi-selective media.

5.2.3 Inoculum applied to callused grapevine cuttings

Inoculum levels applied to vines were determined with two 10ml aliquots from each resuspended inoculum mix. All aliquots were stored at 4°C in an insulated cool box with chiller packs while on site, and transferred to a cold room on return (4°C). Serial dilutions were performed and plated on selective media with incubation at 28°C for 5 days.

5.2.4 Field trial treatment groups and organisation

A total of 18 treatment groups was used to determine the effectiveness of the biocontrol strains against high levels of pathogenic *A. vitis*. Vine variety, thermotherapy and bacterial inoculum were examined. For each treatment group, a total of three plots per treatment (2m in length and 1m wide) for Shiraz and 5 plots per treatment for Ramsey vines were used. Vines were organised using a random block design (Figure 15) with pathogen and biocontrol strains applied at the same rate. All vines treated with a biological control strain were subsequently treated with the pathogen mix for 15 minutes. Extra treatments of pathogen only and control (no inoculum applied) were used to determine the baseline levels of galling and disease development in the field site.

Vines were planted in late October with a polypropylene watering system in place. Watering was performed weekly (more often in hot weather) and fertilised (via the watering system) fortnightly with ammonium nitrate and urea according to current practice (D. Haeusler, Temple Bruer Nursery Manager). Vines were removed from the field plot once full dormancy was reached in September the following year, bagged and returned to the laboratory for analysis (stored at 4°C until required).

5.2.5 Bacterial colonisation of callused grapevine cuttings

After inoculation, samples of each vine treatment were bagged and stored at 4°C until processed. Bacteria were isolated from callus material (Section 2.15.6). Samples were serially diluted and plated on selective media (RS, Biovar 1 and Kings B agars) supplemented with cycloheximide. Aliquots were preserved in equal volumes of glycerol (30%) in nutrient broth and stored at -80°C for future use.

67 Shiraz un cont		68 Shiraz ht cont		69 Ramsey ht cont		
61 H	62 F	63 C1	64 K	65 E	66 C1	**Ramsey Heat treated vines**
55 C1	56 C2	57 K	58 H	59 C2	60 F	
49 E	50 K	51 F	52 K	53 H	54 C2	
43 C2	44 C1	45 K	46 E	47 C1	48 H	
37 F	38 H	39 E	40 F	41 C2	42 E	
31 C1	32 F	33 E	34 H	35 C2	36 K	**Shiraz Heat treated vines**
25 E	26 H	27 C2	28 K	29 C1	30 F	
19 C2	20 K	21 H	22 F	23 E	24 C1	
13 K	14 F	15 C2	16 H	17 C1	18 F	**Shiraz untreated vines**
7 E	8 H	9 C1	10 E	11 C2	12 K	
1 H	2 E	3 C2	4 F	5 K	6 C1	

Figure 15: Design of 1997-98 field trial at Temple Bruer Vineyard, Langhorne Creek, South Australia. Plots 1-18: Shiraz non heat-treated grapevine cuttings, Plot 19-36: Shiraz heat-treated grapevine cuttings, Plot 37-66: Ramsey heat-treated grapevine cuttings, Plot 67: Shiraz non heat-treated, pathogen only treatment, Plot 68: Shiraz heat-treated, pathogen only treatment, Plot 69: Ramsey heat-treated, pathogen only treatment. E: E26 (*A. vitis* biocontrol), F: F2/5 (*A. vitis* biocontrol), H: HLB2 (*A. tumefaciens* biocontrol), K: K315 (*P. fluorescens* biocontrol), C1: no inoculum applied, C2: pathogen mix only applied. All vines were treated with the pathogen mix after the initial application of the biocontrol isolate with three replicates of each treatment.

5.2.6 Effect of biocontrol bacteria on gall frequency, position and size

After harvest vines were washed free of soil and assessed for galling (Section 2.15.7). Each treatment was analysed and the following data recorded: number of galled vines, gall diameter and position on individual rootlings and total vine number for each plot.

5.2.7 Effect of thermotherapy and biocontrol application on vine growth

The effect of thermotherapy on grapevine establishment was evaluated after harvest of the field trial once vines had reached dormancy. This was achieved for each treatment by counting the vines that failed to produce roots or shoots for individual treatments. Vines were visually assessed before planting for the level of callusing that had occurred across heat-treated and non-heat treated cuttings.

5.2.8 Effect of biocontrols, thermotherapy and variety on root development

Dry root weights of galled and non-galled plants for individual plots were determined by measuring root mass. Samples were placed in a drying oven at 80°C for 4 days until no further weight change could be detected.

5.2.9 Pathogen colonisation of grapevine sap in the presence of biocontrol bacteria

Sap samples that had been preserved at -80°C (in 15% w/v glycerol) were recovered by thawing, and inoculating an aliquot (200µl) into 4ml RS broth (containing cycloheximide). Cultures were grown at 28°C (170 rpm) for 5 days. A 2ml aliquot was taken and total DNA extracted using the Ultraclean™ Soil DNA Purification Kit (MoBio101). DNA was eluted in 50µl SDDW and stored at -20°C. PCR was performed using the *pehA* PCR protocol (Section 2.9.2) in 96 well PCR plates (Whatman Polyfiltronics Uni PCR 200µl Thermocycler plates) and sealed with Chill Out Liquid Wax 14 (40µl)(MJ Research Inc.).

Initially reactions were run in duplicate with a set of serially diluted standards included. PCR products were quantified using the Pico Green® ds (double stranded)-DNA quantitation reagent (Molecular Probes, Oregon). A 2µl aliquot of PCR product was resuspended in 48µl 1xTE buffer in a 96 well microtitre tray. Pico Green® reagent was diluted 1:200 in 1xTE buffer with 50µl delivered to each well. Plates were incubated at room temperature for 5 minutes at 100 rpm then assayed using a Victor 2 1420 Multilabel counter (Wallac Workstation). The plate reader was set for fluorescence detection (485nm/535nm).

5.3 RESULTS

5.3.1 Inoculum levels in peat cultures

All biocontrol and pathogen inoculum mixes were prepared in peat by Bio-Care Technology Pty. Ltd. All inocula were viable for use in the field trials and amounts required for inoculation were determined to allow approximately equal bacterial numbers for application to grapevines. No significant difference was observed for all bacterial inoculum levels (Table 13). Levels of contamination were estimated at less than 10^6 $cfu.g^{-1}$ of peat.

Strain	Pathogenicity	Log $cfu.g^{-1}$ (±SD)
E26	-	9.46 (0.3)
F2/5	-	9.48 (0.4)
HLB2	-	9.78 (0.5)
K315	-	9.53 (0.3)
K306	+	8.7 (0.4)
K377	+	9.09 (0.2)

Table 13: Estimation of bacterial levels in peat inoculum preparations from Bio-Care Technology Pty. Ltd. Biocontrol and pathogenic strains were cultured in peat and levels estimated after 2 weeks incubation. Strains used were E26 and F2/5 (*A. vitis* biocontrol strains), HLB2 (*A. tumefaciens* biocontrol strain), K315 (*P. fluorescens* biocontrol strain) and pathogenic *A. vitis* isolates K306 and K377. SD = calculated log standard deviation.

5.3.2 Inoculum applied to callused grapevine cuttings

Bacterial numbers from diluted peat inoculum were estimated from serial dilutions and plating on NA. HLB2 (*A. tumefaciens* biocontrol) had a higher ratio of 16.6:1 of biocontrol bacterium to pathogen applied ($7.5x10^7 cfu.ml^{-1}$: $4.50x10^6 cfu.ml^{-1}$), when compared to other treatments F2/5 (4.3:1), K315 (4.6:1) and E26 (4.9:1) but was not significantly different ($p=0.05$). HLB2 may have had an advantage in colonisation due to this difference. All biocontrol strains were applied at higher rates than the pathogen (greater than 4 times the

amount of pathogen). Pathogen levels were higher than normally encountered in field soils but significantly lower than levels of biocontrol agents applied (p=0.04).

5.3.3 Bacterial colonisation of callused grapevine cuttings

Biocontrol and pathogenic bacteria were reisolated from grapevine callus after inoculation. No significant differences were observed for bacterial levels isolated from Shiraz NHT, Shiraz HT or Ramsey heat-treated vines within individual bacterial treatments except HLB2 (Figure 16). A significant difference was observed for strain HLB2 on NHT Shiraz and HT Ramsey vines (p=0.03) when isolated on RS medium.

Pathogen levels were significantly higher on NHT vines when compared with HT Ramsey vines. This shows that all bacteria used in this study were capable of colonising the callus material.

5.3.4 Efficacy of biocontrol bacteria and thermotherapy on gall reduction

Gall frequency was determined by assessment of all individual grapevine rootlings for galling. An example of galling is given in Figure 17. Gall data was analysed using a mixed logistic regression model (Dr. H. Oakey, Biometry SA). Inoculation of the biocontrol strains F2/5, HLB2 or K315 showed significant decreases in galling for both heat treated and untreated grapevines.

Overall, after biocontrol treatments Shiraz NHT vines had more than 5 times the galls of both Shiraz and Ramsey HT varieties, and galling was significantly reduced by the use of thermotherapy (Figure 18). The removal of indigenous population of *A. vitis* from grapevine

Figure 16: Recovery of bacteria from inoculated callus grapevine material.

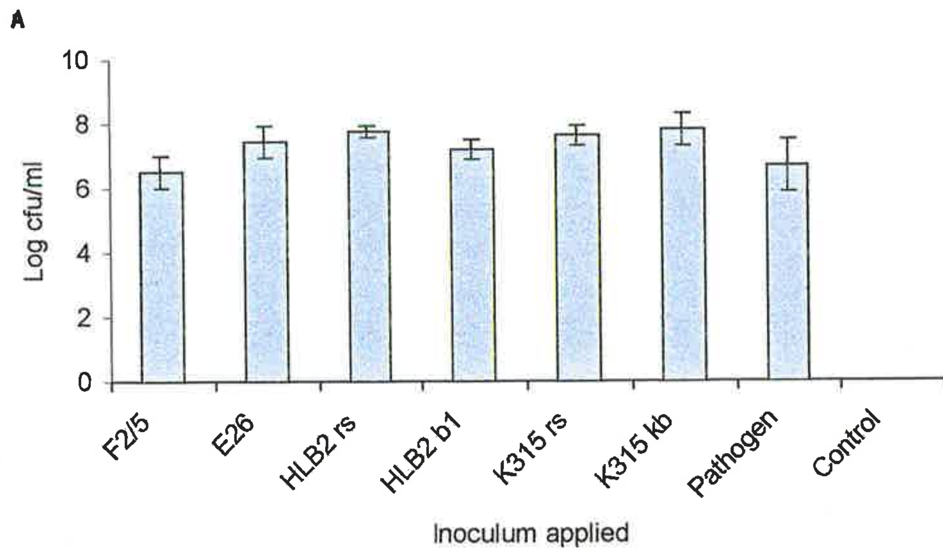
A: Shiraz non heat-treated material recovery levels

B: Shiraz heat-treated material recovery levels

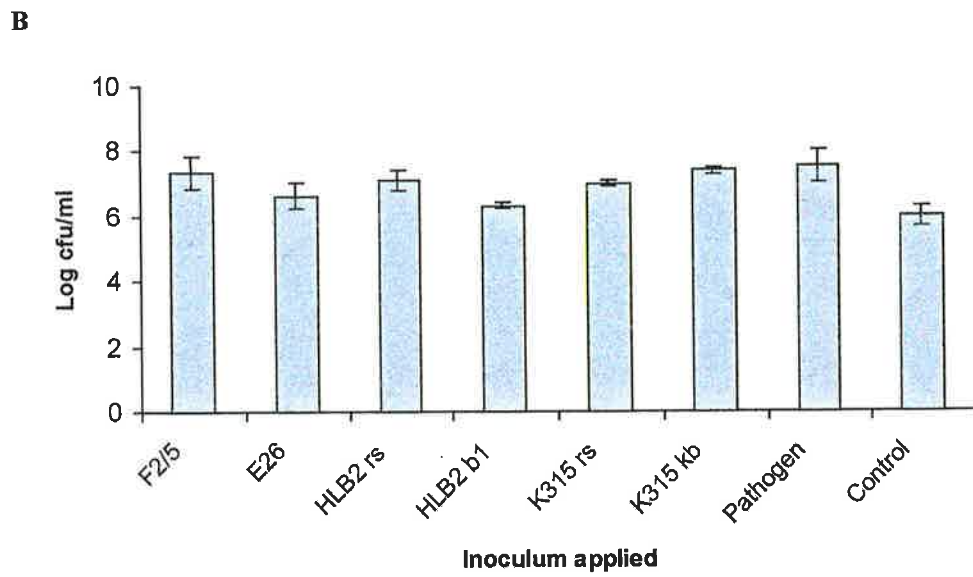
C: Ramsey heat-treated material recovery levels

Bacterial counts were performed on selective media rs-(RS media selective for *A. vitis)* to determine levels of biocontrol bacteria E26 and F2/5 and pathogenic *A. vitis* present of grapevines, b1- (Biovar 1 medium) to determine levels of biocontrol bacterium HLB2 (*A. tumefaciens*) and kb- (Kings B medium) to determine levels of biocontrol bacterium K315 (*P. fluorescens*).

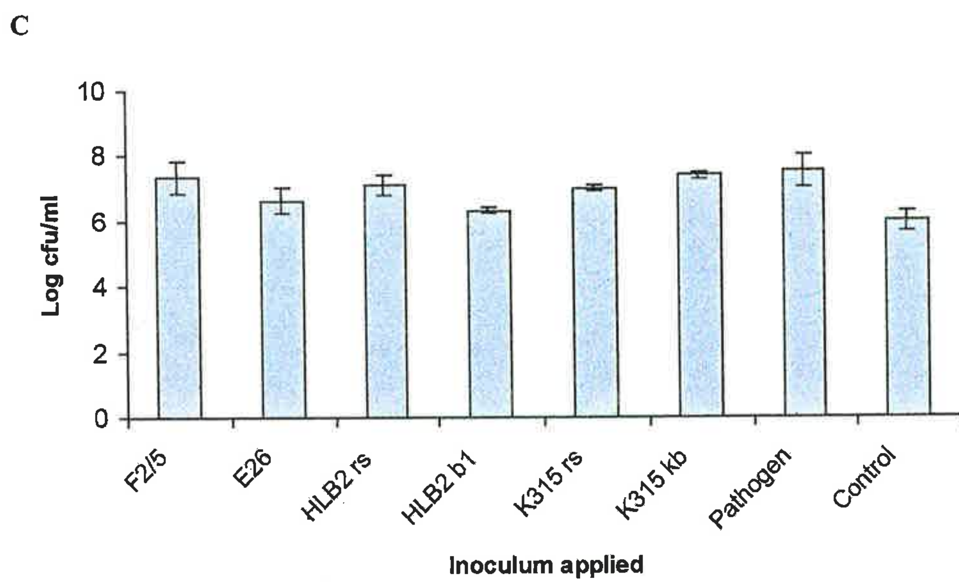
A



B



C



cuttings by thermotherapy was beneficial for the reduction of disease symptoms occurring in the nursery.

Within the Shiraz NHT vines, application of pathogen alone resulted in 41% galling. When biocontrol agents were applied to the vines, significant reductions in galling frequencies were observed with F2/5 (15% galling), HLB2 (20% galling), and K315 (26% galling) given in Figure 18. The effect of E26 application was compared to the pathogen alone application with no significant difference observed (44% galling for E26 treated vines and 40% for pathogen only treated). Control vines (no inoculum applied) had very low levels of galling for each vine type ranging from 0.9 to 4.5%.

Overall, in the Shiraz NHT treatment when F2/5 was applied to grapevine, gall frequency was reduced by 62.5% relative to the pathogen only treatment. HLB2 reduced galling by 42% and K315 reduced galling by 22%.

Significant differences were seen for galling between NHT and HT varieties. Galling on all varieties was significantly reduced when thermotherapy was used. Heat-treated control vine galling was below detection while galling on pathogen treated material had a reduction from 41% (Shiraz NHT) to 12% (Shiraz HT) and 15% (Ramsey HT) (shown in Figure 18).
Gall frequencies with heat-treated varieties (Shiraz and Ramsey) were not significantly different when used in conjunction with the biocontrol agents. The pathogen alone treatment resulted in 11-15% galling frequency, for Shiraz and Ramsey, respectively. Galling frequency was reduced when thermotherapy and F2/5, HLB2 or K315 was applied but were not significantly different from the pathogen treatment ($p = >0.42$) as levels were very low.

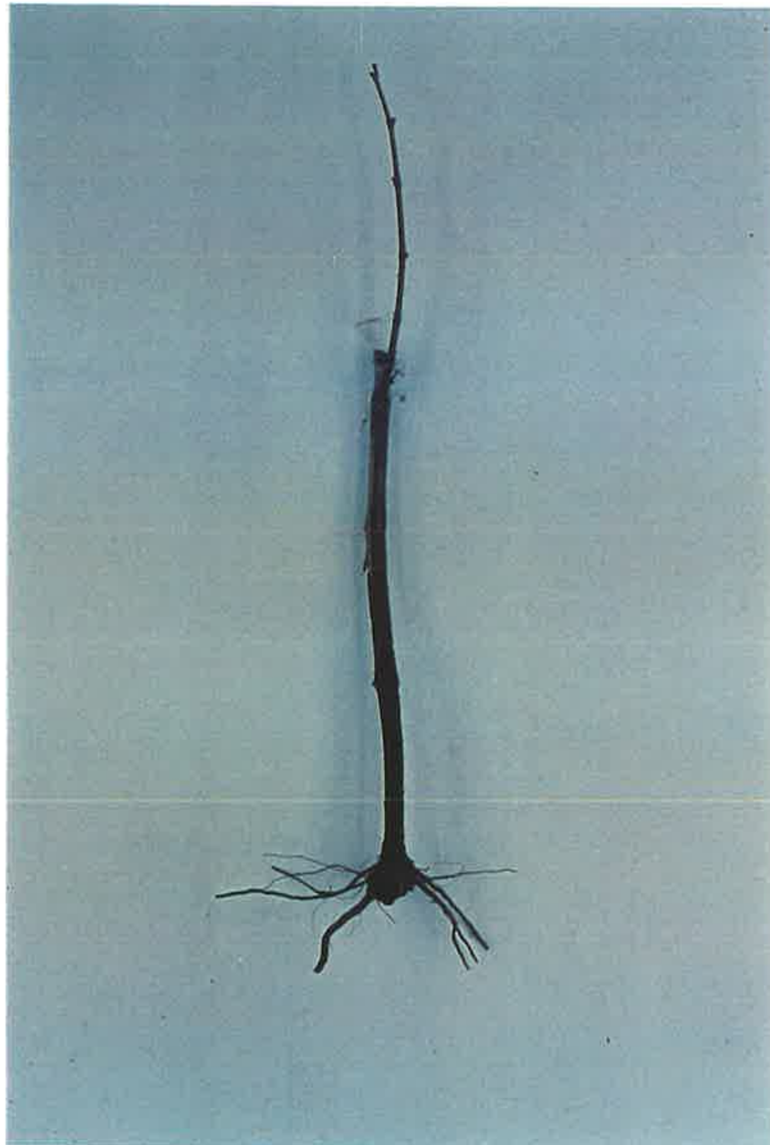


Figure 17: Example of galling on a one-year-old grapevine rootling. Galls positioned at the base and first node of the vine.

Figure 18: Gall frequency observed in the 1997-98 field trial inoculated with biological control agents with pathogen inoculation. Vine galling was assessed after 9 months of growth in the field nursery, Temple Bruer Wines, Langhorne Creek, South Australia.

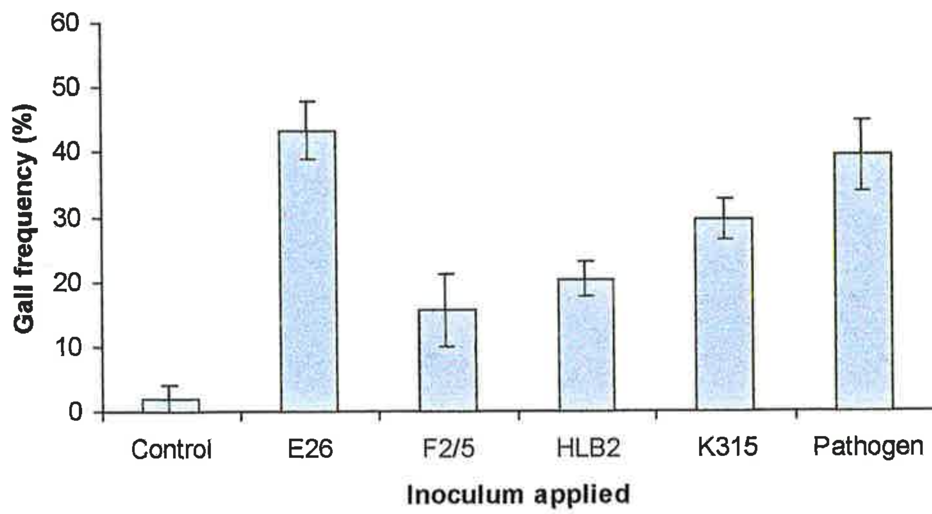
Control - uninoculated, E26 - treated with E26 and pathogen mix, F2/5 - treated with F2/5 and pathogen mix, HLB2 - treated with HLB2 and pathogen mix, K315 - treated with K315 and pathogen mix, Pathogen - treated with pathogen mix alone.

A: Gall frequency on Shiraz non heat-treated vines

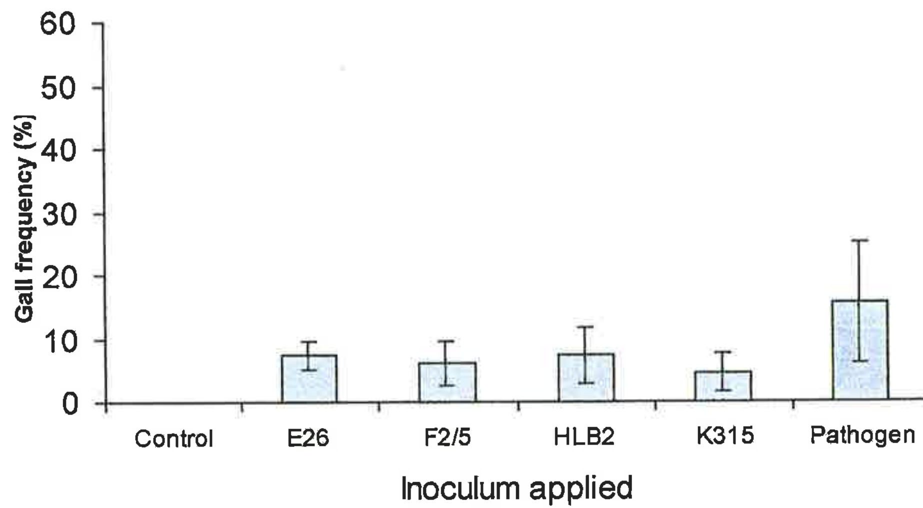
B: Gall frequency on Shiraz heat-treated vines

C: Gall frequency on Ramsey heat-treated vines

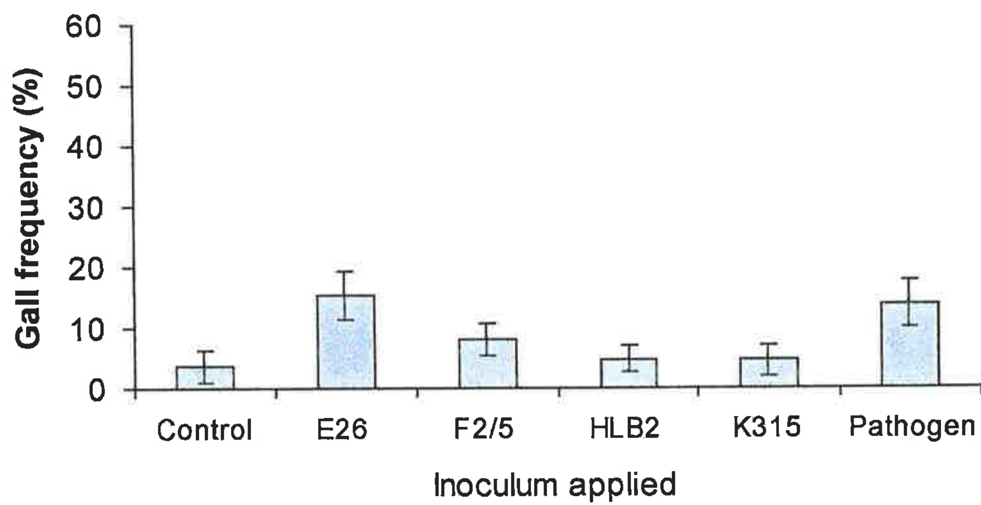
A



B



C



Thermotherapy on control vines was able to reduce galling from 4.5% (NHT) to 0.1% (HT) but was not found to be statistically significant due to the low rate of galls.

Similar reductions in galling were seen for F2/5 (21% to 5%), HLB2 (20% to 5%) and K315 (26% to 6.5%) for NHT and HT vines. The reduction in gall frequency may be due to elimination of pre-existing pathogens within the vines allowing improved colonisation by the biocontrol strains. E26 treated vine galling was not significantly different to the pathogen alone treatment resulting in 44% galling on Shiraz NHT, 13% galling on Shiraz HT and 17% galling on Ramsey HT.

5.3.5 Effect of biocontrol agents on gall position and size

The effect of biocontrol application on gall size and position on the grapevine was determined using a log linear model (Biometry SA). Comparisons were made between pathogen and individual biocontrol strains for galled grapevine rootlings only. The results of modelling found a two-way interaction between gall position on rootlings and biological control ($p=0.007$, $\chi^2=24.214$, $df=10$) and position and thermotherapy ($p<0.007$, $\chi^2=24.1145$, $df=4$) significant at the 5% level. This indicates that both the biological control strain applied and the heat-treatment affect the position of galling (Figure 19). For analysis, positions 3 and 4 (Figure 3) were combined as few galls occurred in position 4 to improve statistical properties.

HLB2, E26, F2/5 and the pathogen treatments followed similar trends with the majority of galls in position 2 (ie. 55-100%) and for the most part similar proportions in position 1 (15-65%) and 3/4 (10-35%). The trend for K315 is similar for gall proportions in positions 2 and 3/4 and much lower proportion in position 1. These results suggest that K315 treatment led to galling in positions higher up the vine relative to other treatments.

Galls on Ramsey and Shiraz heat-treated vines occurred at higher frequencies in position 1 and 2 compared to position 3/4. There were no significant differences in the proportions in each position for Shiraz heat-treated vines compared with Ramsey heat-treated vines ($p=0.536$, $\chi^2=1.248$, $df=2$). The trend for Shiraz non heat-treated vines was different with most galls being found in position 2 and position 3/4. This suggests that the heat treatment tended to result in galls in positions higher up the vine compared to no heat treatment.

The diameter of galls occurring on vines was significantly reduced (in most treatments) when the pathogen was applied in conjunction with a biological control agent. For Shiraz non heat-treated vines significant decreases in gall diameter were observed with all biocontrol treatments when compared to the pathogen alone treatment (Figure 20). Gall diameter was reduced by more than 30% when a biocontrol strain was applied prior to the pathogen mix ($p<0.02$) (using a t-test assuming unequal variances).

When biocontrol strains were applied in conjunction with thermotherapy, no significant reductions in gall diameter were observed. Pathogen inoculated vine galls were significantly reduced when compared to non heat-treated Shiraz vines ($p=0.04$, t-stat=1.81, $df=16$). When a gall did occur there was no difference in gall diameter compared to the pathogen alone treatment. There was a significant increase in gall diameter for F2/5 treated vines when compared to pathogen only heat-treated vines ($p= 0.0024$, t-stst=3.68, $df=65$). Galls on Ramsey vines were an average of 14mm on pathogen treated vines with F2/5 and E26 having significantly smaller galls at 9mm (Figure 20).

5.3.6 Effect of thermotherapy and biocontrol inoculation on vine growth

Assessment of root and bud growth for heat-treated and non heat-treated cuttings allowed determination of the effect of thermotherapy on the growth of vines. Vine survival shown in Figure 21 demonstrated that survival of non heat-treated cuttings was significantly higher than both heat-treated varieties (Shiraz and Ramsey). For all NHT treatments vines survived at 30-60% higher ($p<0.005$) than the heat-treated vines.

The effect of galling, biological control and thermotherapy and variety were assessed for their effects on dry root weight of grapevines using ANOVA and REML analysis (Genstat, Version 5, release 4.1, 4th edition). Explanatory factors were analysed to test for significance. Results showed that the only significant factor was heat treatment, which was significant at the 5% level. The application of the biological control and whether the vine was galled had no significant effect on the dry root weight.

The non heat-treated Shiraz had the highest average dry root weight per vine (2.77 grams) compared to Shiraz heat-treated (2.08 grams) while Ramsey heat-treated vines recorded the lowest average dry root weight per vine (1.45 grams).

Using LSD (least significant difference) it was determined that there were significant differences in mean dry root weight per vine between non heat-treated and heat-treated Shiraz. The heat-treatment caused a reduction in the mean dry root weight per vine ($p= 0.001$). In addition, there was a significant difference between Shiraz heat-treated and Ramsey heat-treated vines with Ramsey (heat-treated) vines showing proportionally lower root weight per vine.

Figure 19: Assessment of gall position for individual biocontrol treatments. Gall position was recorded and analysed to determine the effects of the biocontrol agents. Inoculum applied to vines as follows: Control - no inoculum, Pathogen -Pathogen combination only, E26- E26 *A. vitis* and pathogen combination, F2/5- F2/5 *A. vitis* and pathogen combination, HLB2- HLB2 *A. tumefaciens* and pathogen combination, K315- K315 *P. fluorescens* and pathogen combination.

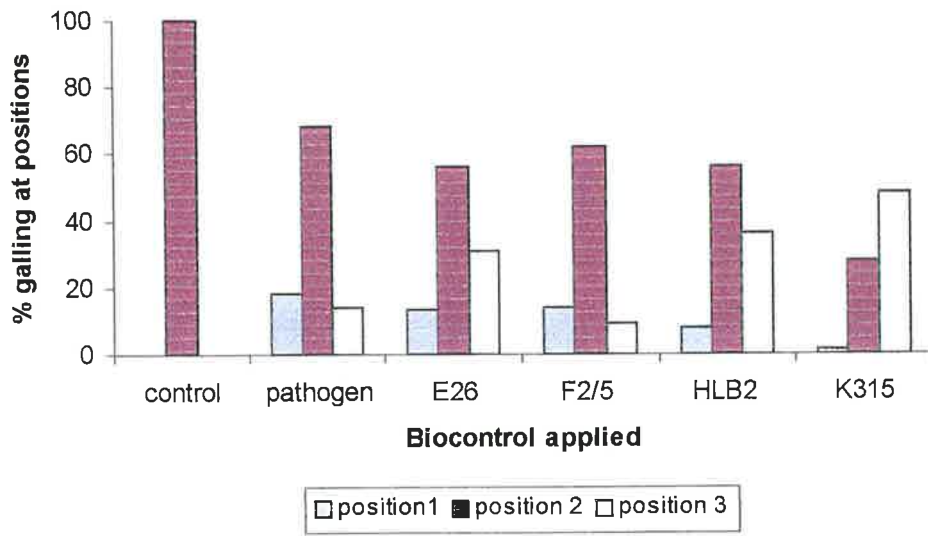
Legend indicates position of gall on grapevine: 1= crown of vine, 2= first node, 3= second node.

A: Shiraz non heat-treated

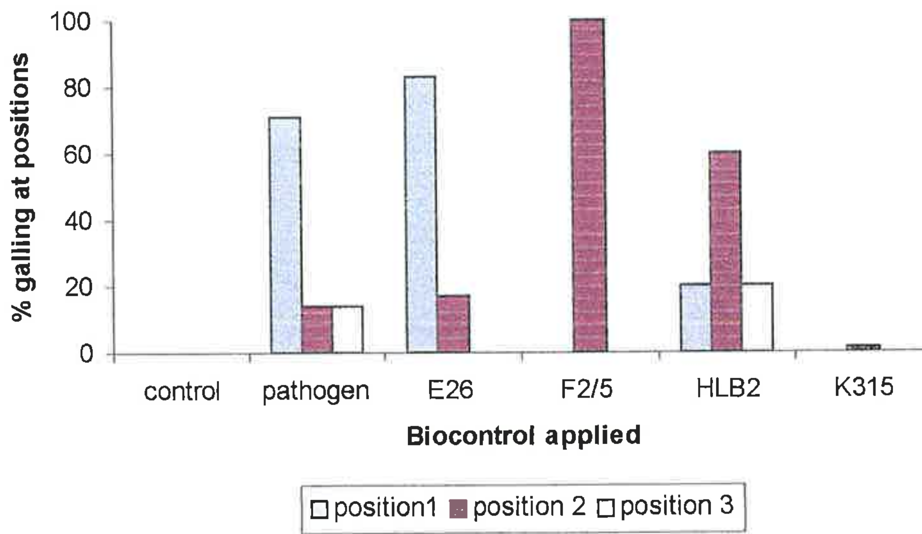
B: Shiraz heat-treated

C: Ramsey heat-treated

A



B



C

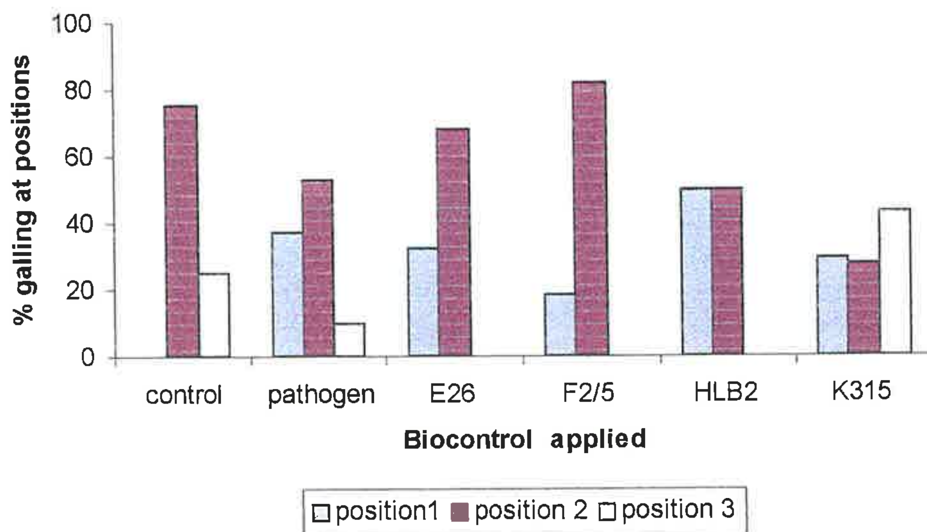


Figure 20: Assessment of gall diameter on A: Shiraz non heat-treated, B: Shiraz heat-treated and C: Ramsey heat-treated, inoculated with vines treated with biocontrol agents and pathogen mix.

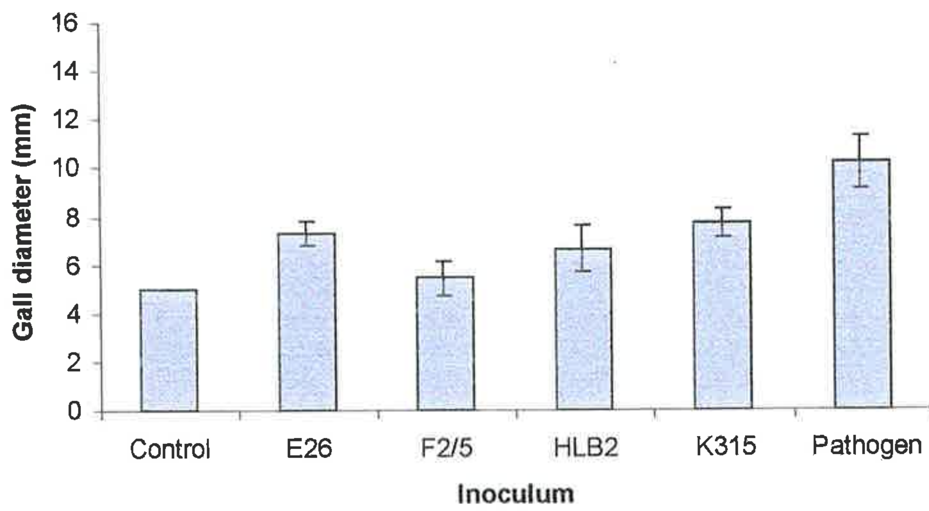
Inoculum applied to vines as follows: Control - no inoculum, Pathogen -Pathogen combination only, E26- E26 *A. vitis* and pathogen combination, F2/5- F2/5 *A. vitis* and pathogen combination, HLB2- HLB2 *A. tumefaciens* and pathogen combination, K315- K315 *P. fluorescens* and pathogen combination.

A: Shiraz non heat-treated

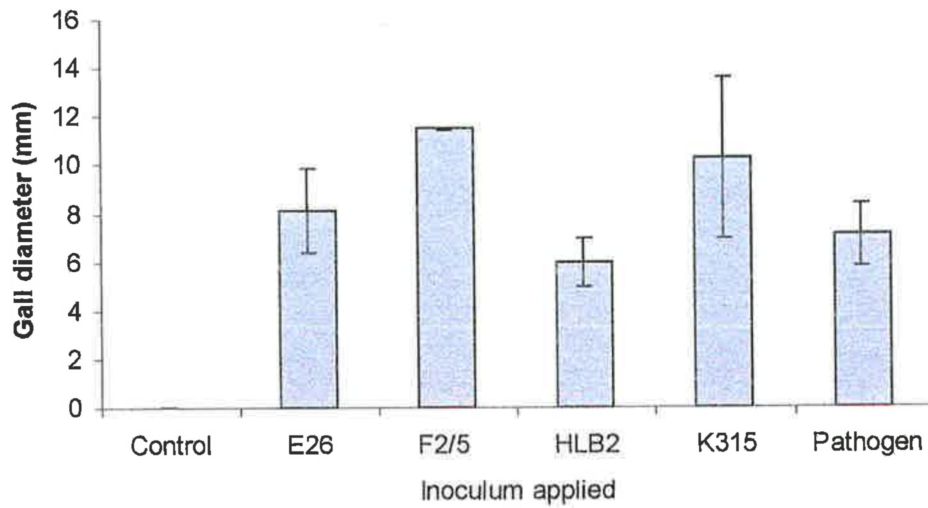
B: Shiraz heat-treated

C: Ramsey heat-treated

A



B



C

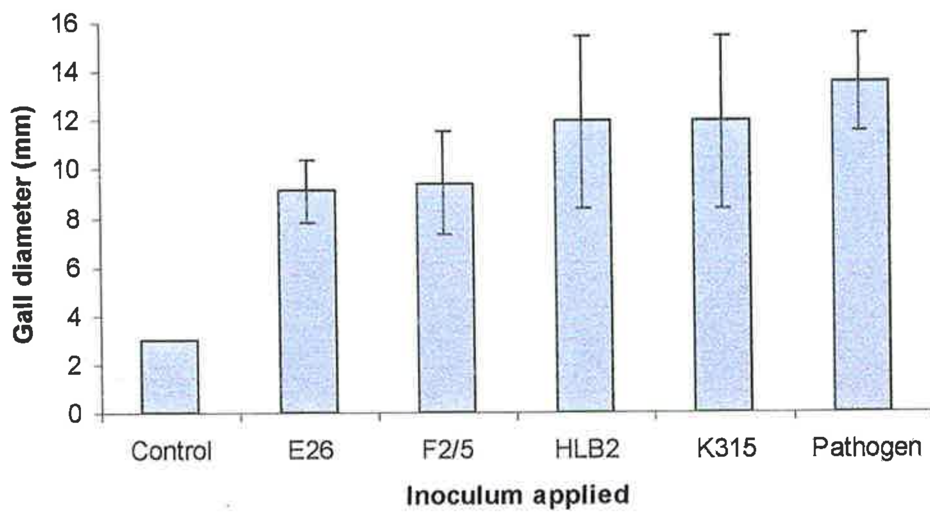


Figure 21: The effect of thermotherapy on vine establishment assessed after 9 months in the 1997-98 field trial with A: Shiraz non heat-treated, B: Shiraz heat-treated and C: Ramsey heat-treated vines. Vines were assessed for growth after this period.

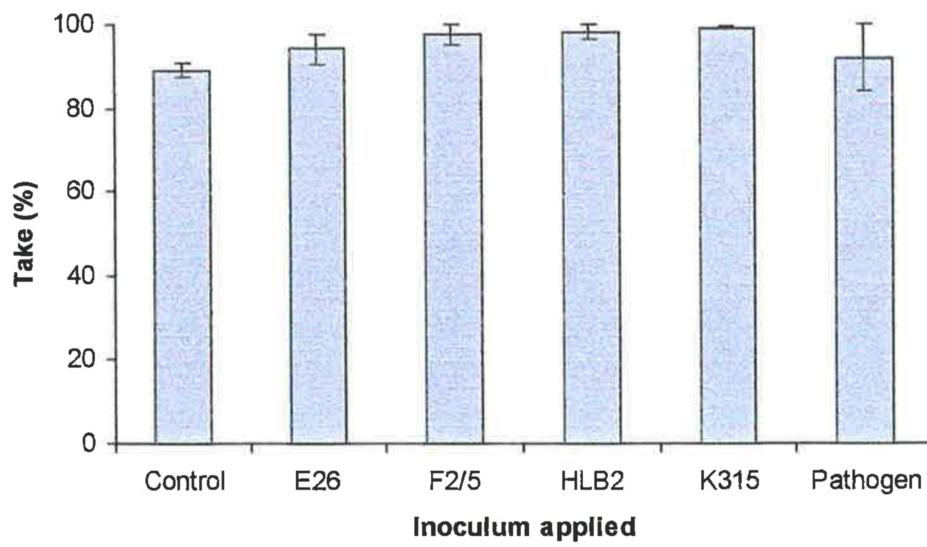
Treatments were as follows: Control - no inoculum, Pathogen -Pathogen mix only, E26- E26 *A. vitis* and pathogen mix, F2/5- F2/5 *A. vitis* and pathogen mix, HLB2- HLB2 *A. tumefaciens* and pathogen mix, K315- K315 *P. fluorescens* and pathogen mix.

A: Shiraz non heat-treated

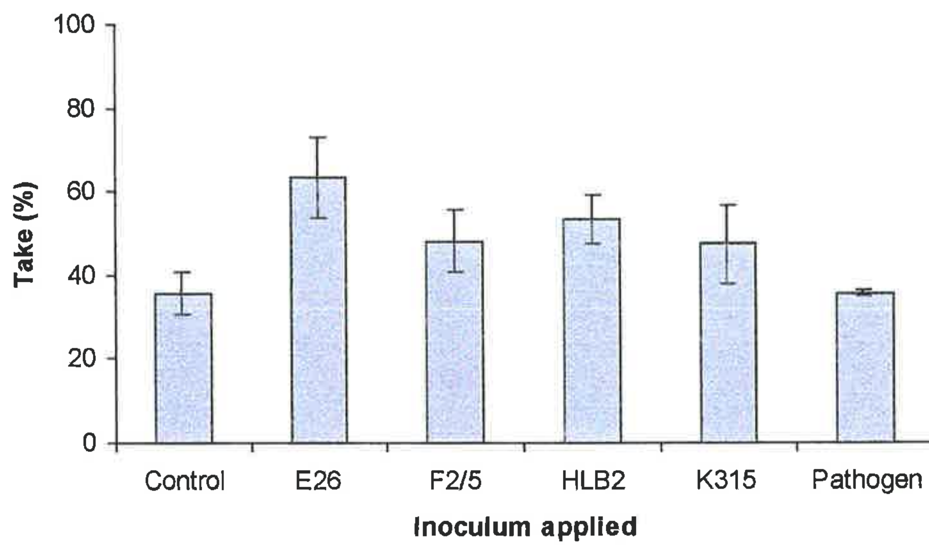
B: Shiraz heat-treated

C: Ramsey heat-treated

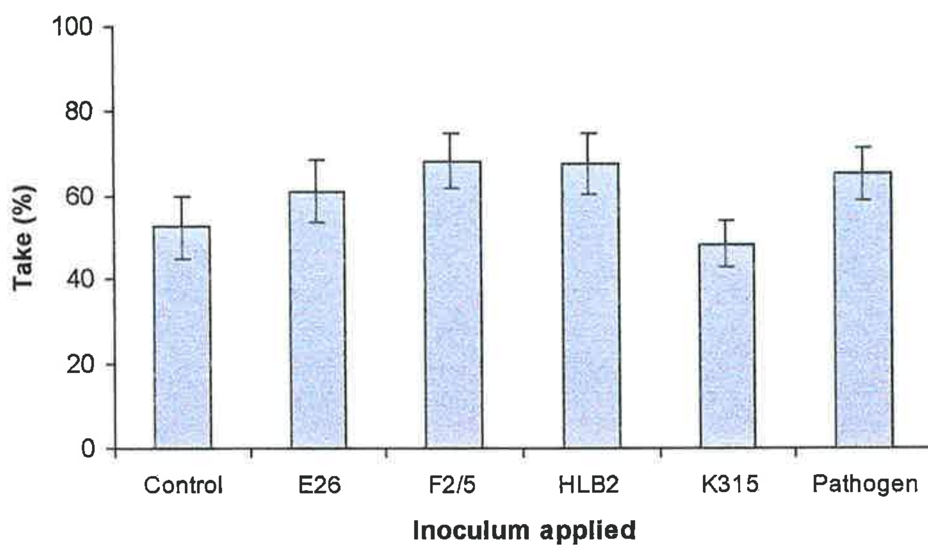
A



B



C



5.3.7 Pathogen colonisation of grapevine sap in the presence of biocontrol strains

Pathogen levels in grapevine sap were compared across treatments by qualitative estimation using PCR amplification of the target sequence from total DNA preparations and Pico Green® Quantitation reagent. Pathogen levels in all vines were significantly higher for vines treated with the pathogen combination in the presence or absence of the biocontrol strains, than those of the control ($p<0.002$) (Figure 22). Shiraz NHT vines treated with E26, F2/5 or K315 applied with pathogen were not significantly different from the pathogen alone treatment, while there was a significant increase in pathogen levels ($p= 0.04$) with HLB2 treatment. This indicates that the pathogen was still able to colonise in the presence of the biocontrol agents.

The use of thermotherapy with Shiraz vines resulted in a significant increase in pathogen levels for control, pathogen and F2/5 treated vines ($p<0.023$) when compared to the NHT vines. No change was observed for HLB2, E26 or K315 treated vines when compared to the pathogen treatment. Ramsey treatments were not significantly different from Shiraz HT vines.

Figure 22: Pathogen levels recovered from the 1997-98 field trial. Sap extractions were performed on individual vines and sap was inoculated into RS broth. DNA was extracted, run through *pehA* PCR and quantified using Pico-Green ds-DNA reagent. Counts: fluorescent signal determined by amount of DS-DNA product present from the PCR allowing comparison of product detected across treatments.

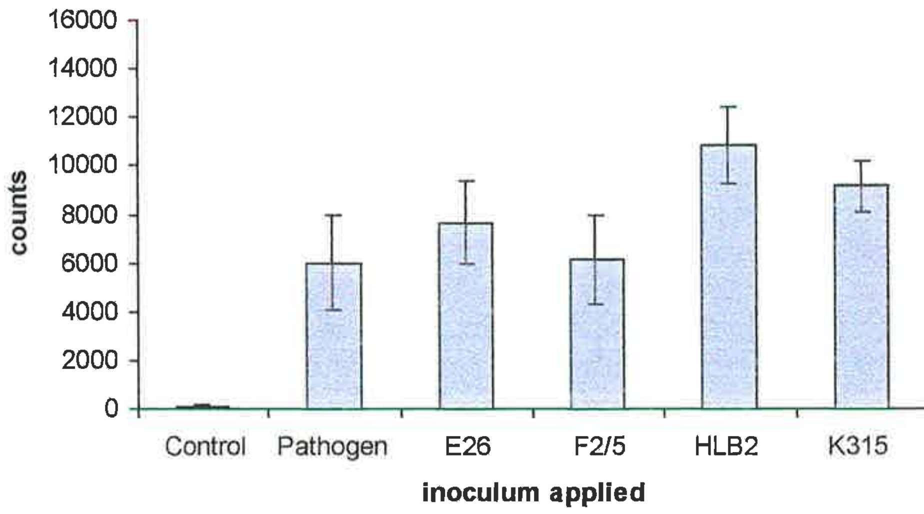
A: Shiraz non heat-treated vines

B: Shiraz heat-treated vines

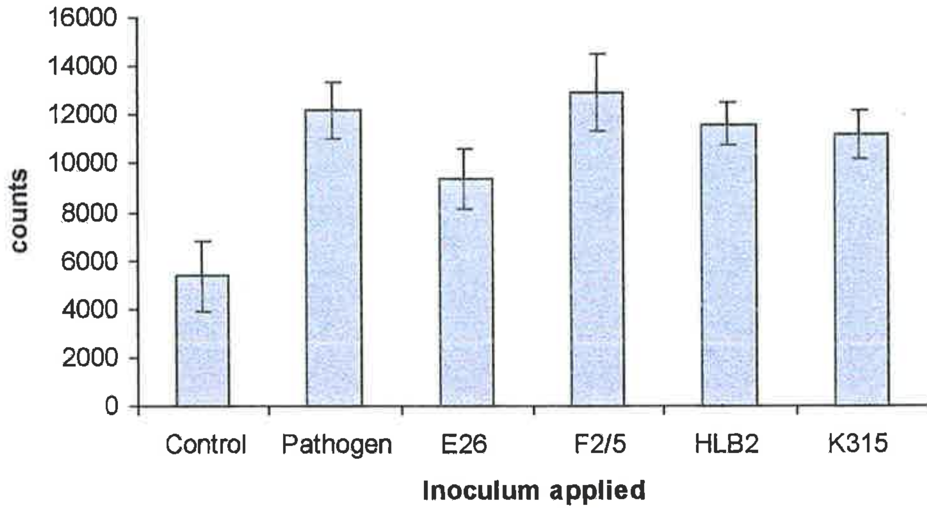
C: Ramsey heat-treated vines

Treatments consisted of Control - no inoculum, Pathogen -Pathogen mix only, E26- E26 *A. vitis* and pathogen mix, F2/5- F2/5 *A. vitis* and pathogen mix, HLB2- HLB2 *A. tumefaciens* and pathogen mix, K315- K315 *P. fluorescens* and pathogen mix.

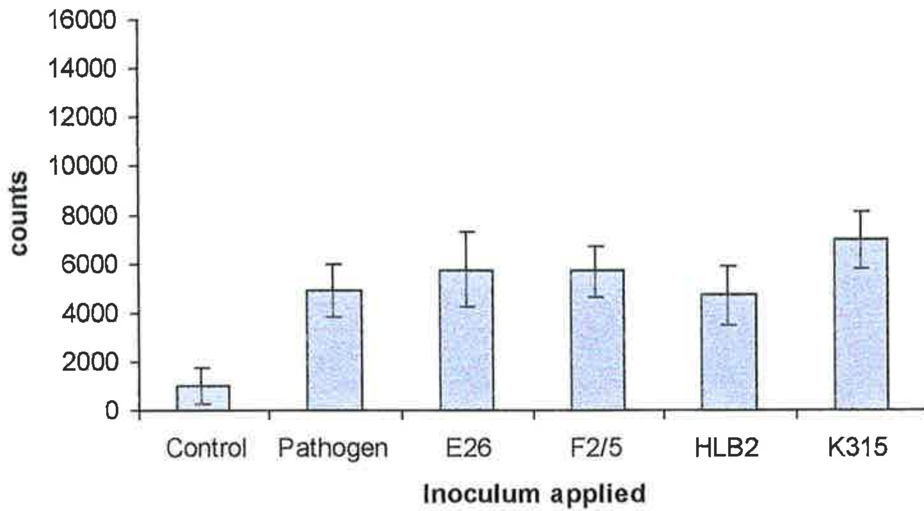
A



B



C



5.4 DISCUSSION

The aim of this study was to determine the efficacy of 4 putative biocontrol strains to control crown gall disease in a grapevine nursery. Modifications were made to the original field trial design (Chapter 4) to allow determination of the effect of the biocontrol strains on heat-treated and non-heat-treated grapevine cuttings with the direct application of a high level of pathogenic *A. vitis.*

Significant reductions in galling frequencies were observed for the biocontrol isolates F2/5 (*A. vitis*), HLB2 (*A. tumefaciens*) and K315 (*P. fluorescens*), with 62.5%, 42% and 22% reduction in galling observed, respectively, for non heat-treated vines. A similar trend was not observed with heat-treated cuttings as galling was significantly reduced on all treatments when compared to non heat-treated cuttings. This demonstrated that these three biocontrol strains were effective at reducing crown gall under field conditions when challenged with pathogenic *A. vitis,* at levels much higher than those normally encountered in the field. The fourth biocontrol strain tested, E26 (*A. vitis),* was not effective at reducing gall induction in this trial.

Significant reductions were observed in gall diameter on non heat-treated vines with biocontrol strains F2/5, HLB2 and K315. The decrease in gall diameter is advantageous to the grapevine, as the total vine nutrient supply is not concentrated into the gall. Schroth *et al.*, 1988, found that galling was only deleterious to grapevines when greater than 50% of the trunk was covered with gall. Any attempt to decrease gall incidence and gall diameter would be useful for the proliferation and function of the grapevines to produce grapes. The decrease in gall diameter over the first growing year of the vine may be due to the ability of the biocontrol isolates to delay galling. As vines were not replanted and observed for galling

over a 2-3 year period, it is unknown whether galling was delayed or inhibited by the biocontrol isolates. The predominance of galls at the site of inoculation, the crown or first node, was also observed and this appeared to preclude galling further up the trunk of the vine. Treatment of vines with HLB2, F2/5, E26 or pathogen alone resulted in the majority of galls occurring at position 2 while K315 application resulted in formation of galls mainly in position 2 or 3. Heat-treated vines had higher proportions of galls occurring in position 1 and 2 rather than higher up the vine trunk.

This field trial has demonstrated that there were significant inhibitory effects of thermotherapy on vine establishment and development after one year of growth. Callus development rates were observed to be slower for heat-treated grapevines when compared to the non heat-treated vines after 8 weeks of callusing and vine establishment was significantly lower for heat-treated vines when compared to non-heat treated grapevines. The heat-treatment was shown to reduce callus development. This would be overcome by allowing vines to callus for longer periods when thermotherapy has been utilised. The amount of callus present on vines would influence the amount of bacteria able to colonise callus surface and thus the amount of biocontrol agent present on individual cuttings. All vines were planted at the same time to avoid problems of lack of continuity between treatments. The callusing period can be easily optimised using heat-treated canes packed in vermiculite and placed in an incubation room to mature. The canes are maintained at a constant temperature and can be planted when the callus is at its optimal growth stage. The effect of thermotherapy on grapevine establishment requires a further field trial using optimal conditions for the heat-treatment and callusing of grapevine cuttings.

The majority of previous research with these biological control strains involved inoculation of bacteria onto wounds caused by holes drilled into grapevine stems. In this case, the biocontrol strain was applied directly to fresh callus and then pathogen was applied (similar to normal field conditions). The biocontrol bacteria should be capable of occupying the majority of binding sites on the callus before the pathogen is introduced. The results from this trial indicate that F2/5 and HLB2 were the most effective strains in controlling crown gall disease.

Levels of pathogen within vine sap demonstrated that the pathogen is capable of colonising the vascular system of the vine in the presence of the biocontrol strains. The use of PCR allowed detection and quantification of pathogenic *A. vitis* in sap extracted from asymptomatic vines after harvesting. Pathogenic *A. vitis* were present at higher levels on heat-treated vines than non heat-treated Shiraz grapevines. The levels of pathogenic *A. vitis* did not vary between galled or non-galled grapevines indicating that the pathogenic *A. vitis* are able to colonise grapevine in the presence of the biocontrol strains but are precluded from causing galling. It has been shown previously that the inhibition of galling is not due to agrocin production by the biocontrol strain and attachment of the biocontrol strain is clearly not inhibiting the attachment and colonisation of the pathogenic *A. vitis.* This implies that another method of control is acting to prevent initiation of the disease (Burr *et al.*, 1997). The prevention of T-DNA transfer from the pathogen into the plant host genome has been suggested (Burr *et al.*, 1997). The PCR method requires further development with the incorporation of an internal positive control DNA. This allows determination of the presence of inhibitory compounds that amay not be completely removed in the DNA purification process and inhibit the PCR.

Production of peat inoculum allows bacteria to enter into the soil environment in a metabolic state similar to the culturing medium (G. Bullard, pers. comm.). As peat has a limited nutrient resource when compared to nutrient rich broths, this may allow better survival of the bacteria on application to the grapevine callus. Peat culture is used for the production of No Gall® (the commercially available strain of K1026) and has been very successful for *Rhizobium* inoculations.

The use of inoculated pathogen allowed good differentiation in terms of disease control and biocontrol treated vines in the second field trial. A site was selected that had no prior exposure to grapevines and pathogenic *A. vitis* was applied directly to the vines. The use of an industrial thermotherapy unit allowed all vines to be treated in a single batch that reduced variation of thermotherapy treatment between batches of vines. This ensures all heat-treated vines were at 50°C for the same amount of time and any inhibitions seen in plant growth are due to the biocontrol agents applied for individual treatments.

Further testing is required to confirm the efficacy of the biological control strains as a preventative measure for crown gall disease on grapevine to confirm the activity of the biocontrol agents on the grapevine and reductions in disease and gall frequencies. The interpretation of the interaction is complex but results suggest that the position of galls will change depending on both heat-treatment and biological control agent. Ideally, extended field trials that allow vines to be planted into vineyard for a number of years are required to determine the effects of the biocontrol bacteria on vines for longer term disease control.

Although the level of galling in the control groups appears insignificant at 4.6%, for nurseries producing large volumes of grapevine rootlings it becomes financially costly to

lose the vines after the growing season. The use of the biocontrol could allow planting of callused cuttings into *A. vitis* infested soils reducing the requirements for soil that has been out of rotation for over 5 years or buying pasteurised soil for use in the biodegradable pot planting systems.

CHAPTER 6

ASSESSMENT OF THE EFFICACY OF BIOCONTROL AGENTS IN A GRAPEVINE NURSERY: TRIAL 3 (1998-1999)

6.1 INTRODUCTION

A third field trial was completed in 1998-99 following the methods used in the 1997-98 field trial (Chapter 5). Variable thermotherapy treatments were used with 0, 10 and 20-minute treatments at 50°C to determine whether shorter periods of heat treatment would be beneficial. Vines were callused (in vermiculite) at Orlando-Wyndham Vine Nursery. Biocontrol inoculum included F2/5 (non-pathogenic *A. vitis*), HLB2 (non-pathogenic *A. tumefaciens*) and a combination of both isolates (F2/5 and HLB2). Pathogenic *A. vitis* were a combination of K306 and K1072 (grown in peat by Bio Care Technology Pty. Ltd.). K315 (*P. fluorescens)* and E26 (non-pathogenic *A. vitis*) were eliminated from the experimental design, as performance in the previous trial indicated they were less successful at biocontrol than F2/5 and HLB2. Based on the design of Trial 2 (Chapter 5), pathogen from peat culture was applied directly to the callused grapevine cuttings. F2/5 and HLB2 were further studied under field conditions to determine the effectiveness of the biological control isolates in conjunction with thermotherapy treatments.

6.2 Experiments

6.2.1 Field trial organisation

6.2.1.1 Vine variety selection and treatment

A single variety of *Vitis vinifera* Shiraz BVRC12 was supplied by Riverland Vine Improvement Association (RVIA), Victoria. Shiraz vines were heat-treated or untreated for the experiments as described previously (Section 2.15.3). Cuttings were callused before planting (Section 2.15.4).

6.2.1.2 Inoculum preparation and application

Inoculum was prepared in peat by Bio-Care Technology Pty. Ltd. (Section 2.7.2) and prepared for inoculation onto callused grapevine cuttings (Section 5.2.1.2).

6.2.1.3 Site selection and preparation

The 1998-99 field site was located at Temple Bruer Wines Pty. Ltd., Langhorne Creek (South Australia). The site had no previous exposure to grapevine propagation and was a clay-based soil. The site was prepared as per section 4.2.1.3.

6.2.2 Inoculum levels in peat cultures

Biocontrol and pathogen levels in peat inoculants were estimated using serial dilution and plating onto selective media (Sections 2.7.2 and 5.2.2).

6.2.3 Inoculum applied to callused grapevine cuttings

Inoculum levels applied to vines were determined with two 10ml aliquots from each resuspended inoculum mix (Section 5.2.3).

6.2.4 Field trial treatment groups and organisation

A total of 15 treatment groups were used to determine the effectiveness of the biocontrol strains against high levels of pathogenic *A. vitis*. The effect of thermotherapy and bacterial inoculum was examined. For each treatment group a total of four plots (2m in length and 1m wide) was used. Plots of vines were organised using a random block design (Figure 23) with pathogen and biocontrol strains applied at the same rate. All vines treated with a biological control strain (one-hour) were subsequently treated with the pathogen combination (15 minutes). Extra treatments of control (no inoculum applied) and pathogen combination only were used to determine the baseline galling levels. Vines were planted in late October (1998) with a polypropylene watering system in place (Section 5.2.4). Vines were removed from the field plot once full dormancy was reached in September (1999), bagged and returned to the laboratory for analysis (stored at 2°C until required).

6.2.5 Bacterial colonisation of callused grapevine cuttings

Inoculated callused cuttings were assessed for colonisation of biocontrol and pathogenic bacteria (Section 5.2.5).

6.2.6 Efficacies of biocontrol bacteria on gall frequency, position and size

After harvest vines were washed free of soil and assessed for galling. Each treatment was analysed and the following data recorded: gall frequency per plot with total number of vines, gall diameter for individual galls and position of gall(s) on individual rootlings (Figure 3).

Figure 23: 1998-99 field site arrangement at Temple Bruer Vineyards (Langhorne Creek, South Australia. A random block design was used with 4 replicate plots. Included were three heat treatments and five bacterial treatments

0 CONTROL: un-inoculated, nil HT; 10 CONTROL: un-inoculated, 10 mins HT; 20 CONTROL: un-inoculated, 20 mins HT; 0 PATH ONLY: Pathogen combination, nil HT; 10 PATH ONLY: Pathogen combination, 10 mins HT; 20 PATH ONLY: Pathogen combination, 20 mins HT; 0 F2/5: *A. vitis* F2/5 biocontrol +pathogen, nil HT; 10 F2/5: *A. vitis* F2/5 biocontrol +pathogen, 10 mins HT; 20 F2/5: *A. vitis* F2/5 biocontrol +pathogen, 20 mins HT; 0 HLB2: *A. tumefaciens* HLB2 biocontrol + pathogen, nil HT; 10 HLB2: *A. tumefaciens* HLB2 biocontrol + pathogen, 10 mins HT; 20 HLB2: *A. tumefaciens* HLB2 biocontrol + pathogen, 20 mins HT; 0 F/H: *A. vitis* F2/5 biocontrol, *A. tumefaciens* HLB2 + pathogen, nil HT; 10 F/H: *A. vitis* F2/5 biocontrol, *A. tumefaciens* HLB2 + pathogen, 10 mins HT; 20 F/H: *A. vitis* F2/5 biocontrol, *A. tumefaciens* HLB2 + pathogen, 20 mins HT.

Random allocation of treatments as follows

heat	**biological control**
1=10	1=PATH ONLY
2=NHT	2=HLB2
3=20	3=CONTROL
	4=F/H
	5=F215

plot	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 4**
1	20 HLB2	10 PATH ONLY	20 CONTROL	NHT F215
2	NHT F/H	20 F/H	10 CONTROL	NHT F/H
3	10 PATH ONLY	10 CONTROL	NHT PATH ONLY	20 PATH ONLY
4	10 F/H	20 F215	20 PATH ONLY	20 HLB2
5	NHT PATH ONLY	NHT CONTROL	20 F215	20 F215
6	20 F/H	20 HLB2	10 F/H	20 CONTROL
7	NHT HLB2	10 F/H	10 PATH ONLY	10 F215
8	20 CONTROL	10 F215	10 HLB2	NHT CONTROL
9	10 F215	10 HLB2	NHT CONTROL	NHT HLB2
10	20 F215	NHT HLB2	20 HLB2	10 CONTROL
11	NHT F215	NHT PATH ONLY	NHT F/H	10 PATH ONLY
12	NHT CONTROL	20 PATH ONLY	20 F/H	20 F/H
13	10 HLB2	NHT F215	10 F215	10 HLB2
14	10 CONTROL	NHT F/H	NHT F215	10 F/H
15	20 PATH ONLY	20 CONTROL	NHT HBL2	NHT PATH ONLY

20 vines per plot planted in single row

6.2.7 Effect of thermotherapy and biocontrol application on vine growth

The effect of thermotherapy on grapevine growth was evaluated in the field trial. This was achieved for each treatment by counting the vines that failed to produce roots or shoots for individual treatments. Vines were visually assessed before planting for the level of callusing that had occurred across heat-treated and non-heat treated cuttings and assessed by wet weight of callus material on individual vines. Callus material was aseptically removed from cuttings and weighed to within 0.001g. Five replicate vines were used per thermotherapy treatment.

6.2.8 Effect of biocontrol strains on canopy development

Dry shoot weights of galled and non-galled grapevines were determined for individual vines by measuring aerial growth of vine shoots after harvest. Growth from vines was placed in separate paper bags and dried in an oven at 80°C for 4 days until no further weight change could be detected. Dry shoot weight was measured to within 0.01g.

6.3 RESULTS

6.3.1 Inoculum levels in peat cultures

Bio-Care Technology Pty. Ltd produced biocontrol and pathogen peat cultures. Estimates of bacterial levels in peat were made using serial dilution and plating on selective media. No significant difference was observed for the biocontrol and pathogen levels in peat cultures with F2/5 ($1.3x10^9 cfu.g^{-1}$), HLB2 ($2.96x10^9$ $cfu.g^{-1}$), combined F2/5 and HLB2 ($7.2x10^8 cfu.g^{-1}$:$3.98x10^8 cfu.g^{-1}$) and pathogen combination K306 and K1072 ($7.2x10^8$ $cfu.g^{-1}$: $1.3x10^9$ $cfu.g^{-1}$). Contamination in the peat inoculum was estimated at lower than 10^6 $cfu.g^{-1}$ of peat.

6.3.2 Inoculum applied to callused grapevine cuttings

Bacterial levels applied to grapevine cuttings were estimated using serial dilution and plating on selective media. Each strain (biocontrol or pathogen) was applied at 10^7 $cfu.ml^{-1}$ in the resuspended peat inoculum. Ratios of biocontrol to pathogen were calculated resulting in the following ratios of biocontrol to pathogen: F2/5 *A. vitis* (1.17:1), HLB2 *A. tumefaciens* (2.1:1) and combined F2/5 and HLB2 (1.64:1). Levels of biocontrol strains F2/5 and F2/5+HLB2 were not significantly different to pathogen levels in the inoculum. Levels of HLB2 alone were significantly higher than pathogen levels ($p=0.046$). No significant differences were observed for the levels of biocontrol agents used for vine inoculation. As 80 vines were used for each treatment, when immersed in 10L of inoculum, individual vines had greater than $1x10^9$ bacteria available to colonise the callus.

6.3.3 Bacterial colonisation of callused grapevine cuttings

Bacteria were reisolated from callus material of grapevines after inoculation for planting in the field trial. No significant differences were observed for bacterial levels recovered from callus material for individual biocontrol treatments except F2/5 and the F2/5/HLB2 combination. A significantly higher level of biocontrol strain was recovered from F2/5 vines heat-treated for 20 minutes when compared to 0 and 10 minutes heat-treatment (p=0.034) on RS media. Bacteria were recovered at $5x10^7 cfu.g^{-1}$ callus on vines treated for 0 and 10 minutes, while vines treated for 20 minutes had $5x10^9 cfu.g^{-1}$ callus. A similar trend was observed for the combined F2/5/HLB2 treated vines. Pathogen levels remained constant over the three heat-treatments with control vines at 10^6-10^7 colonies isolated on the medium.

6.3.4 Efficacies of biocontrol bacteria on gall frequency, position and size

Gall frequency was determined by assessment of individual grapevine rootlings for galling. Gall data was analysed using a mixed logistic regression model. All thermotherapy treatments resulted in the same proportion of galls whether heat-treated for 0, 10 or 20 minutes (Figure 24).

6.3.4.1 Non heat-treated grape vines

Vines inoculated with biocontrol strains and pathogen showed a significantly higher level of galling when compared to control (un-inoculated) vines. Gall frequency for uninoculated vines (not treated with pathogen) were less than 5%. This was compared to pathogen treated vines that resulted in a 40% gall frequency. Analysis of galling on biocontrol treated vines using pathogen treated vines as the baseline at 40%, showed significant decreases in galling for the biocontrol isolate F2/5 (14%

galling) and combination of biocontrol isolates F2/5+ HLB2 (20% galling) treated vines (Figure 24A). This was a reduction in galling frequency of 65% for F2/5 and 50% for the combined treatment (F2/5+HLB2). Gall frequency for vines treated with HLB2 (32% galling) were not significantly different to the pathogen alone treatment (40%). Table 14 summarises the efficacy of the biocontrol agents in reducing gall frequency.

	Control	Pathogen		F2/5		HLB2		F2/5 and HLB2	
Therm.	% gall	% gall	Eff.	% gall	Eff.	% gall	Eff.	% gall	Eff.
NHT	2	40	0	14	65	32	20	20	50
HT10	0	40	0	16	60	27	32.5	13	68
HT20	3	35	0	7	80	34	3	24	40

Table 14: Efficacy of thermotherapy and biological control treatments on galling efficiencies for the 1998-99 field trial. Treatments were NHT= non heat-treated; HT10= heat-treated at 50°C for 10 minutes; HT20= heat-treated at 50°C for 20 minutes. Bacterial treatments were control (no inoculum applied), Pathogen (pathogen combination applied); F2/5 (*A. vitis* F2/5 biocontrol and pathogen applied); HLB2 (*A. tumefaciens* HLB2 biocontrol and pathogen applied); F2/5 and HLB2 (*A. vitis* F2/5 biocontrol and HLB2 biocontrol and pathogen applied). Eff.= efficacy of biocontrol isolate at reducing gall formation.

$$\text{Efficacy} = \frac{\text{\%Galled (pathogen only)} - \text{\% Galled (treated)}}{\text{\% Galled (pathogen only)}} \times 100$$

Figure 24: Gall frequency observed in the 1998-99 field trial of Shiraz BVRC12 vines to compare heat treatment and inoculation with biological control agents to control crown gall disease. Vines were assessed at 9 months for galling.

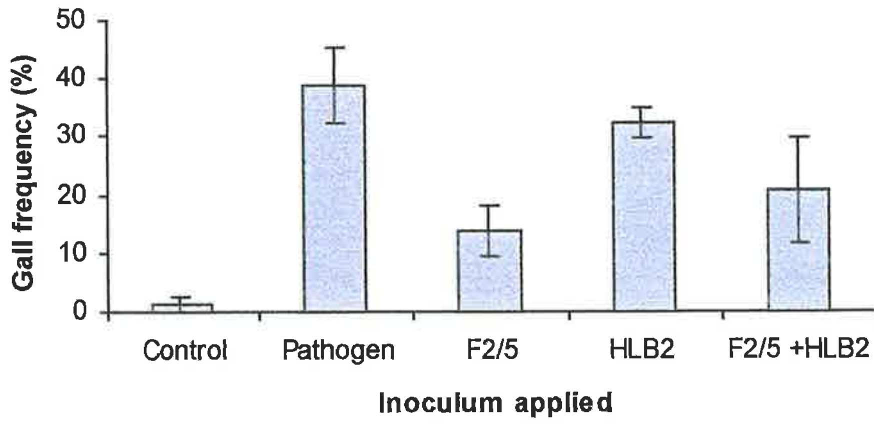
Inoculum applied: Control: no inoculum added, Pathogen alone: combination of K306 and K1072 *A. vitis* pathogens, F2/5: *A. vitis* F2/5 biocontrol agent and pathogen, HLB2: *A. tumefaciens* biocontrol agent and pathogen, F2/5+HLB2: combines F2/5 and HLB2 and pathogen

A: Gall frequency on Shiraz non heat-treated vines

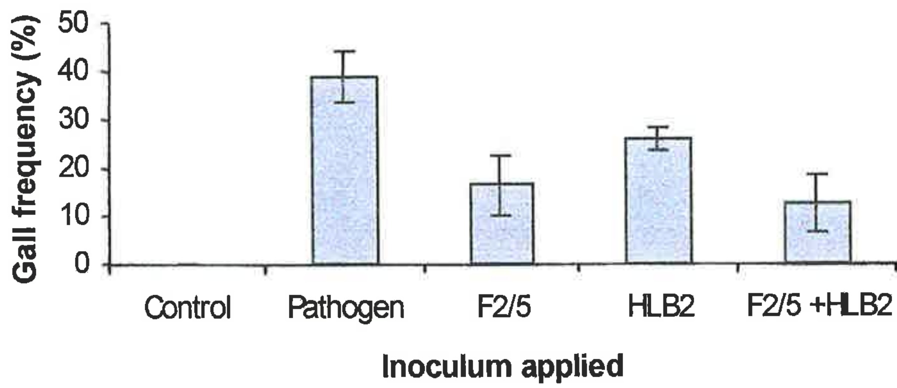
B: Gall frequency on Shiraz heat-treated vines (10 minutes)

C: Gall frequency on Shiraz heat-treated vines (20 minutes)

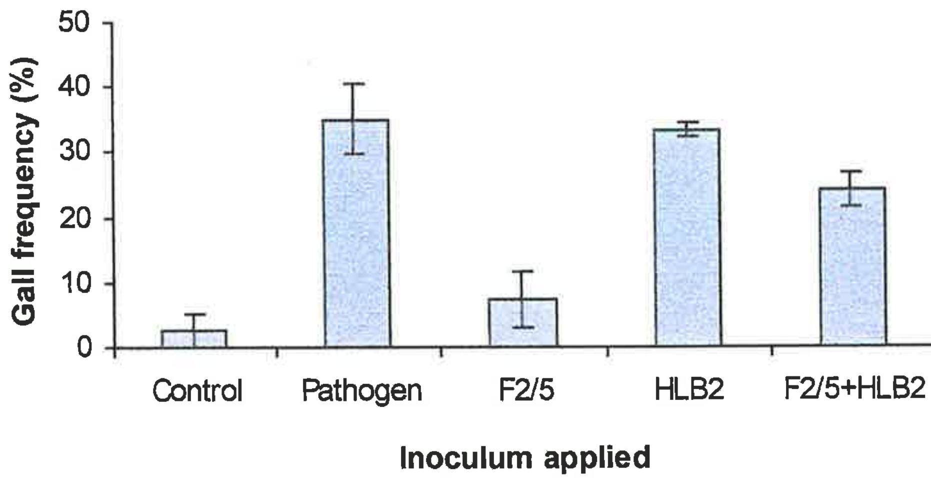
A



B



C



6.3.4.2 Heat-treated grapevines (10 mins)

Vines heat-treated for 10 minutes showed similar galling frequencies when compared to the non heat-treated variety (Figure 35, B). The level of galling for pathogen treated vines was 40% (baseline) with a significant decrease in galling was observed with F2/5 (16%) and F2/5+HLB2 (13%) treated vines. Gall frequency was reduced by 60% for F2/5 treated vines while HLB2 treated vine galling was not significantly different to the pathogen baseline (27%). When compared to non heat-treated vines F2/5 galling frequency was not significantly different while HLB2 treated vines had significantly less galling, reduced from 32% to 25% galling.

6.3.4.3 Heat-treated grapevines (20 mins)

Heat-treatment of vines for 20 minutes resulted in a reduction in gall frequency for F2/5 treated vines to 7%, an 80% reduction in galling compared to the pathogen treatment (Figure 24 C). The baseline level of gall frequency for pathogen treated vines was 35%. All other treatments were not significantly different to the non heat-treated vines. HLB2 galling frequencies (34%) were not significantly different to the pathogen alone treatment (35%) while treatment with the combines F2/5 and HLB2 resulted in 24% galling.

Galling on control vines was assessed and found not to be significantly different for the three heat-treatments (1%, 0% and 3% for non heat-treated, heat-treated 10 mins, and heat-treated 20 mins, respectively).

6.3.5 Assessment of biocontrol effect on gall position and size

The effect of biocontrol application on gall position and size was determined using a Residual Maximum Likelihood (REML) analysis to fit a linear model with fixed and random effects. Comparisons were made between pathogen and individual biocontrol strains for galled grapevine rootlings only. The resultant analysis showed that galling for all treatments was concentrated at the base of the vine regardless of heat-treatment and biocontrol agent applied (Figure 25). Only five control vines had galls present, making it inappropriate to make comparisons between control vines and other treatments. Galling at positions other than the crown of the plant was below 20% for all treatments except control vines (40%). This suggests that none of the biocontrol treatments significantly altered the position of galls in this trial. Uninoculated control vines had galls more evenly spread over the base, first node and roots, whereas the pathogen inoculated vines had galling present at the point of inoculation on the base of the vine. This is very different to what was observed in the 1997-98 field trial where the majority of galls were at position 2 (the first node above the crown).

6.3.5.1 Gall diameter

The effect of biocontrol and thermotherapy on gall diameter was analysed using the REML analysis. The predicted gall size showed that the smallest average gall size was for control, F2/5 and F2/5+HLB2 treated vines in comparison to HLB2 and pathogen treated vines, indicating F2/5 was capable of reducing or delaying gall initiation. No significant difference was observed for HLB2 and pathogen treated vines ($p= 0.752$, $\chi^2=0.1$, $df=1$) which suggests that HLB2 had no effect on gall size. When comparing F2/5 and F2/5+HLB2 no significant difference was observed for predicted gall size ($p= 0.527$, $\chi^2=0.4$, $df=1$) and there was no interaction between

Figure 25: Assessment of gall position for individual biocontrol treatments. Gall position was recorded and analysed to determine the effects of the biocontrol agents on gall position.

Inoculum applied: Control (no inoculum), Pathogen: combination of K306 and K1072 *A. vitis* pathogens, F2/5: *A. vitis* F2/5 biocontrol agent and pathogen, HLB2: *A. tumefaciens* biocontrol agent and pathogen, F2/5+HLB2: combines F2/5 and HLB2 and pathogen

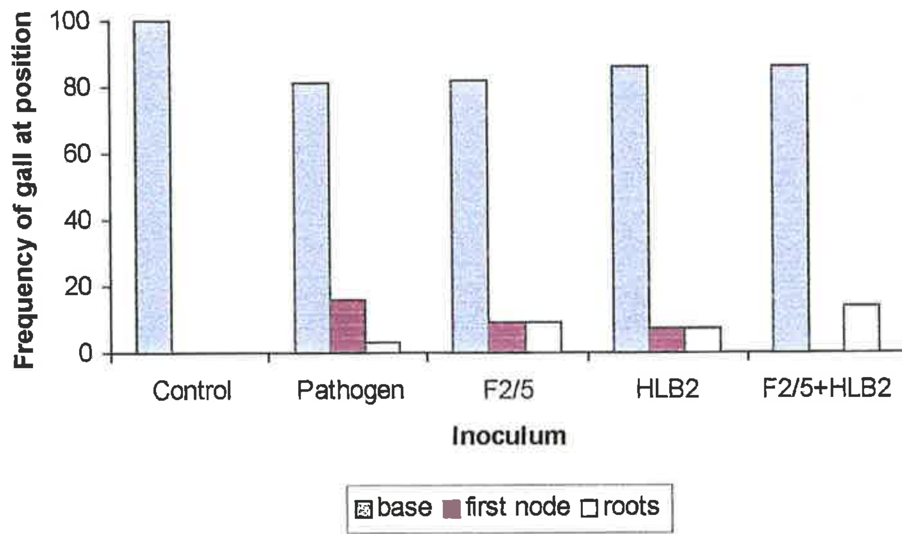
A: Gall frequency on Shiraz non heat-treated vines

B: Gall frequency on Shiraz heat-treated vines (10 minutes)

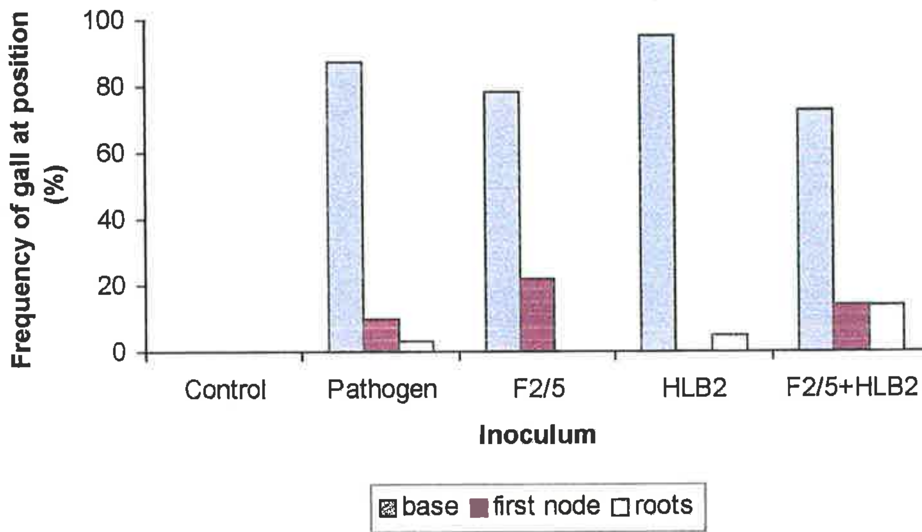
C: Gall frequency on Shiraz heat-treated vines (20 minutes)

Legend indicates gall position: 1.Crown/base of vine, 2. First node, 3. Vine roots.

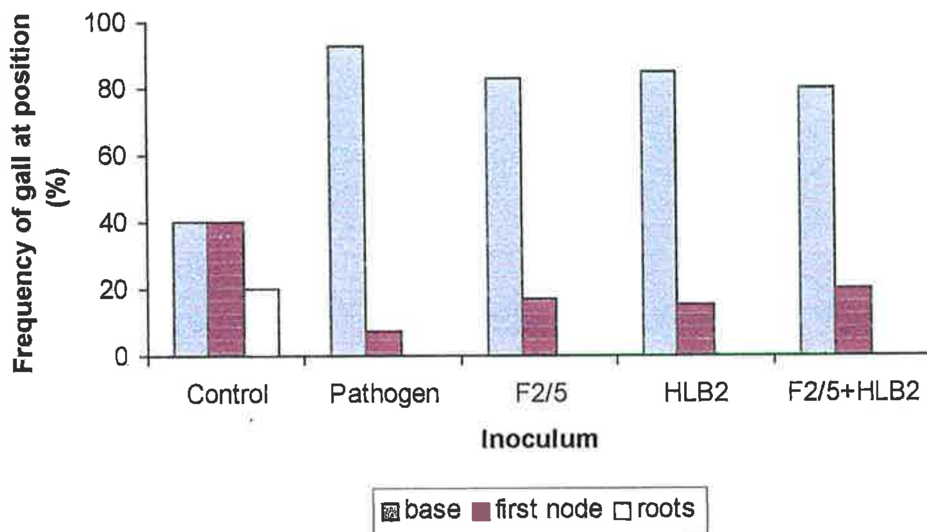
A



B



C



HLB2 and F2/5. This showed that the addition of HLB2 to the inoculum mixture had no statistically significant effect on gall size when F2/5 was present.

The interaction between biocontrol and thermotherapy was found to be non-significant ($p= 0.856$, $\chi^2=3.3$, $df=7$). The main effect of thermotherapy was also found to be non-significant ($p= 0.183$, $\chi^2=3.4$, $df=2$) after allowing for the biocontrol effects. The main effect for biocontrol was significant at the 5% level ($p= 0.039$, $\chi^2=10.1$, $df=4$) after allowing for heat-treatment. The variation between replicates tended towards zero indicating the differences in replicates was small.

6.3.6 Effect of thermotherapy and biocontrol application on vine growth

The effect of thermotherapy and the interaction of biocontrol bacteria with grapevine cuttings were assessed. Vine growth (Table 15) demonstrated that thermotherapy did not adversely affect the growth of vines with no significant difference between heat treatment groups. Both thermotherapy treatments did not affect vine growth with survival at 100% for most treatments while non heat-treated HLB2 and F2/5+HLB2 were slightly lower at 94% and 87% (significant at the 5% level).

Assessment of the effect of thermotherapy on callus development showed that there was significantly less callus development on heat-treated cuttings (Figure 26). ANOVA analysis showed that, non heat-treated cuttings had significantly larger callus (0.43g per cane) present on vines when compared to the heat-treated vines, heat-treated 10 minutes (0.29g per cane) and heat-treated 20 minutes (0.22g per cane).

Significant differences were observed between non heat-treated and both heat-treatments (heat treatment 10 mins, p= 0.048, t-statistic=2.495, df =18) (heat treatment 20 mins p=0.011, t-statistic=2.475, df =18). No difference in callus weight was observed between cuttings heat-treated for 10 or 20 minutes. This showed that the rate of callus development was reduced when thermotherapy was used on Shiraz BVRC12 grapevine cuttings.

	Relative vine growth for bacterial treatments (%)				
Thermotherapy	Control	Pathogen	F2/5	HLB2	F2/5 +HLB2
NHT	100	100	100	94	85
HT10	99	100	100	98	95
HT20	100	99	100	98	100

Table 15: Assessment of vine survival on recovery of vines from the field trial at 9 months growth. Vines were treated with thermotherapy and application of biological control strains F2/5 and HLB2 and pathogenic *A. vitis* for the 1998-99 field trial. Treatments were NHT- (no HT), HT10 (HT at 50°C for 10 mins), HT20 (HT at 50°C, 20 mins), Control (no inoculum), Pathogen: (combination of K306 and K1072 *A. vitis* pathogens), F2/5: (*A. vitis* F2/5 biocontrol agent and pathogen), HLB2: (*A. tumefaciens* biocontrol agent and pathogen), F2/5+HLB2: (F2/5 + HLB2 and pathogen).

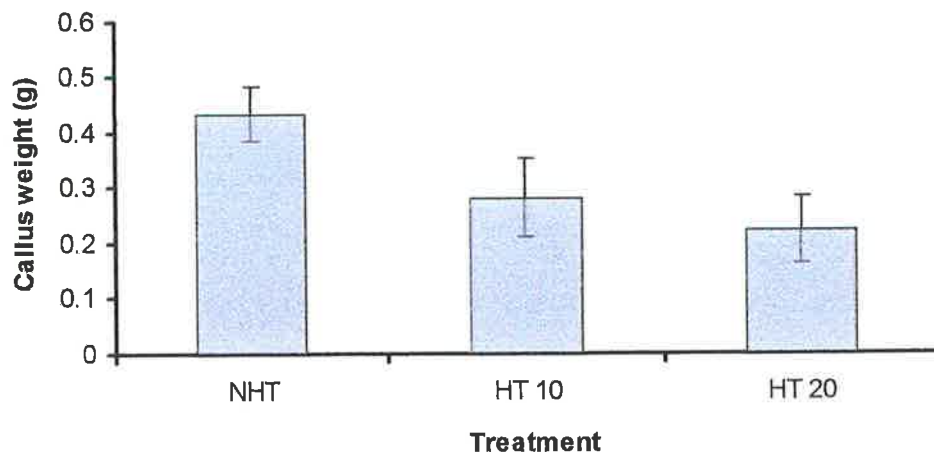


Figure 26: Mean callus weight of grapevine cuttings (g per vine) with non heat-treated, heat-treated (10 minutes) and heat-treated (20 minutes) on Shiraz BVRC12 cuttings. Vines were heat-treated then callused in vermiculite for 2 weeks in a controlled temperature room (Orlando-Wyndham Vine Nursery, Rowland Flat, South Australia) (±SD).

Treatments:

NHT = no heat-treatment

HT10 = thermotherapy for 10 minutes at 50°C

HT20 = thermotherapy for 20 minutes at 50°C

6.3.7 Effect of biocontrol strains on canopy development

The effect of biocontrol application on canopy development was assessed to determine if biocontrol strains were affecting plant growth. Assessment of canopy development was completed using the dry weight of all canopy material on vines. Data was transformed using a square root transformation. Results showed that shoot weight was unaffected by galling, biological controls or heat treatment (Figure 27).

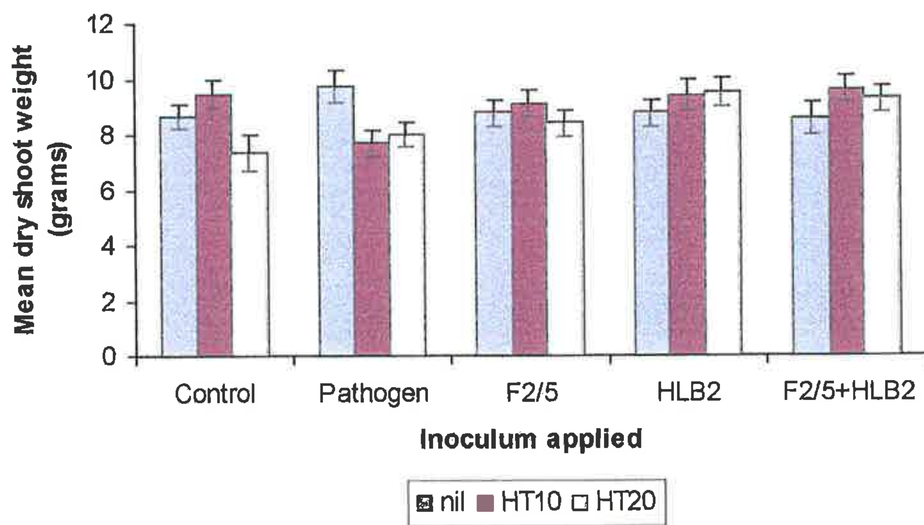


Figure 27: Mean dry shoot weight for grapevines inoculated with biological control agents and thermotherapy. Heat-treatment (10 mins, 20 mins) and non heat-treated vines (±SE).

6.4 DISCUSSION

The third field trial confirmed the effectiveness of the biocontrol bacterium F2/5 (*A. vitis*) to control crown gall disease on grapevine cuttings in a field nursery. The effectiveness of the biocontrol strain F2/5 (*A. vitis*) in the presence of a high level of pathogenic *A. vitis* is of major significance. The ability of this bacterium to control crown gall disease in soil that contains the pathogen indicates that application of the biocontrol strain to clean grapevines will protect the vines from infection. This would allow planting from nurseries into soil containing pathogenic *A. vitis*. This result is significant for the grapevine nursery industry as in the past as land used for grapevine propagation was not reused for greater than five years. The application of a biocontrol strain that is effective in the presence of the pathogen would allow reuse of the land, provided that the risks of other grapevine diseases that are soil borne are minimised.

As biocontrol agents were applied at half the rate applied in the 1997-98 trial, F2/5 was successful at a lower application rate. The ratio of F2/5 to pathogen was 1.17:1 (1998-99 trial) compared to 4.3:1 in the 1997-97 trial. This suggests that lower doses may be sufficient to control infection from natural populations of *A. vitis* in soil.

Gall frequency for the HLB2 treatment was not significantly different from the pathogen alone treatment. The ratio of HLB2 to pathogen in 1997-98 was 16.6:1 compared to 2.1:1 in 1998-99. This suggests that higher levels of HLB2 may be required to allow good control of crown gall on grapevines.

The assessment of vine growth using dry shoot weight for individual treatments showed that the application of the biocontrol bacteria was not inhibitory to plant growth. The main effects of these treatments were seen with thermotherapy that led to reduced callus production on heat-treated cuttings. This did not affect plant growth over a 9-month period. Callus production was improved in 1998-99 as vines were heat-treated for only 10 or 20 minutes and then cooled for 1 hour to ambient temperature. The callusing method allowed for production of larger callus on all vines although some variation was seen with heat-treated material. This improved callus development for the heat-treated vines, but a significant difference still existed compared to non heat-treated vines. If heat-treated vines are stored correctly and treated with care, grapevine establishment can be more successful, with better take and growth of vines.

The use of thermotherapy for 20 minutes in conjunction with the application of the biological control strain F2/5 was able to reduce galling in a field nursery to 20% when pathogen was applied directly to the grapevine callus. When used with callusing in vermiculite in a temperature-controlled incubator, vine take was most successful.

Gall induction in this study was maintained at approximately 40% for pathogen treated control grapevines under field conditions. In previous glasshouse studies performed, most galling efficiencies were near 100% (Burr and Reid, 1994: Pu and Goodman, 1993). These studies concentrated on the application of pathogen and biocontrol bacteria to fresh wounds caused by drilling into the grapevine trunk. In this study the bacteria were applied directly to callused cuttings before planting into

the field nursery. The presence of wound sites on callus material would allow the introduction of pathogenic *A. vitis* into the grapevine.

Gall position was not affected by the application of the biocontrol agents. Galls occurred mainly at the crown of the vine (greater than 80% frequency) and at the first node or on roots the remaining 20%. This was not unexpected because the callus at the base of the grapevine cutting was inoculated with pathogen. Gall diameter was decreased when cuttings were treated with F2/5 or F2/5 +HLB2 while the largest galls occurred on HLB2 or pathogen only treated vines. This demonstrates that F2/5 was effective at reducing or delaying gall initiation. When galls did occur they were reduced in size. No synergistic effect was seen when F2/5 and HLB2 were combined in a single inoculum, which was not unexpected, as HLB2 was not effective at controlling disease when used alone in this trial.

Biocontrol strain F2/5 has been shown to be the most effective strain tested to control crown gall disease on grapevine. The use of F2/5 in conjunction with thermotherapy of dormant canes prior to planting into a nursery would allow the most effective preventative treatment to reduce crown gall disease in grapevines. Improved callusing was obtained on grapevine cuttings heat treated for the shorter period of 10 minutes but this does not allow the internal grapevine tissue to reach the desired temperature of 50°C. Provided care is taken during the callusing period, the 20-minute heat-treatment should allow effective destruction of the majority of pathogens within the grapevine and good levels of establishment of the grapevines. If the biocontrol treatment is omitted, the potential exists for grapevines to become reinfected with the pathogen.

CHAPTER 7

THE ROLE OF BACTERIAL ATTACHMENT IN THE BIOLOGICAL CONTROL OF CROWN GALL ON GRAPEVINE

7.1 INTRODUCTION

Research into the mechanisms of biological control by non-pathogenic agrobacteria has focussed on agrocin production and antagonism towards pathogens. The role of agrocins has been demonstrated as a primary mechanism of biocontrol by *A. rhizogenes* K84 (agrocins 84 and 434) (Kerr, 1989). The production of inhibitory compounds is significant but effective biological control requires additional factors. Other factors involved in the disease control process for K84 are competition for nutrients and binding sites within the plant host. Strain K434 was still capable of disease control when the plasmid encoding agrocin 84 production was removed (McClure *et al.*, 1994). The plasmid cured strain of K84 was still able to control the disease showing that chromosomal traits were also involved in biocontrol (Penãlver *et al.*, 1994). The potential exists for competition for binding sites on the plant root surface between non-pathogenic K84 (K1026) and pathogenic agrobacteria that are controlled by the biocontrol strain.

The mechanism of action of the biocontrol strains investigated in this study are currently unknown but a number of research groups have investigated the role of agrocin production (Burr *et al.*, 1997, Bell *et al.*, 1995), bacterial attachment (Burr *et al.*, 1997, Brisset *et al.*, 1991, Pu and Goodman, 1993), and DNA transfer (Burr *et al.*, 1997). Attachment of pathogenic *A. vitis* has been investigated using wounded

grapevine rootlings and tissue cultured callus and shoot tips without microscopic observation of the interactions occurring between bacterial cell and plant tissue. Inhibition of gall induction by blocking specific sites with non-pathogenic bacteria was demonstrated by Lippincott and Lippincott, (1969). As part of the *Agrobacterium* infection cycle the bacterium must first attach to the plant host cell before DNA transfer can occur. This is followed by DNA integration into the plant host cell. Studies investigating this have shown that attachment is an essential step in pathogenesis and isolates lacking *att* (attachment) genes are not capable of causing disease (Matthysse, 1987; Matthysse *et al.*, 1995). The *att* genes have been linked to genes encoding cellulose production. *Agrobacterium tumefaciens* has been shown to produce cellulose fibrils to remain bound to the plant cell surface (Matthysse *et al.*, 1981). Bacterial isolates with mutations in these genes have significantly reduced virulence (Matthysse and McMahan, 1998).

In this study the attachment of biological control strains and pathogenic *A. vitis* was investigated to determine the interaction between pathogen and biocontrol strain on grapevine roots. All pathogens were selected for resistance to agrocins produced by the biocontrol strains. This was to determine if pre-inoculation of roots with biocontrol strains was capable of preventing pathogen colonisation. This is a potential point of entry for pathogenic bacteria due to the presence of wounded tissue as the root extend into the soil and may be damaged by insects or prone to protozoan attack. Competition for binding sites is also of interest as the potential exists for biocontrol strains to out compete pathogenic *A. vitis* by application to vines before planting into pathogen infested soils.

In the investigation of bacterial attachment, grapevine, *Arabidopsis* and tomato were used as host plants to determine if biocontrol strains were capable of preventing pathogenic *A. vitis* from attaching to the plant roots. In all cases tissue culture roots were used and all were shown to be free of pathogenic *A. vitis.*

The Green fluorescent protein (GFP) marker system was selected to allow microscopic observation of the interaction between pathogenic *A. vitis* and biocontrol bacteria. The marker plasmid p519*ngfp* was constructed by Matthysse *et al.*, (1996). The *gfp* gene from the jellyfish *Aquoria victoria* (Chalfie *et al.*, 1994) was altered to allow high levels of expression of the gene product in Gram negative bacteria (Cormack *et al.*, 1996). The mutant gene GFP*mut*2 was incorporated to allow correct folding of the protein which remained soluble within the bacterial cell enhancing GFP expression by 100 fold when compared to the wild-type protein expressed in *E. coli* (Cormack *et al.*, 1996). The GFP*mut*2 was inserted directly behind the *lac* promoter in the plasmid pDSK519. The promoter *pnpt*2 was inserted directly in front of the *gfp* (creating p519*ngfp*) to ensure the *gfp* gene was switched on. The helper plasmid pNJ5000 (Grinter, 1983) was included in the donor strain to allow successful transfer of the plasmid (p519*ngfp*) into the host strain.

7.1.1 Aims

1. To determine levels of attachment of wild type bacterial strains to tissue culture root material

2. To determine whether pathogenic and biocontrol strains compete for binding sites on plant roots

3. To determine whether biocontrol bacteria are capable of preventing pathogen colonisation of the plant root surface

7.2 EXPERIMENTS

7.2.1 Genetic modification of bacteria

Bacteria were genetically modified for use in attachment assays. Plasmid p519*ngfp* was introduced into the bacteria used in this study via plate mating of individual isolates with *E. coli* DH5α (p519*ngfp*, pNJ5000) (Section 2.6.2). Spontaneous rifampicin mutants were also generated from the wild type strains (Section 2.6.1). All strains generated from these methods were tested for fitness against the parent strains.

Fitness tests included the following:

1. Plasmid stability (Section 2.6.3.1)
2. Antagonistic activity with a modified Stonier's assay (Section 2.4)
3. Growth curves (Section 2.2)
4. Plasmid content of p519*ngfp* modified strains (Section 2.6.3.4)

7.2.2 Survival of bacteria in CAS medium

All bacteria were initially tested in the bacterial attachment assay system without plant root material. Bacteria were cultured in broth (Section 2.1.1) and diluted in PBS to a concentration of 10^5 cfu.ml^{-1}. Using 36mm petri dishes, 2ml Calcium sucrose (CAS) broth was added and an aliquot (100µl) of diluted bacterial suspension. At 0 and 60 minutes samples were taken, diluted in PBS and spread onto NA. Plates were incubated at 28°C for 2 days and colonies counted. The assay was modified to ensure bacterial survival at greater than 95%. The assay was performed all strains for wild type, green fluorescent protein (*ngfp*) tagged and spontaneous rifampicin resistant (rif^r) mutant bacteria to be used in the study. This was to ensure characteristics of the wild type strain were retained.

7.2.3 Microscopic observation of bacterial attachment on plant root surfaces

Cut root segments of *Vitis vinifera* (cv. Cabernet Sauvignon and Riesling), *Arabidopsis thaliana* (eco. Landsberg erecta) and tomato (*Lycopersicon esculentum* cv. Floridade) tissue culture plantlets were incubated in the presence of 10^8 cfu of bacteria. Bacterial attachment to roots was scored after 24, 48 and 72 hours (Section 2.14). For light microscopy, live preparations were examined in an algal counting chamber (Thomas Scientific, Pty. Ltd.) and photographed with a Zeiss Photoscope 2 using Nomarski optics (University of North Carolina) or an Olympus Vanox microscope (CSIRO Division of Land and Water, Adelaide, South Australia). Bacterial attachment on the root surface was scored for segments along the root including root hairs, epidermis, cut ends and root tips.

7.2.4 Bacterial attachment on plantlet roots

Bacteria were cultured in broth (Section 2.1.1) and diluted in PBS to a concentration of 10^5 cfu.ml^{-1}. CAS broth (2ml) was inoculated with an aliquot (100µl) of bacterial suspension with equal lengths of tissue culture plantlet roots (*V. vinifera* cv. Riesling or Cabernet Sauvignon, *A. thaliana* (eco. Landsberg erecta) or *L. esculentum* (cv. floridade). At 0 and 60 minutes samples were taken, diluted in PBS and spread onto NA. Plates were incubated at 28°C for 2 days and colonies counted. Assays were performed in triplicate and repeated a minimum of four times.

Attachment was calculated by percentage:

$$\% \text{ attached} = \frac{\text{final count}}{\text{Initial count}} \times \frac{100}{1} \ (\pm \text{SE}).$$

7.2.5 Interaction of biocontrol and pathogenic bacteria

Bacterial isolates were selected to allow detection of pathogenic and biocontrol strains in a single system using a GFP marked pathogen and wild type antagonist. Paired biocontrol and pathogen combinations used are listed in Table 16. It was previously determined that the pathogens were resistant to antagonistic compounds produced by the biocontrol isolates. Biocontrol strains were inoculated into the assay system (after dilution to 10^5 cfu.ml^{-1} in PBS) with pathogenic strain added after 15 minutes incubation time. Samples were taken at 0 and 60 minutes to determine the level of attachment. All samples were serially diluted in PBS and plated onto NA and NA containing kanamycin (25µg.ml^{-1}) and incubated at 28°C for 2 days before counting. All plates were done in triplicate and individual assays were completed with 5 replicates.

Bacterial antagonist	Agrocin resistant pathogen
E26*ngfp* 1	K306
F2/5	K1072*ngfp*1
K315	K1072*ngfp*1
HLB2	K377*ngfp*2

Table 16: Bacterial strains selected for resistance to inhibitory compound(s) produced by antagonistic biocontrol strains for use in attachment assays. Resistance was determined using the modified Stonier's plates (Section 2.4). For use in competition assays, a combination of wild type and p519*ngfp* strains were selected. Strains are listed in Table 1.

7.2.6 Microscopic observation of bacterial interaction on grapevine roots

Interactions between biocontrol isolates and pathogenic *A. vitis* on grapevine roots (*V. vinifera* cv. Cabernet Sauvignon) were completed using attachment assays (Section 2.14) with a green fluorescent protein tagged pathogens and wild-type biocontrol isolates (Table 16). Plantlets were removed from the medium and rinsed in SDW. Grapevine roots in 2ml CAS broth were inoculated with an aliquot (100µl) of biocontrol bacteria (cultured in NB and diluted in PBS to 10^5 cfu.ml^{-1}), 15 minutes prior to the addition of an aliquot (100µl) of pathogenic *A. vitis* (10^5 cfu.ml^{-1}) and incubated for 24, and 48 hours. Assessment of interactions between biocontrol and pathogenic bacteria were done using an epifluorescence microscope (Olympus Vanox). Photographs were generated using either Nomarski (differential interference contrast) or epifluorescence (excitation 488nm, emission 520nm) optics, using 100x and 200x magnification. Individual isolates and competition assays were observed to determine the effects of pathogenic *A. vitis* in the presence or absence of a biocontrol agent.

7.2.7 Bacterial colonisation of *Vitis vinifera* plantlets

Rifampicin resistant bacterial isolates were cultured in broth (Section 2.1.1) and diluted in PBS to 10^5cfu.ml^{-1}. Tissue culture *V. vinifera* (cv. Cabernet Sauvignon) plantlets (Section 2.18) were removed from the culture medium and washed in SDW. Intact plantlets were inoculated with prepared bacteria for 1 minute then planted into sterile 50ml Falconer tubes (with 3 drainage holes in the base) in microwave sterilised UC soil (Section 2.14.4). Plantlets were allowed to grow for 0, 2, 5 and 10 days then removed and assayed to determine levels of bacteria loosely, tightly or irreversibly bound to the grapevine roots using a series of washing steps (Section 2.14.5.1).

Supernatants from the washing steps were combined and serially diluted for plating on selective media (NA with Km: 25$\mu g.ml^{-1}$).

Root material was spread onto NA and covered in 0.7% water agar to determine levels of irreversibly bound bacteria. Bacterial counts were performed and estimates of bacterial numbers of both loosely and tightly bound bacteria present on the root surface were calculated. All samples were plated in triplicate and each assay was replicated 5 times. Where competition between biocontrol and pathogen was tested, a rifampicin resistant isolate was paired with a *gfp* expressing isolate to allow enumeration of both isolates by plating on selective media (NA supplemented with rifampicin or kanamycin).

7.3 RESULTS

7.3.1 Fitness of genetically modified bacteria

Isolates carrying the GFP plasmid p519*ngfp* were tested for fluorescence using a UV light box with 485nm-wavelength filter. Selected isolates were assessed for the stability of the introduced plasmid (p519*ngfp*) by growth in non-selective broth. Results of strain fitness are summarised in Table 17. Plasmid loss due to non-selective growth media was determined by calculation of the percentage of bacterial cells retaining kanamycin resistance. Results indicated that greater than 95% of isolates of E26*ngfp*1, F2/5*ngfp*1, K377*ngfp*2 and K1072*ngfp*2 retained the plasmid under non-selective growth conditions over 10 days (Table 17). These isolates were tested further for fitness characteristics.

Isolate	% plasmid stability (± SE)	Antagonistic activity	Growth rate	Plasmid p519*ngfp*
E26*ngfp* 1	100 (5.2)	-	++	+
E26 rif^R	-	+	++	-
F2/5*ngfp* 1	100 (1.1)	+	++	+
F2/5 rif^R	-	+	++	-
K377*ngfp* 2	97 (2.1)	+	++	+
K377 rif^R	-	+	++	-
K1072*ngfp* 2	98(2.4)	+	++	+
K1072rif^r	-	+	++	-

Table 17: Summary of fitness testing on genetically modified bacterial isolates. Fitness tests included plasmid stability in tagged isolates, level of antagonism and growth compared to the parent isolate, and the presence of the plasmid DNA p519*ngfp* in the transformed isolates. + = Not significantly different in zone of inhibition when compared to the parent strain. ++ = Not significantly different growth rate when compared to the parent strain.

Antagonistic activity of newly constructed isolates was tested against the parent isolate. Both F2/5 and E26 modified strains were tested as antagonists while pathogenic strains (K377 and K1072) were tested in the overlays. Significant reductions in antagonism in the marked strains differences between wild type and genetically marked individuals were observed for E26*ngfp*1, ($p=0.003$), F2/5*ngfp*2 ($p=0.017$), and K1072*ngfp*1 ($p=0.04$) and these were not used further. No differences were observed for other isolates tested and these were tested further.

Growth of wild type and genetically modified bacteria was assessed using growth curves. K1072 rif^R grew significantly slower than the parent strain (Figure 28). No difference was observed for K1072*ngfp*2 and modified and parent strains of F2/5 and HLB2.

Plasmid DNA was extracted from isolates and run on an agarose gel to confirm the presence of plasmid p519*ngfp* from the plate mating. A plasmid of the expected size was observed in all GFP tagged strains tested and was absent in wild-type parent strains (Figure 29).

7.3.2 Bacterial survival in CAS broth

All isolates (wild type, *ngfp* tagged and spontaneous rifampicin mutants) were tested in the attachment assay system without roots to ensure optimal survival of the bacteria. All samples were diluted in PBS and plated on NA, NAKm ($25\mu g.ml^{-1}$) or NA rifampicin ($100\mu g.ml^{-1}$) as required.

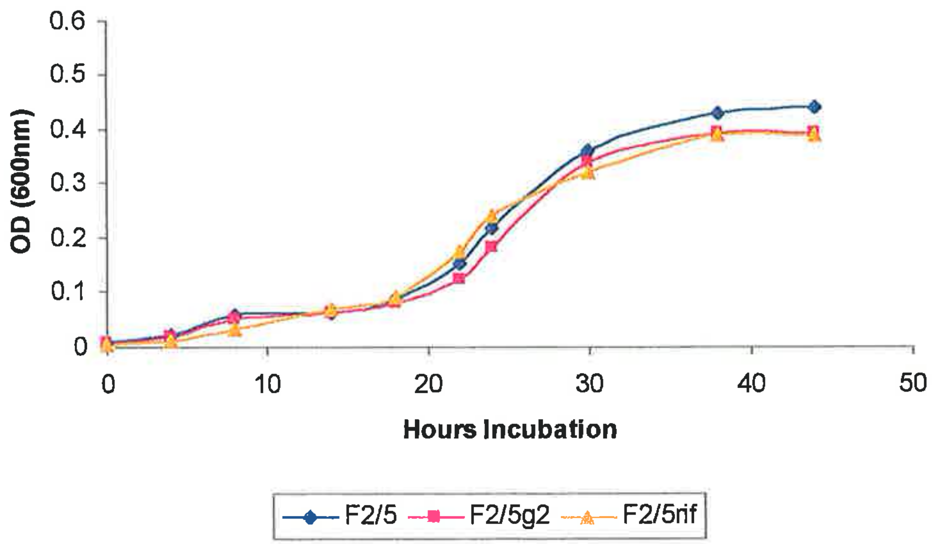
Figure 28: Comparisons of growth curves of wild type and genetically modified bacteria. Bacteria were modified using either insertion of the plasmid p519*ngfp* or spontaneous rifampicin mutation.

A: F2/5 wild type strain, F2/5*gfp*2 and F2/5rif^r.

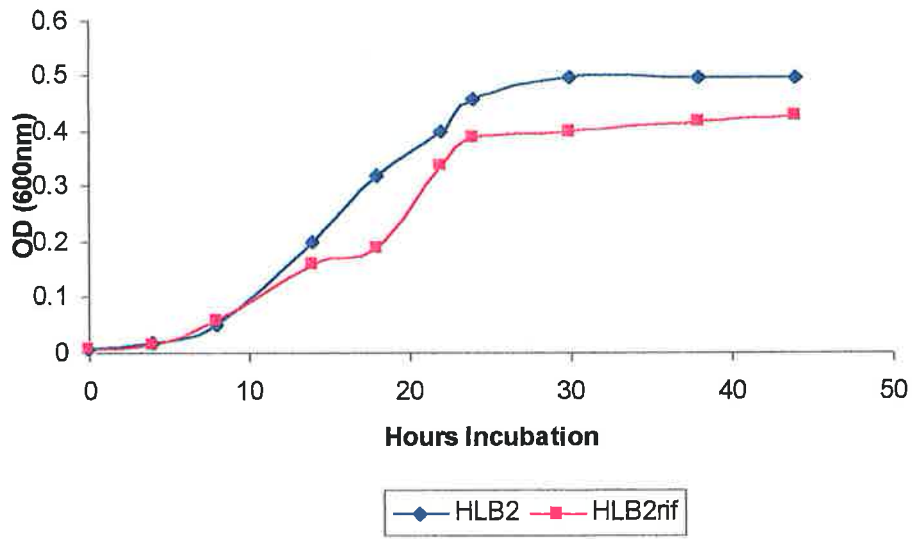
B: HLB2 wild type strain and HLB2rif^r.

C: K1072 wild type strain, K1072*gfp*2 and K1072rif^r.

A



B



C

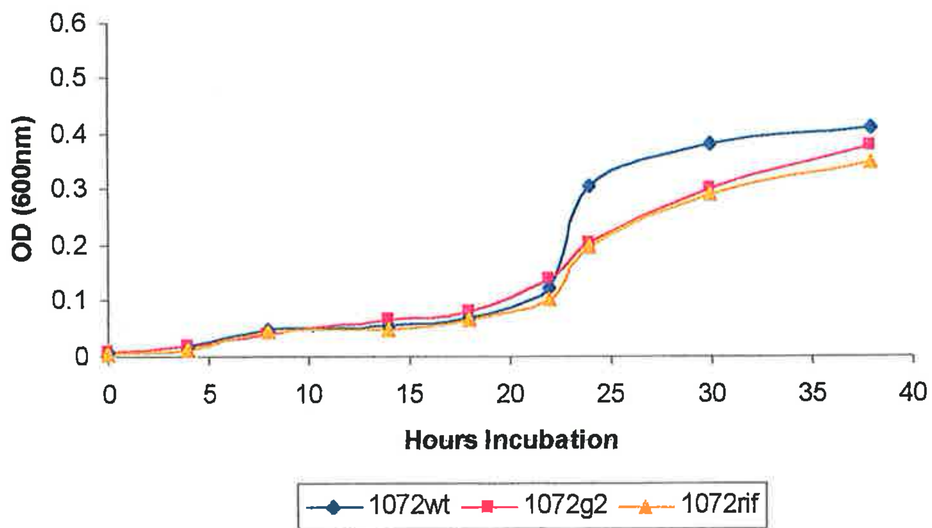


Figure 29: Plasmid DNA extracted from wild-type and genetically modified agrobacteria using transformation with plate mating to incorporate p519*ngfp* as a green fluorescent protein marker. DNA was run on agarose gel and visualised with ethidium bromide staining and UV detection. Lane 1: F2/5, 2: F2/5*gfp*1, 3: F2/5*gfp*2, 4: E26, 5: E26*gfp*1, 6: E26*gfp*2, 7: K377, 8: K377*gfp*1, 9: K377*gfp*2, 10: K1072, 11: K1072*gfp*1, 12: K1072*gfp*2. Refer to figure 7 for correct plasmid profile of F2/5.

Biocontrol strains F2/5, HLB2 and pathogenic *A. vitis* isolates had a survival rate of greater than 95% over the one-hour period in CAS broth. Significant death rates were encountered in the CAS broth only (Table 18). K315 was significantly inhibited in CAS solution. Alternatives were tested incorporating glycerol and magnesium sulphate into the broth that significantly improved survival from 72% to 84% and 82% respectively. However, this was not enough to allow quantification of attachment to roots.

Isolate	Pathogenicity	% survival in CAS (±SE)
A. vitis F2/5	-	95 (0.6)
F2/5*ngfp*1	-	96 (2.1)
F2/5rif^{r}1	-	96 (2.5)
A. vitis K377	+	98 (2.0)
K377*ngfp*1	+	95 (3.6)
K377*ngfp*2	+	95 (2.0)
A. vitis K306	+	100 (0.4)
K306 rif^{r}1	+	100 (0.6)
A. tumefaciens HLB2	-	100 (0.3)
HLB2*ngfp*1	-	95 (1.2)
HLB2 rif^{r}1	-	97 (1.5)
P. fluorescens K315	-	72 (5)
K315 (10g.L^{-1} glycerol)	-	84 (2.5)
K315 (6mg.L^{-1} $MgSO_4.7H_2O$)	-	82 (9.8)

Table 18: Bacterial survival in CAS broth (Section 7.3.2) attachment assays over 1 hour test period. All isolates were required to survive in the system at greater than 95% to allow use in grapevine attachment assays. *ngfp* = green fluorescent protein tagged bacteria, rif = spontaneous rifampicin mutant

7.3.3 Colonisation of root surfaces

All isolates were tested for attachment to grapevine tissue cultured root material in CAS broth for 24, 48 and 72 hours. Wild type bacteria bound to cut roots of *V. vinifera* (cv. Cabernet Sauvignon and Riesling) after 24 hours incubation. Large numbers of bacteria were observed on the cut ends, root hairs and epidermis of each root segment (Figure 30). The root surfaces were uniformly covered with no apparent preferential sites of attachment. All pathogenic and biocontrol strains tested were capable of colonising the plant root surface at high levels. The biocontrol *A. vitis* strains F2/5 and E26 were able to colonise the root surfaces. HLB2 was observed in the mucilagenous layer (Figure 30) and was capable of colonising root segments at a very high density. K315 did colonise the root surface but populations were less dense than those of *A. vitis* isolates. It was capable of entering into the plant root hair cells. All *A. vitis* isolates, regardless of pathogenicity, were capable of colonising tissue cultured plant root surfaces. No differences were observed for wild-type strains and genetically modified progeny with *ngfp* or strains carrying spontaneous rifampicin resistance.

7.3.4 Bacterial attachment on tissue cultured plant roots

Attachment of bacteria to plant root surfaces was assessed using three plantlet types, grapevine, tomato and *A. thaliana*. Comparisons were made of the level of attachment of individual strains on the tissue cultured plant roots. Results showed that the interaction between plant and bacteria is variable (Table 19) but most bacteria bind at approximately the same rate (% attachment) when applied individually. Biocontrol strain F2/5 (*A. vitis*) did not differ in attachment between each plant type (15-21%). Experiments replicated at least 3 times (usually the mean of 5 replicates).

Similar results were also observed for pathogens K377 and K1072 (16-20%), while K306 attachment levels were higher on *Arabidopsis* (25%) than on grapevine roots (15%). HLB2 attached at a higher rate on *Arabidopsis* (26%) while the levels attached on grapevine roots was significantly reduced to 7%. This may be due to host specificity as the strain was originally isolated from hops, not grapevine material. *P. fluorescens* K315 was able to attach to all plant roots, although a high level of bacterial death was observed in the CAS broth. This made evaluation of K315 difficult.

	% attachment on:		
Isolate	*V. vinifera*	*L. esculentum*	*A. thaliana*
E26	15	22	13
F2/5	19	15	21
HLB2	7	12	26
K315	32*	27*	17*
K306	15	19	25
K377	17	18	20
K1072	16	17	21

Table 19: Attachment assays over a one hour period of bacterial isolates with *V. vinifera* (cv. Cabernet Sauvignon), *Lycopersicon esculentum* (cv. Floridade) and *Arabidopsis thaliana* (ecotype Landsberg erecta) in CAS broth. All assays were replicated at least 3 times. * Corresponds to partial death rate of *P. fluorescens* in CAS broth.

7.3.5 Interaction between biocontrol agents and pathogens

Bacteria were tested in the attachment assays using a rifampicin resistant pathogen. Biocontrol strains were added prior to the pathogen and all pathogens selected were

resistant to inhibitory compound(s) produced by the biocontrol strain. The colonisation of cut root surfaces by pathogens in the presence of biocontrol isolates were compared to control assays performed with individual isolates.

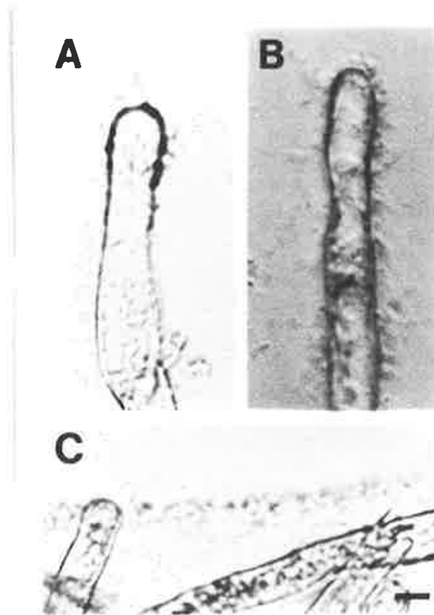
7.3.5.1 Bacterial colonisation of tomato roots

Colonisation of tomato roots showed variable levels of attachment of pathogens in the presence of a biocontrol isolate (Figure 31). Pathogen K377rif^R, was able to attach to the root surface at a level of 18% when inoculated alone. When biocontrol isolate HLB2 was added to the assay, pathogen attachment was significantly reduced from 18% to 7%, a 61% reduction in the level of pathogen able to attach to the plant root. When biocontrol strain F2/5 was added prior to the pathogen, no reduction in attachment was observed ($p=0.86$), indicating that the biocontrol strain did not prevent colonisation of the root surface by pathogenic *A. vitis*. A similar result was obtained for E26 and K315 with no reduction in pathogen attachment in the presence of a biocontrol bacterium.

7.3.5.2 Bacterial colonisation of grapevine roots

Attachment assays were performed on tissue cultured grapevine roots with the addition of biocontrol isolate and a pathogenic *A. vitis*. When HLB2 (*A. tumefaciens* biocontrol) was applied prior to pathogen K377 it reduced pathogen attachment to grapevine roots from 18 to 7 percent (61% reduction) (Figure 32). In all other competition assays, the biocontrol strain did not preclude pathogen attachment to the plant roots. F2/5 and E26 (*A. vitis* biocontrol strains) and K315 (*P. fluorescens* biocontrol strain) did not significantly reduce the level of pathogen attachment to the grapevine roots when compared to the pathogen alone treatments.

I



II

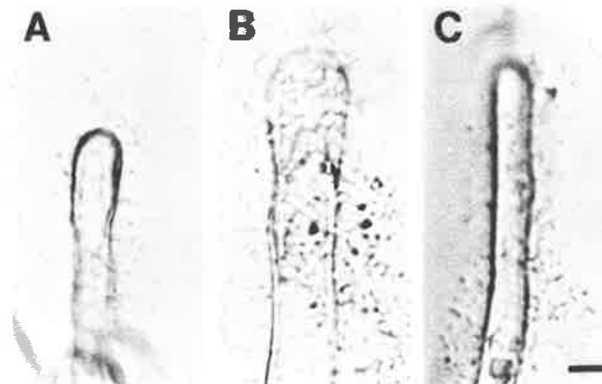


Figure 30 (I and II): Bacterial colonisation of *V. vinifera* (cv. Riesling) plantlet roots demonstrating binding of biocontrol and pathogenic bacteria on root hairs. Bar = 100µm.

I: Biocontrol bacteria A: F2/5 (*A. vitis*), B: K315 (*P. fluorescens*) and C: HLB2 (*A. tumefaciens*).

II: Pathogenic *A. vitis* isolates A: K306, B: K377 and C: K1072.

7.3.6.1 Microscopic observation of bacterial interactions on grapevine

The use of green fluorescent protein (*ngfp*) tagged bacteria allowed the interaction between pathogen and biocontrol to be further investigated. Observed with Nomarski optics, the attachment of K1072*ngfp*2 on grapevine roots (Figure 33) showed little of the trends of attachment of the bacteria and fibril production. When observed using fluorescence microscopy, attachment was displayed with numerous strands of bacteria coating the root tip surface and the high level of attachment (Figure 33). Similar fibrils were observed for other pathogenic *A. vitis* and F2/5 (biocontrol *A. vitis)* but not for E26, or K315. HLB2 (*A. tumefaciens* biocontrol) covered the entire root surface with no observable pattern of colonisation.

When competition assays were performed, roots were observed with fluorescence microscopy. The attachment of K377*ngfp* (pathogen) in the presence of HLB2 (biological control) was observably decreased (Figure 34) when compared to the attachment level of K377 alone. HLB2 reduced attachment of the pathogenic *A. vitis* on grapevine roots. The prevention of attachment was not observed microscopically or reflected in the bacterial counts with F2/5, E26 or K315 biocontrol isolates. All grapevine roots treated with a biocontrol and pathogenic *A. vitis* (F2/5, E26 or K315) had a high level of pathogen present on the root surface.

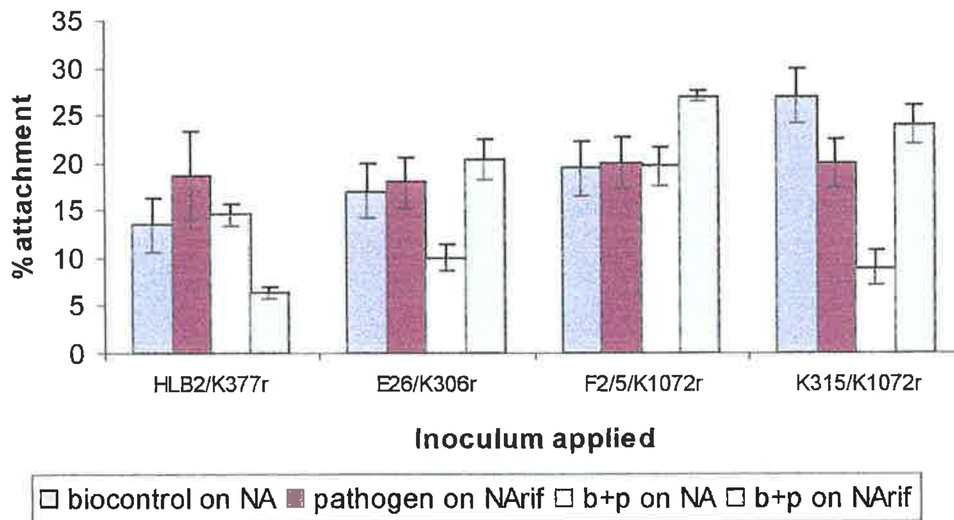


Figure 31: Attachment of biocontrol and pathogenic bacteria in *in vitro* an assay system. Bacteria were inoculated into CAS broth in the presence of tomato 4-day-old roots. Attachment was assessed by determining initial and final bacterial levels in the assay system over a one-hour period (± standard error).

Bars correspond to: 1. Individual biocontrol strain assay plated on NA, 2. Individual rifampicin resistant pathogen plated on NA rifampicin ($100\mu g.ml^{-1}$), 3. Competition combining biocontrol and pathogen in a single assay, plated on NA, 4. Competition combining biocontrol and pathogen in a single assay, plated on NA rifampicin ($100\mu g.ml^{-1}$)

Biocontrol and pathogen combinations

1. HLB2 and K377R
2. E26 and K306R
3. F2/5 and K1072R
4. K315 and K1072R

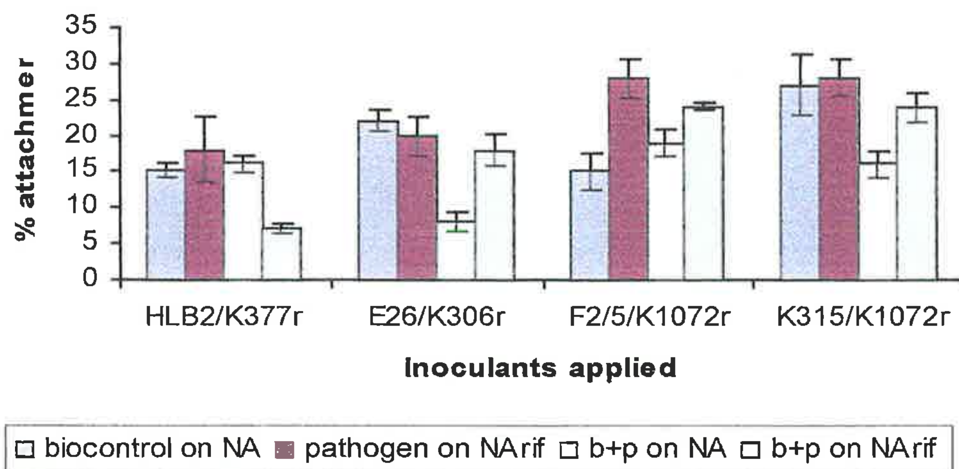


Figure 32: Attachment of biocontrol and pathogenic bacteria in an *in vitro* assay system. Bacteria were inoculated into CAS broth in the presence of *Vitis vinifera (*cv. Cabernet Sauvignon) roots. Attachment was assessed by determining initial and final bacterial levels in the assay system over a one-hour period.

Bars correspond to: 1. Individual biocontrol strain assay plated on NA, 2. Individual rifampicin resistant pathogen plated on NA rifampicin (100µg.ml^{-1}), 3. Competition combining biocontrol and pathogen in a single assay, plated on NA, 4. Competition combining biocontrol and pathogen in a single assay, plated on NA rifampicin (100µg.ml^{-1}) ± standard error.

Biocontrol and pathogen combinations

1. HLB2 and K377R
2. E26 and K306R
3. F2/5 and K1072R
4. K315 and K1072R

Figure 33: Microscopy observation of bacterial colonisation of *Vitis vinifera* (cv. Cabernet Sauvignon) using (a) Nomarski optics and (b) fluorescence microscopy at 485nm excitation wavelength to observe colonisation patterns of K1072*ngfp*.

Figure 34: Microscopic observation of bacterial competition for colonisation of grapevine root material (Cabernet Sauvignon) using a biocontrol isolate HLB2 (*A. tumefaciens) and* K377*ngfp (gfp* tagged K377 *A. vitis* pathogen). Application of HLB2 significantly reduced attachment of K377*ngfp* to the grapevine material.

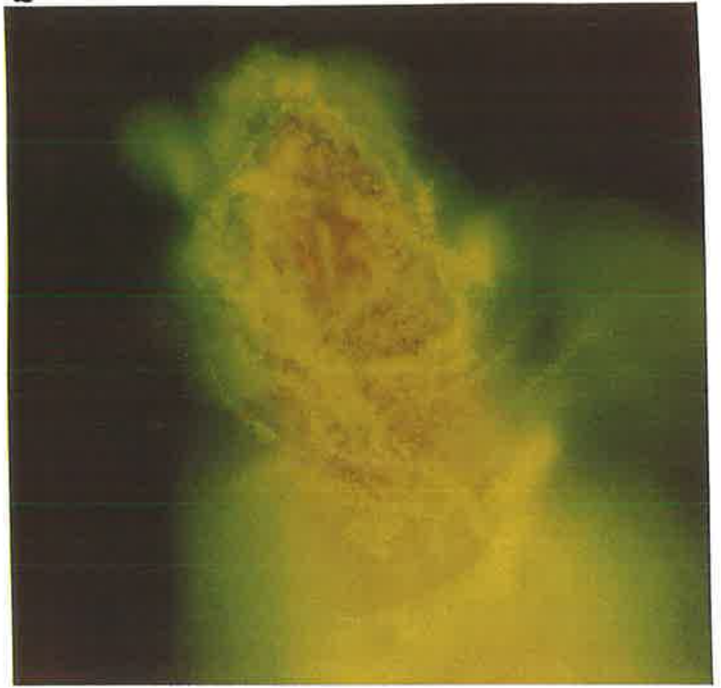
A: K377*ngfp*

B: HLB2

C: Combination of HLB2 and K377*ngfp*.

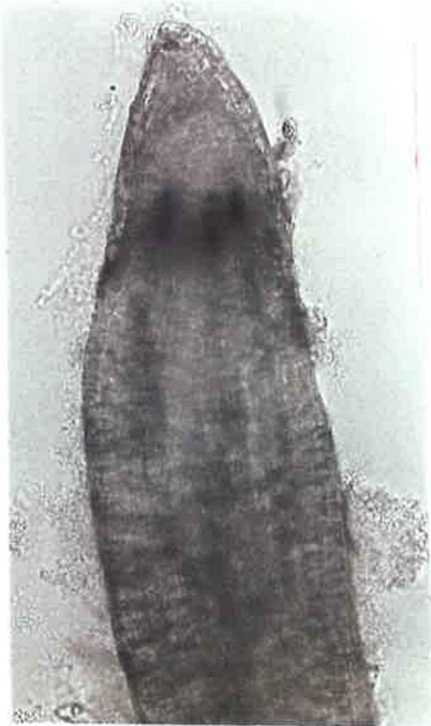
33 a

b



34

a



b

c

7.3.7 Bacterial colonisation of *V. vinifera* plantlets

7.3.7.1 Individual bacterial isolates on plantlets

Biocontrol strains F2/5 (*A. vitis*) and HLB2 (*A. tumefaciens)* and pathogenic *A. vitis* K1072 colonised *V. vinifera* roots over a 10-day period. Each isolate had a lag phase of up to 2 days. After 10 days incubation in the pasteurised soil system, bacterial counts were not significantly different for F2/5 (biocontrol), HLB2 (biocontrol) and K1072 (pathogen) with all bacteria colonising at greater than 10^7 bacteria cm root (Tables 20, 21 and 22). There was a 1,000 to 10,000 times increase in colonisation of loosely bound and tightly bound bacteria for all three strains over 10 days while irreversibly bound levels remained constant from 2-10 days.

After 10 days incubation of plantlets in the presence of F2/5 (biocontrol *A. vitis*) bacterial numbers colonising the plant root surface increased from 10^2 to 10^8 loosely bound bacteria per centimetre of root. An increase in tightly bound bacteria was observed from 10^2 to 10^7 bacteria per centimetre of plant root (Table 20). After roots were washed they were placed on petri-dishes and covered in soft buffered agar. Irreversibly bound bacteria were uniformly distributed along the length of the root with an increase from day 0 to day 10 of 0.07 to 1.55 log units per cm root.

There was a 10,000 times increase bacterial levels over the 10 day period for loosely bound bacteria (/cm root) for HLB2 (Table 21). Numbers of tightly bound bacteria increased 1000 fold, while irreversibly bound bacteria increased from 0.66 log units to1.6 log units per cm root.

Colonisation of the root surface by pathogenic *A. vitis* K1072 resulted in a 10,000 fold increase in both loosely and tightly bound bacteria over the 10 day period (Table 22).

	No. of bacteria.cm^{-1} root (mean log_{10}(±SD))		
Day	Loosely bound	Tightly bound	Irreversibly bound
0	2.18 (0.2)	2.01 (0.4)	0.07 (0.5)
2	4.42 (0.35)	4.14 (0.70)	1.39 (0.11)
5	5.25 (0.22)	4.46 (0.21)	1.42 (0.7)
10	7.38 (0.35)	6.65 (0.19)	1.55 (0.05)

Table 20: Bacterial recovery of strain F2/5 (*A. vitis*) bacteria associated with *V. vinifera* (cv. Cabernet Sauvignon) roots after incubation in pasteurised soil for up to 10 days (± log standard deviation).

	No. of bacteria.cm^{-1} root (mean log_{10}(±SD))		
Day	Loosely bound	Tightly bound	Irreversibly bound
0	3.56 (0.27)	3.10 (0.4)	0.66 (0.1)
2	4.97 (0.18)	3.93 (0.13)	1.4 (0.05)
5	5.31 (0.19)	4.33 (0.28)	1.61 (0.02)
10	7.41 (0.04)	6.8 (0.36)	1.6 (0.03)

Table 21: Bacterial recovery of strain HLB2 (*A. tumefaciens*) bacteria associated with *V. vinifera* (cv. Cabernet Sauvignon) roots after incubation in pasteurised soil for up to 10 days (± log standard deviation).

	No. of bacteria.cm^{-1} root (mean log_{10}(±SD))		
Day	Loosely bound	Tightly bound	Irreversibly bound
0	3.52 (0.19)	3.17 (0.5)	0.72 (0.11)
2	4.93 (0.07)	4.20 (0.11)	1.47 (0.07)
5	5.35 (0.02)	4.48 (0.04)	1.42 (0.02)
10	7.54 (0.27)	7.29 (0.20)	1.33 (0.12)

Table 22: Numbers of strain K1072 (*A. vitis* pathogen) bacteria associated with *V. vinifera* (cv. Cabernet Sauvignon) roots after incubation in pasteurised soil for up to 10 days (± log standard deviation).

Irreversibly bound bacterial levels increased from 0.72 to 1.33 log units per cm root over the 10-day period.

7.3.7.2 Co inoculation of grapevines with biocontrol and pathogenic *A. vitis*

Colonising ability of pathogenic *A. vitis* in the presence of a biocontrol bacterium was tested. When F2/5 was coinoculated with K1072 a significant decrease of 1 to 2 log units was observed for the levels of F2/5 (biocontrol) attachment on the root surface (Table 23) compared to the inoculation of F2/5 alone (Table 20). This was seen in the loosely and tightly bound categories while the irreversibly bound bacterial counts remained constant (Table 23). The level of pathogen attachment decreased by 1 log unit within the loosely and tightly bound bacteria groups (Table 23) when compared to the inoculation of pathogen alone (Table 22). The levels of irreversibly bound bacteria were not significantly different for both biocontrol F2/5 and pathogenic K1072.

Co-inoculation of HLB2 (*A. tumefaciens* biocontrol) and K1072 (*A. vitis* pathogen) showed no significant differences for attachment levels for loosely bound and irreversibly bound bacteria (Table 24) when compared to the inoculation of the strains alone (Tables 21 and 22). Levels of tightly bound bacteria increased by 0.5 log units.

Day	No. of bacteria.cm^{-1} (mean log_{10}(±SD))					
	Loosely bound		Tightly bound		Irreversibly bound	
	F2/5rif	K1072*ngfp*	F2/5rif	K1072 *ngfp*	F2/5rif	K1072 *ngfp*
0	1.44 (0.1)	3.23(0.28)	0.94 (0.1)	2.55(0.1)	0.87(0.2)	0.78(0.1)
2	3.33(0.28)	4.33(0.15)	2.68(0.18)	3.64(0.18)	1.41(0.01)	1.4(0.13)
5	4.39(0.17)	5.43(0.30)	3.71(0.59)	4.96(0.42)	1.4(0.08)	1.29(0.01)
10	6.62(0.33)	7.19(0.18)	5.88(0.34)	6.66(0.26)	1.5(0.05)	1.27(0.1)

Table 23: Bacterial colonisation of *V. vinifera* roots in soil for up to 10 days. Intact plantlets were co-inoculated with biocontrol *A. vitis* (F2/5) and pathogenic *A. vitis* K1072 (± log standard deviation).

Day	No. of bacteria.cm^{-1} (mean log_{10}(±SD))					
	Loosely bound		Tightly bound		Irreversibly bound	
	HLB2rif	K1072*ngfp*	HLB2rif	K1072*ngfp*	HLB2rif	K1072*ngfp*
0	3.2 (0.11)	3.36(0.1)	2.10 (0.1)	2.43(0.2)	0.95(0.2)	0.85(0.2)
2	5.05(0.19)	4.78(0.2)	4.0(0.34)	3.98(0.21)	1.53(0.01)	1.41(0.11)
5	5.24(0.24)	5.10(0.31)	4.01(0.62)	4.05(0.47)	1.53(0.05)	1.28(0.06)
10	7.9(0.41)	7.69(0.34)	7.06(0.36)	6.98(0.2)	1.43(0.09)	1.26(0.06)

Table 24: Bacterial colonisation of V. vinifera roots in soil for up to 10 days. Intact plantlets were co-inoculated with biocontrol *A. tumefaciens* (HLB2) and pathogenic *A. vitis* K1072. Bacteria were recovered from the plantlet roots and counted. (± Log standard deviation).

7.4 DISCUSSION

Bacterial attachment assays were performed on tissue cultured grapevine, tomato and *Arabidopsis*. All biocontrol and pathogenic bacterial strains used in this study demonstrated effective attachment to cut and intact roots of all plant types using the assay system described by Matthysse and McMahan, (1997). Although the rate of attachment varied between plant type and bacterial strain, the preliminary assays using cut tissue culture plantlet roots in calcium sucrose broth, showed a specificity of interaction between plant and bacterium. F2/5 colonisation was highest (based on percentage attachment) on *V. vinifera* and *A. thaliana*, while E26 attachment was highest on tomato. HLB2 showed the greatest affinity for *Arabidopsis* and limited attachment (7% total) on *V. vinifera,* which may be due to its original isolation from hops. All pathogenic *A. vitis* attached at the highest rate to *A. thaliana*, with attachment to grapevine and tomato not significantly different.

The method developed for assaying bacterial attachment to plant hosts gave reproducible results for grapevine, *Arabidopsis* and tomato. All plant systems were useful in determining the level of attachment but ultimately, attachment to grapevine was the most important system for looking at bacterial interaction in crown gall disease on grapevine. Tomato seedlings gave results similar to those of *V. vinifera* and could therefore be utilised as a rapid screening method for studying bacterial interactions on plant roots. These experiments should be followed by assays looking at the specific interaction of the bacterial strains on the plant type of interest, grapevines in this study.

Wild type and rifampicin resistant mutants of the biocontrol and pathogenic *A. vitis* strains were capable of colonising tissue cultured grapevine roots (*Vitis vinifera* cv. Cabernet Sauvignon) at comparable rates when inoculated as a pure culture or in competition assays with plant roots or intact plantlets.

When inoculated in the presence of the biocontrol isolate F2/5, K1072 was able to colonise the plant root system at a comparable rate to F2/5. The pathogen K1072 is resistant to antagonistic compounds produced by F2/5. This indicates that the mode of action of biological control for F2/5 does not rely on antagonistic compounds and that prior colonisation of the grapevine root system by a biocontrol bacterium does not prevent pathogen colonisation. This correlates with previous work published by Burr *et al.*, (1997) that found pathogen colonisation in the presence of a biocontrol strain was not inhibited on wounded grapevine stems for strain F2/5.

Colonisation of the pathogen (K1072) in the presence of the biocontrol isolate HLB2 was significantly reduced on cut grapevine roots (by 61%). K1072 has been previously shown to be susceptible to antagonistic compounds produced by HLB2 indicating that the result extrapolates to *in-planta* and *in-vitro* tests. Pu and Goodman, (1993a and 1993b) showed HLB2 was able to colonise callus culture and cut grapevine roots. When virulent *A. vitis* were included in the system, significant decreases in pathogen levels were observed in a limited exposure time to the biocontrol and pathogen.

Bacteria were able to colonise different parts of the plant root surface including root hairs, epidermis and very extensive colonisation of wound sites. The incorporation of

the green fluorescent protein (GFP) tagging system allowed rapid and unequivocal detection of the bacteria directly on the plant root surface when used in combination with virulent and avirulent bacteria.

Further investigation into the competition between pathogen and biocontrol strains was performed using the GFP genetic marker. The GFP is constitutively expressed and is easily detected using fluorescence microscopy. Competition assays were performed with a combination of tagged and untagged biocontrol and pathogenic bacteria. This assay allowed microscopic observation of the interaction between pathogenic and biocontrol bacteria on cut roots of grapevine and tomato. Exclusion of attachment of the pathogen K377*ngfp* from grapevine and tomato roots was observed when incubated in the presence of HLB2 for up to 48 hours. The surface of the root was covered in non-fluorescent bacteria HLB2 (using fluorescence microscopy). Visualisation of the competing bacteria using Nomarski optics allows the observer to detect bacterial attachment on the root surface but fails to detect different strains applied. The incorporation of the GFP marker allowed direct observation of both biocontrol and pathogen on the plant root surface. Exclusion of the pathogenic strain was not observed for the biocontrol strains F2/5, E26 and K315 when challenged with high levels of the pathogen. In the presence of each of these strains, a high level of pathogen was found on the root surface.

The GFP marker was used for microscopic observation of the interaction between bacteria. The system was also used to perform plate counts using the antibiotic resistance contained on the GFP plasmid. The data observed in plate counts readily

reflected the microscopic observations, with the pathogen present at high levels for F2/5, E26 and K315, while at reduced levels for HLB2 using the cut root assay.

Bacterial colonisation of a growing plant system over a 10 day period was investigated. Much of the previous research done on bacterial attachment of pathogen and biocontrol strains alone or in competition have focussed on short time exposures (0 to 3 hours). A longer period of 10 days was investigated using intact tissue culture plantlets to determine the level of attachment and colonisation of grapevine root systems. Biocontrol strains were tested alone or in competition with pathogenic *A. vitis* on tissue cultured grapevine plantlets. All strains were able to initiate colonisation of the root system with significant increases in bacterial numbers observed (up to a 7 log unit increase over 10 days). The majority of bacteria counted on the root system were loosely or tightly bound to the root surface with a small percentage being irreversibly bound to the root system. Colonisation of F2/5 at the 0 time point showed that a minority of bacteria were irreversibly bound when inoculated alone. When co-inoculated with K1072, the levels of F2/5 increased by 0.8 log units. These levels had equalised when assessed after 2 days incubation. This may demonstrate that F2/5 is able to bind at a higher rate when challenged with a pathogen, although pathogen colonisation of the plant roots is not inhibited by the presence of this biocontrol strain.

The competition assays between HLB2 and the pathogen showed that the pathogen was able to colonise the root system in the presence of HLB2. This is contrary to much of the published research and earlier work in this study that showed when HLB2 was present, pathogen levels were significantly decreased (Pu and Goodman,

1993a, Pu and Goodman, 1993b) in short term experiments. The length of the experiment and the plant type used (intact or cut roots) appears to affect bacterial colonisation. Using a system that more closely reflects planting of callused cuttings into pathogen infested soil has demonstrated that not all biocontrol strains affect colonisation of the root system by pathogens.

The potential exists for many bacteria to be caught in bacterial aggregates as the majority of bacteria are loosely or tightly bound on the grapevine root, not irreversible bound. Initially it was shown that HLB2 covers the entire surface of the grapevine roots in a mucilaginous layer. This layer may prevent pathogen colonisation initially but may not provide ongoing protection to the grapevine. As two of the biocontrol strains are *A. vitis*, there may be the potential for formation of cellulose like fibrils seen in some *A. tumefaciens* (Deasey and Matthysse, 1984) which entrap bacteria close to the root surface. Through the use of the GFP assay system it may be possible to determine the interaction between biocontrol and pathogen on the plant root with further investigation.

F2/5 has been demonstrated to be an effective biocontrol strain, and as such mechanism(s) of biocontrol exists. The prevention of disease initiation may be by alternative modes of action or it may be due to colonisation of specific entry sites which weren't detected in these studies. Studies have shown that prior inoculation of this biocontrol strain protects the grapevine and prevents disease development. Virulence genes have been shown to be induced (Burr *et al.*, 1997) but incorporation of the bacterial DNA into the plant host genome is inhibited. The potential exists for the induction of a plant defence mechanism (systemic induced resistance) for the

control of crown gall. This has not previously been investigated on grapevine. Induced resistance has been demonstrated on grapevine for resistance to powdery mildew under practical cultural conditions (Schoenbeck *et al.*, 1980, Schoenbeck *et al.*, 1982, Weltzien, 1989). Immunisation of the plant is possible through inoculation with pathogens, non-pathogens and treatment with chemical substances which are immunity signals produced by immunised plants or chemicals which release such signals (Kuc, 1990). The potential exists for the induced resistance to protect the plants from a wider range of pathogens than the initial pathogen intended. This requires further investigation into the potential for induced resistance for the control of crown gall disease on grapevine.

CHAPTER 8

GENERAL DISCUSSION

This is the first study of biocontrol of crown gall to be completed in a field nursery site using these potential biocontrol agents. The biocontrol strain F2/5 was shown to be successful in reducing crown gall incidence in the grapevine nursery.

The efficacy of several biocontrol strains in reducing the incidence of crown gall disease was assessed in field trials in a grapevine nursery. The initial trial allowed methods to be developed for subsequent trials. The two successive field trials demonstrated that the *A. vitis* biocontrol strain F2/5 was able to reduce gall frequency by up to 80% over a nine-month period when used together with thermotherapy or by up to 62.5% when used on non heat treated grapevine cuttings. The level of pathogen applied to the callused grapevine cuttings was high (relative to populations normally encountered in the field) with biocontrol strains applied at a higher level with a ratio of >4.3:1 (biocontrol:pathogen). The pathogens were applied directly to the grapevine after treatment with the biocontrol strain. Levels of *A. vitis* in naturally infested soils vary between 10^3 and 10^7 cfu.g^{-1} soil (Burr *et al.*, 1987a) so the levels colonising the callus were significantly higher in these trials. The total number of bacteria attaching to the callus of grapevines in this study was between 10^7 and 10^9 cfu.ml^{-1}. Previous trials have utilised 10^8 cfu.ml^{-1} with an aliquot applied to holes bored in the living woody stem of the plant (Burr and Reid, 1994, Burr *et al.*, 1997) or an aliquot of 10^9 cfu.ml^{-1} bacterial cells when applied to wounds of intact plants (Pu and Goodman, 1993). This trial has used callused cuttings and applied bacteria directly to the callus material, imitating a situation similar to the initial contact

between pathogen free grapevines and pathogenic *A. vitis* may occur in a normal nursery setting.

Previous trials have used plants inoculated with the pathogen at a wounded site on the woody stem, resulting with between 75% and 100% infection depending on the pathogen used and mode of application (Burr and Reid, 1994, Burr *et al.*, 1997, Pu and Goodman, 1993). Many of these trials were maintained under glasshouse conditions or where green shoots or cuttings were used, maintained in the laboratory. Biggs *et al.*, (1994) used similar conditions with vines maintained under glasshouse conditions and this obtained a maximum of 47% gall frequency on pathogen treated vines. This is similar to the results in this study, with the highest rate of galling on pathogen treated vines that was maintained over the life of the trial was 40%. The plants were maintained in a grapevine nursery and subjected to a nursery maintenance regime and environmental stresses not encountered in the glasshouse. The substantial difference between frequency of gall induction in Australia and results gained by Burr and Reid, (1994), Burr *et al.*, (1997) and Pu and Goodman, (1993) may be due to method of application, environmental conditions or climate. Such environmental stresses include freeze injury and the production of aerial galls commonly seen in North America which are generally absent in Australia. Most commonly bacteria were inoculated into a fresh wound site which may allow faster establishment of the infection within the grapevine. Vines in this study were tested for presence of the pathogen within the vascular system. This was confirmed using PCR but vines had no visible symptoms of crown gall disease.

Other biocontrol strains tested reduced gall frequency but were not as reliable or as effective as strain F2/5. HLB2 (*A. tumefaciens* biocontrol) reduced galling by 50% as compared with F2/5 at 62.5% reduction in gall frequency in the 1997-1998 field trial, but did not significantly affect the disease incidence in the 1998-1999 trial where gall frequency was not significantly different to the pathogen only treatment. Pu and Goodman, (1993) found when using HLB2 as a biocontrol agent, inoculation levels must be significantly higher than the pathogen for effective control. To reduce gall formation by >80% the ratio of biocontrol to pathogen was 10,000:1. When lower ratios of biocontrol:pathogen were used, gall frequency was significantly increased (Pu and Goodman, 1993). *A. vitis* E26 had no significant effect on gall frequency in 1997-1998 and so was omitted from further field trial testing. Previous trials had indicated that E26 may be a useful biocontrol strain by reducing gall frequency from 46% to 0% in glasshouse studies (Biggs *et al.*, 1994). *P. fluorescens* K315 reduced galling in the 1997-98 field trial by 36%, and although this was a significant result, it was deemed problematic for general release into the environment as a biocontrol strain (G. Bullard, pers. comm..) due to its links with the human pathogen *P. aeruginosa* and cystic fibrosis infection (Hanna *et al.*, 2000). Only F2/5 and HLB2 biocontrol strains were chosen for further trials because of their significant effect in reducing gall frequency. A combination of the biocontrol strains F2/5 and HLB2 was tested in the third trial (1998-99) but showed no improvement of the protection of the vines from attack by pathogenic *A. vitis* compared to either of the strains when inoculated individually. This combined approach has not previously been attempted and appears that the concentration of HLB2 was the critical factor controlling the success or failure of the combined inoculum, based on the studies of Pu and Goodman

(1993). If the level of HLB2 were to be significantly increased while keeping F2/5 the same, an increase in disease protection might be observed.

Thermotherapy of dormant grapevine cuttings has been shown to reduce the incidence of crown gall disease on grapevine (Ophel *et al.*, 1990). The 1997-98 trial demonstrated that thermotherapy significantly reduced the level of galling by more than 50% on grapevine rootlings in comparison to the non heat-treated grapevines. Although thermotherapy was effective in controlling gall formation in short term experiments, it is known that when planted into pathogen infested soils (10^6 $cfu.g^{-1}$ soil), grapevines quickly become systemically infected within 10 weeks (Bishop *et al.*, 1988). Therefore, thermotherapy is capable of reducing galling provided the vines are planted into pathogen free soil. When planted into pathogen infested soil the vines may become infected in as short a period as 10 weeks, demonstrating the requirement for application of a biological control strain in conjunction with thermotherapy for the most efficient control of the disease. The use of F2/5 in combination with thermotherapy in the 1998-1999 trial resulted in a 15% reduction in gall frequency when compared to non heat-treated grapevines. This further reduction in gall frequency would improve commercial aspects of the nursery by having fewer discards and a higher percentage of marketable material (Bazzi *et al.*, 1999).

Although significant reductions in galling were observed when thermotherapy was used, the grapevine is not protected once it is planted into *Agrobacterium*-infested soil. Based on the data from field trials reported here, the application of the biocontrol strain F2/5 in conjunction with thermotherapy should decrease the

likelihood of crown gall disease development although the pathogen is still able to colonise the grapevine.

Differences in gall distribution on the root system (as measured by scoring the predominant galling position) were observed between the two successful field trials (1997-1998 and 1998-1999). In the 1997-98 trial, the majority of galls occurred mainly at the first node on grapevines treated with a biocontrol strain. When retested in the 1998-99 trial, galls mainly occurred at the crown in the treatments where biocontrol strains were inoculated. With pathogen only treated vines, the galls occurred at the crown in both trials. This difference may be due to the method of application of the pathogen to the vine and the method of callusing. In the 1997-1998 trial grapevine cuttings were callused in sand for a period of greater than 3 months. At planting, many of the vines had started growing by sending out the initial shoots, which is commonly observed on cuttings callused in this manner (induced by heat and allowed to grow for an extended period). When vines were inoculated and planted, the shoots died due to sensitivity to ultra violet light (from the sun) or were damaged through handling. This may have allowed the pathogen to attach to wounded sites on the grapevines and allowed initiation of infection at the first node. This effect was not observed in the 1998-99 trial, as vines were callused for only 2 weeks, inoculated and planted immediately. Plant growth and shoot development were not induced as the callus development time was significantly less than in previous trials.

A recommendation arising from this work is that the biocontrol strain F2/5 should be used in conjunction with thermotherapy treatment of 50°C for 20 minutes for control of crown gall disease. The biocontrol should be applied to heat treated callused

cuttings immediately prior to planting. This method significantly reduced galling levels by up to 80% (15% greater reduction than observed with non heat-treated or 10 minute heat-treated vines). Thermotherapy ensures the removal of any pathogens (to undetectable levels) contained within the grapevine material and allows the development of disease free rootlings when the biocontrol strain F2/5 is applied. Other pathogens that may be present within the vascular system or on the bark of the cutting (eg. fungal spores) may be inactivated by the thermotherapy treatment reducing the pathogen load carried by the grapevine. Although 20% of vines treated with F2/5 and thermotherapy had galls, the dose of pathogen encountered in the third field trial was significantly higher than normally encountered in the field. Therefore, treatment under 'normal' conditions should result in a significant decrease in losses due to crown gall disease in grapevine nurseries. Grapevine cuttings treated with F2/5 can be planted into soil with previous exposure to *A. vitis* as the biocontrol strain F2/5 is able to protect vines from induction of the disease process. This is important for both nursery sites and replanting on sites where diseased plants previously stood. Nursery sites can be replanted reducing the necessity to obtain land without prior exposure to grapevines. Vines that were removed due to crown gall disease can be replaced by new vines that are protected from crown gall disease by the presence of the biocontrol F2/5.

In Australia, potential infection of the vines may occur through injury on aerial sections of the vines though pruning injury. Much of the disease seen overseas is due to freezing injury which is not a common occurrence in Australia. The maintenance of a biocontrol strain within the vascular system is of greater importance in areas where freeze injury occurs. Currently, the length of time that the biocontrol strain

remains within the grapevine is unknown. Further testing is required using a long term field trial that incorporates the biocontrol strains on grapevines that can be grown for a number of years to determine the extent of protection. Inoculated vines could be easily tested for the presence of the biocontrol strain by testing vines after spring growth has occurred. Bacteria are known to travel within the vascular system of the grapevine and are present in older shoots (Burr *et al.*, 1987). Ideally replanting of vines used in these field trials would give a good indication of whether the biocontrol strains were maintained within the grapevine vascular system.

Testing of mother vines for *A. vitis* before cuttings are taken would help to ensure vines are disease free on planting. This would reduce the risk of transferring crown gall disease between vineyards. Vines can be tested using the PCR test (developed by Eastwell *et al.*, 1995) to detect pathogenic *A. vitis* in dormant vines. Although no vitopine strains have been detected in Australia to date, this requires further investigation as it would be anticipated that these strains do exist in Australian vineyards. If these strains are detected an alternative set of primers would be required that can identify vitopine strains such as those described by Burr and Otten, (1999).,. The incorporation of an internal positive control (IPC) fragment into the PCR would confirm the PCR reaction where negative results were obtained showing that the reaction was not inhibited by the presence of inhibitory compounds. This would require the selection of an appropriate IPC and this would need to be optimised within the PCR reaction. The potential exists to incorporate standards for the PCR to determine the severity of infection within the grapevine cuttings and soil. Suitable vines could then be callused and inoculated with the biocontrol strain for protection from infection and reduce stock losses from crown gall. The development of the PCR test allows faster and more reliable detection of *A. vitis* within grapevine samples.

The advantage of PCR is the ability to avoid the need to culture onto semi-selective media then positively identify bacteria using the monoclonal antibody.

Attachment and the interaction of biocontrol and pathogenic strains on plant systems were investigated as potential mechanisms of control. Pathogen colonisation in the presence of a biocontrol strain was tested on cut plant roots including grapevine, tomato and *Arabidopsis* with one strain genetically marked with GFP. When F2/5 was used as the antagonist, pathogen levels were not significantly different to pathogen alone application showing that the mode of action was not blocking of potential binding sites. This is in agreement with the work of Burr *et al.*, (1997) who used sections of green actively growing shoots. When HLB2 was applied a significant reduction in pathogen levels was observed indicating that HLB2 prevented pathogen colonising of the cut plant root surface.

This method was also applied to inoculation of intact tissue cultured plantlets using a rifampicin resistant mutant with some conflicting results. F2/5/pathogen colonisation was similar to that seen on the cut roots. HLB2/pathogen inoculation resulted in no observable difference between pathogen and biocontrol strains, contrary to results in the cut root assays. This indicated a difference between the cut root and intact plants assay systems. Further investigation into the colonisation of the root system with competing biocontrol and pathogenic strains using the GFP genetic marker would give valuable information regarding the colonisation of the plant root system over the 10day period. The GFP assays thus far were performed using cut roots and microscopically observing the bacterial interactions with Nomarski and fluorescence

microscopy. Application of this method to a soil system would allow a greater understanding of the biocontrol/pathogen interaction.

The presence of high levels of pathogen in HLB2 treated vines was reflected in results obtained from sap extractions and quantitation of the PCR product. All vines tested had high levels of pathogenic *A. vitis* present in the sap in the presence of the biocontrol strains.

Further investigation into the mechanism of control of F2/5 is required as it has been shown that attachment, competition for binding sites (this study, Burr *et al.*, 1997) and agrocin production are not the major mechanisms for biocontrol activity (Burr *et al.*, 1997). As disease control is optimised by prior inoculation of the vines with the biocontrol strain, the role of systemic induced resistance also requires some investigation. Prior inoculation of the vine with a non pathogenic strain may protect the plant by induction of a plant defence mechanism which so far has not been examined for crown gall of grapevine. If this is the case the potential exists for the induced resistance to protect the plant from a wider range of pathogens than crown gall alone. This would be tested on F2/5 inoculated vines through a series of challenges with other typical grapevine diseases such as fungal spores.

Through this study F2/5 has been tested in field trials and shown to be extremely effective at controlling crown gall disease in grapevine nurseries. The potential exists for this strain to be further evaluated by testing biocontrol efficacy in a number of field trials Australia wide. As a direct result of this research, a peat inoculum is currently available from Bio-Care Technology Pty. Ltd. (Somersby, N.S.W.

Australia) for trial on grapevines for the protection from crown gall disease. This would show the efficacy of the biocontrol strain for crown gall disease control through a range of environmental conditions.

Before commercial scale is investigated it would be necessary to determine the stability of the plasmids within F2/5, and to determine whether plasmid(s) could be transferred to sensitive *A. vitis* pathogens allowing them to become resistant to biocontrol activity. It would also be necessary to ensure that plasmid transfer into the biocontrol does not occur to prevent it becoming pathogenic. This could be done by checking ERIC-PCR profiles or other characteristics and plasmid content of strains isolated from tumours obtained in trials performed with this strain and *A. vitis* pathogens of known pattern. The use of this biocontrol strain may allow replanting of vines into pathogen infested soils and in conjunction with thermotherapy develop a stock of grapevines within Australia that are disease free.

APPENDIX 1: CULTURE MEDIA

1. Nutrient Broth/Agar

Difco nutrient broth	
Beef extract	$3gL^{-1}$
Yeast extract	$5\ gL^{-1}$

For agar add $15gL^{-1}$ Difco Bacto agar

2. Tryptone Yeast agar

Tryptone	5g
Yeast extract	3g
$CaCl_2.6H_2O$	1.3g
Bacto agar	15g

Distilled water to 1 litre

3. Luria Bertani broth

NaCl	5g
Yeast extract	10g
Bacto tryptone	10g

Distilled water to 1 litre

4. AB minimal agar

Solution1:

Bacto agar	15g
Glucose	10g

Make up to 900ml with distilled water

20x Buffer solution adjusted to pH 7.27

K_2HPO_4	20g
$NaH_2PO_4.2H_2O$	23g

Make up to 1 litre with distilled water

20x salts solution

NH_4Cl	20g
$MgSO_4.7H_2O$	12.3g
KCl	3g
$CaCl_2.2H_2O$	0.2g
$FeSO_4.7H_2O$	0.5g

Sterilise all solutions. For 1 litre of AB medium add 900ml solution1, 25ml 20x buffer solution and 25ml 20x salts solution aseptically. Allow to cool to 50°C and add biotin at $2\mu gml^{-1}$.

5. RS medium (Roy and Sasser, 1983)

$MgSO_4.7H_2O$	0.2g
K_2HPO_4	0.9g
KH_2PO_4	0.7g
Adonitol	4.0g
Yeast extract	0.14g
NaCl	0.20g
Boric acid	1.0g
Bacto agar	15g
pH to 7.2	

6. Biovar 1 medium (Kerr and Brisbane, 1983)

L-arabitol	3.04g
NH_4NO_3	0.16g
KH_2PO_4	0.54g
K_2HPO_4	1.04g
$MgSO_4.7H_2O$	0.25g
Sodium taurocholate	0.29g
Crystal violet (0.1%)	2ml
Bacto agar	15g
Distilled water to 1L	

Per 100ml add:

Cycloheximide (2%)	1ml
$Na_2SeO_3.5H_2O$ (1.0%)	1ml

7. Biovar 2 medium (Kerr and Brisbane, 1983)

erythritol	3.05g
NH_4NO_3	0.16g
KH_2PO_4	0.54g
K_2HPO_4	1.04g
$MgSO_4.7H_2O$	0.25g
Sodium taurocholate	0.29g
Yeast extract (1%)	1ml
Malachite green (0.1%)	5ml
Bacto agar	15g
Distilled water to 1L	

Per 100ml add:

Cycloheximide (2%)	1ml
$Na_2SeO_3.5H_2O$ (1.0%)	1ml

8. Biovar 3 medium ((Kerr and Brisbane, 1983)

A. sodium tartrate 5.75g
D-glutamic acid (4% solution, pH7.0) 15ml
$NaH_2PO_4.2H_2O$ 6.25g
Na_2HPO_4 4.26g
NaCl 5.84g
$MgSO_4.7H_2O$ 0.25g
Sodium taurocholate 0.29g
Yeast extract (1%) 1ml
Congo red (1%) 2.5ml
Distilled water to 500ml

B. $MnSO_4.4H_2O$ 1.12g
Bacto agar 5g
Distilled water to 500ml

Autoclave separately. Add 50ml A to 50ml B and add:
Cycloheximide (2%) 1ml
$Na_2SeO_3.5H_2O$ (1.0%) 1ml

9. Kings B medium (King *et al.*, 1954)

Difco proteose peptone No.3 20g
K_2HPO_4 0.2g
$MgSO_4.7H_2O$ 1.5g
Glycerol 10ml
Bacto agar 15g
Distilled water to 1 litre, pH to 7.2

10.Potato Dextrose agar

11. Stonier's medium (Stonier, 1960)

potassium citrate 10g
NH_4NO_3 2.7g
L-glutamic acid 2.0g
K_2HPO_4 0.88g
NaH_2PO_4 0.3g
$MgSO_4.7H_2O$ 0.2g
NaCl 0.2g
$CaSO_4$ 0.1g
$Fe(NO_3)_3$ 5.0mg
$MnCl_2$ 0.1mg
$ZnCl_2$ 0.5mg
Biotin 0.2g
Bacto agar 15g
Distilled water to 1 litre, pH to 7.0

12. Water Agar

Difco Bacto agar 7g
Distilled water to 1 litre

13. *Arabidopsis* tissue culture media

MS salts 1x	
Gambourg's B5 vitamin mixture 1x	
Sucrose	2%
Phytagar	0.8%

14. Grapevine tissue culture rooting media

½ MS Salts and MS vitamins (Sigma)	
Phytagel	2.5-3.0g
Sucrose	2%
α-napthaleneacetic acid	0.05μM

APPENDIX 2: SOLUTIONS AND BUFFERS

1. Solutions for plasmid isolation and visualisation
    a. TE8 buffer: 50mM Tris-HCl, 20mM EDTA, pH8.0.
    b. Solution 1: 50mM glucose, 25mM Tris, 10mM EDTA, pH8.0.
    c. Solution 2: 0.2N NaOH, 1% SDS, made immediately before use.
    d. Solution 3: 3M sodium acetate: 60ml 5M KCH3COO, 11.5ml glacial acetic acid, 28.5ml water.
    e. Electrophoresis buffer: Tris 10.78g, boric acid 5.5g, disodium EDTA 0.93g to 1 litre of water.

2. Phosphate buffered saline: 8.5g NaCl,100ml phosphate buffer, add water to 1 litre.

3. Phosphate buffer: 39ml 0.2M NaH_2PO_4, 61ml 0.2M Na_2HPO_4, pH to 7.3.

Bibliography

Astrom, B. (1991) Role of bacterial cyanide production in differential reaction of plant cultivars to deleterious rhizosphere pseudomonads. *Plant and Soil* 133: 93-100.

Baldini, E., and Interneri, C. (1982) Current position on mechanisation of harvesting and pruning in grapevine. *Informatore Agrario* 38(39): 22701-22737.

Bauer, C., Schulz, T.F., Lorenz, D. Eichhorn, K.W., and Plapp, R. (1994) Population dynamics of *Agrobacterium vitis* in two grapevine varieties during the vegetation period. *Vitis.* 33:25-29.

Bazzi, C., Alexandrova, M., Stefani, E., Anaclerio, F., and Burr, T.J. (1999) Biological control of *Agrobacterium vitis* using non tumorigenic agrobacteria. *Vitis* 38(1): 31-35.

Bazzi, C., and Rosciglione, B. (1982) *Agrobacterium tumefaciens* biotype 3, causal agent of crown gall on *Chrysanthemum* in Itali. *Phytopathologische Zeitscrift* 103: 280-284.

Bazzi, C., Piazza, C., and Burr, T.J. (1987) Detection of *Agrobacterium tumefaciens* in grapevine cuttings. *Bulletin OEPP/EPPO* 17:105-112.

Bazzi, C., Stefani, E., Gozzi, G., Burr, T.J., Moore, C.L., and Anaclerio, F. (1991) Hot water treatment of dormant grapevine cuttings: Its effects on *Agrobacterium tumefaciens* and on grafting and growth of
vines. *Vitis* 30: 177-187.

Belanger, C., Canfield, M.R.L., Moore, L.W. and Dion, P. (1995) Genetic analysis of non-pathogenic *Agrobacterium tumefaciens* mutants arising in Crown Gall tumors. *J. Bacteriol.* 177(13): 3752-3757.

Bell, C. R., Dickie, G.A., and Chan, J. W. (1995) Variable response of bacteria isolated from grapevine xylem to control grape crown gall disease *in planta. Am. J. Enol. Vitic.* 46(4): 499-508.

Bertani, G., (1952) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli. Journal of Bacteriology* 62: 293-300.

Biggs, J. G., (1994) Ecology and biological control of *Agrobacterium vitis*, the grapevine crown gall pathogen. PhD Thesis, The University of Adelaide, (Faculty of Agriculture and Natural Resources) South Australia, Australia.

Biggs, J.G., McClure, N.C., Murphy, P.J., Ryder, M. H., and Kerr, A. (1994) Monitoring effects of different potential biocontrol strains on gall formation and population density of a transposon- tagged *Agrobacterium vitis* pathogen. In: Ryder, M.H., P.M. Stephens and G.D. Bowen (eds.) Improving plant productivity with

rhizosphere bacteria. International workshop on plant growth-promoting rhizobacteria (3rd: 1994: Adelaide, S. Aust.). pp 195-197.

Binns, A.N., and Thomashow, M.F. (1988) Cell biology of *Agrobacterium* infection and transformation of plants. *Annu. Rev. Microbiol.* 42: 576-606.

Bishop, A. L., Katz, B.H., and Burr, T.J. (1988) Infection of grapevines by soil-borne *Agrobacterium tumefaciens* biovar 3 and population dynamics in host and non-host rhizospheres. *Phytopath.* 78(7): 945-948.

Bishop, A.L., Burr, T.J., Mittak, V.L., and Katz, B.H. (1989) A monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3 and its utilisation for indexing grapevine propagation material. *Phytopathology* 79: 995-998.

Bouzer, H., Chilton, W.S. Nesme, X., Dessaux, Y., Vaudequin, V., Petit, A., Jones, J.B., and Hodge, N.C. (1995) A new *Agrobacterium* strain isolated from aerial tumors on *Ficus benjamina* L. *Appl. Environ. Microbiol.* 61(1): 65-73.

Brisbane, P.G., and Kerr, A. (1983) Selective media for three biovars of *Agrobacterium. J. Appl. Bacteriol.* 54: 425-431.

Brisset, M-N., Rodriguez-Palenzuela, P., Burr T.J., and Colmer, A. (1991) Attachment, chemotaxis, and the multiplication of *Agrobacterium tumefaciens* Biovar 1 and Biovar 3 on grapevine and Pea. *Appl. Environ. Microbiol.* 57(11): 3178-3182.

Buckholtz, W.B., and Thomashow, M.F. (1984) Host range encoded by the *Agrobacterium tumefaciens* tumor-inducing plasmid can be expanded by modifications of its T-DNA oncogene complement. *J. Bacteriol.* 160: 327-332.

Burr, T. J., Bishop, A.L., Katz, B.H., and Mittak, L. (1988) Production and use if a monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3. *Phytopath.* (Abstr.): 1506.

Burr, T. J., Reid, C.L., Yoshimura M., Momol, E.A., and Bazzi, C. (1995) Survival and tumorigenicity of *Agrobacterium vitis* in living and decaying grape roots and canes in soil. *Plant Disease.* 79:677-682.

Burr, T.J., and Hurwitz, B. (1981) Occurrence of *Agrobacterium radiobacter* pv. *tumefaciens* (Smith and Townsend) Conn biotype 3 on grapevine in New York State. (Abstract) *Phytopathology* 71: 206.

Burr, T.J., and 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., and Katz, B.H. (1983) Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap, and from vineyard soil. *Phytopath.* 73(2): 163-165.

Burr, T.J., and Otten, L. (1999) Crown gall of grape: Biology and disease management. *Annu. Rev. Phytopathol.* 37: 53-80.

Burr, T.J., and Reid, C.L. (1994) Biological control of grape crown gall with tumorigenic *Agrobacterium vitis* strain F2/5. *Am. J. Enol. Vitic.* 45(2): 213-219.

Burr, T.J., Bishop, A.L., Katz, B.H., Blanchard, L.M., and Bazzi, C. (1987b) A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. *Phytopath.* 77: 1424-1427.

Burr, T.J., Katz, B.H., and Bishop, A. L. (1987a) Populations of *Agrobacterium* in vineyard and non-vineyard 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., and Mittak, L. (1987c) Effect of shoot age on systemic infestation of grape by *Agrobacterium tumefaciens* biovar 3 and production of pathogen-free vines by shoot-tip culture. *Am. J. Enol. Vitic.* 39: 67-70.

Burr, T.J., Norelli, J.L., Katz, B.H., and Bishop, A.L. (1990) Use of Ti plasmid DNA probes for determining tumorigenicity of *Agrobacterium* strains. *Appl. Environ. Microbiol.* 56(6): 1782-1785.

Burr, T.J., Ophel, K., Katz, B.H., and Kerr, A. (1989) Effect of Hot water treatment on systemic *Agrobacterium tumefaciens* biovar 3 in dormant grape cuttings. *Plant Disease* 73(3): 242-245.

Burr, T.J., Reid, C.L., Splittstoesser, D.F., and Yoshimura, M. (1996) Effect of heat treatment on grape bud mortality and survival of *Agrobacterium vitis in vitro* and in dormant grape cuttings. *Am. J. Enol. Vitic.* 47(2): 119-123.

Burr, T.J., Reid, CL., Tagliati, E., Bazzi, C., Sule, S. (1997) Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. *Phytopathology* 87(7): 705-711.

Chalfie, M., Tu, Y., Euskirchen, G., ward, W.W., and Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science* 263: 802-805.

Chen, X., and Xiang, W. (1986) A strain of *Agrobacterium radiobacter* inhibits growth and gall formation by biotype 3 strains of *A. tumefaciens* from grapevine. *Acta Microbiologica Sinica* 26(3): 193-199.

Chilton, M.D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P., and 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., (1993) *Agrobacterium*: Biological Plant Disease Control. In: Kim, L. (ed.) Advanced Engineered Pesticides. Marcel Dekker, Inc. New York. pp129-146.

Clare, B.G., Kerr, A. and 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.

Cook, J.R. (1994) Biological control of plant pathogens: Theory to application. *Phytopath.* 75(1): 25-29.

Cook, R.J., and Baker, K.F. (1983) The nature and practice of biological control of plant pathogens. Am. Phytopath. Soc., St Paul, MN.

Cooksey, D.A., and Moore, L.W. (1980) Biological control of crown gall with fungal and bacterial antagonists. *Phytopathol.* 70: 506-509.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996) FACS optimised mutants of the green fluorescent protein (GFP). *Gene* 173: 33-38.

Das, P.K., Basu, M., and G.C. Chatterjee (1978) Studies on the mode of action of agrocin 84. *J. Antibiotics* 31: 490-492.

de Bruijn, F.J., (1987) *Tn5* mutagenesis to map genes. *Methods in Enzymology* 154: 175-196.

de Bruijn, F.J., (1992) Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergeneric Consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl. Environ. Microbiol.* 58(7): 2180-2187.

De Cleene, M., and De Ley, J. (1976) the host range of Crown Gall. *The Botanical Review* 42(4): 389-466.

DeLorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. Mini Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J. Bacteriol.* 172(11): 6568-6572.

Donner, S.C., Jones, D.A., McClure, N.C., Rosewarne, G.M., Tate, M.E., Kerr, A., Farjardo, N.N., and 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.

Eastwell, K.C., Willis, L.G., and Cavileer, T.D. (1995) A rapid and sensitive method to detect *Agrobacterium vitis* in grapevine cuttings using the polymerase chain reaction. *Plant Disease* 79: 822-827.

Ellis, J.G., and Murphy, P.J. (1981) Four new opines from crown gall tumours-Their detection and properties. *Mol. Gen. Genet.* 181: 36-43.

Elmer, W.H. (1995) Association between Mn-reducing root bacteria and sodium chloride applications in suppression of *Fusarium* crown rot of asparagus. *Phytopath.* 85: 1461-1467.

Fajardo, N.N., Tate, M.E., and Clare, B.G. (1994) Agrocin 434: an additional biological control component for crown gall. In. Improving plant productivity with rhizosphere bacteria. International workshop on plant growth-promoting

rhizobacteria (eds. Ryder, M.H., P.M. Stephens and G.D. Bowen) CSIRO Division of Soils, Adelaide, Australia. pp128-130.

Farrand, S.K., (1990). *Agrobacterium radiobacter* strain K84: A model biocontrol system. New Directions in Biological Control: Alternatives for suppressing Agricultural Pests and Diseases, pp 679-691. Alan R. Liss, Inc.

Farrand, S.K., Slota, J.E., Shim, J.-S., and Kerr, A. (1985) Tn5 insertions in the agrocin84 plasmid: the conjugal nature if pAgK84 and the locations of determinants for transfer and agrocin 84 production. *Plasmid* 13: 106-117.

Farrand, S.K., Wilson, M., Lindow, S.E., and Savka, M.A. (1994) Modulating colonisation by plant associated microbes. In: Improving plant productivity with rhizosphere bacteria. International workshop on plant growth-promoting rhizobacteria (eds.Ryder, M.H., P.M. Stephens and G.D. Bowen) CSIRO Division of Soils Adelaide, Australia. pp223-237.

Fermin, J.L., and Fenwick, R.G. (1978) A major new plasmid determined metabolite in crown gall tumours. *Nature* (London) 276: 842-844.

Ferriera, J.H.S., and van Zyl, F.G.H. (1986) Susceptibility of grapevine rootstock's to strains of *Agrobacterium tumefaciens* biovar 3. *S. Afr. J. Enol. Vitic.* 7(2): 101-104.

Ferris, R.S. (1984) Effects of microwave oven treatment on microorganisms in soil. *Phytopath.* 74: 121-126.

Fortin, C., Marquis, C., Nester, E.W., and Dion, P. (1993) Dynamic structure of *Agrobacterium tumefaciens* Ti plasmids. *J. Bacteriol.* 175(15): 4790-4799.

Fridlender, M., Inbar, J., and Chet, I. (1993) Biological control of soil borne pathogens by a B-1,3-glucanase producing *Pseudomonas cepacia. Soil Biology and Biochemistry* 25: 1211-1221.

Gallie, D.R., Zaitlin, D., Perry, K.L., and Kado, C.I. (1984) Characterisation of the replication and stability regions of *Agrobacterium tumefaciens* plasmid pTAR. *J. Bacteriol.* 157(3): 739-745.

Gerard, JC., Canaday, J., Szegedi, E., de la Salle, H. and Otten, L. (1992) Physical map of the vitopine Ti plasmid pTiS4. *Plasmid* 28: 146-156.

Gillings, M., and Ophel-Keller, K. (1995) Comparison of strains of *Agrobacterium vitis* from grapevine source areas in Australia. *Australasian Plant Pathol.* 24: 29-37.

Goodman, R.N., Butrov, D., and Tarbah, F. (1987) The occurrence of *Agrobacterium tumefaciens* in grapevine propagating material and a simplified indexing system. *Am. J. Enol. Vitic.* 38(3): 189-193.

Grinter, N.J., (1983) A broad host range cloning vector transposable to various replicons. *Gene* 21: 133-143.

Hamdan, H., Weller, D.M., and Thomashow, L.S. (1991) Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. tritici by *Pseudomanas fluorescens* 2-79 and M4-80R. *Appl. Environ. Micro.* 57: 3270-3277.

Hanna, S.L., Sherman, N.E., Kinter, M.T., and Goldberg, J.B. (2000) Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary columns liquid chromatography0tandem mass spectrometry. *Microbiology* 146: 2495-2508.

Hemstad, P.R., and Reisch, B.I. (1985) *In vitro* production of galls induced *by Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* on *Vitis* and *Rubus. J. Plant Physiol.* 120: 9-17.

Herrero, M., De Lorenzo, V., and Timmis, K. (1990) Transposon vectors containing non- antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* 172(11): 6557-6567.

Holmes, B., and Roberts, P. (1981) The classification, identification and nomenclature of agrobacteria. *J. Appl. Bact.* 50: 443-467.

Huber, D.M., and McCay-Buis, T.S. (1993) A multiple component analysis of the take-all disease of cereals. *Plant Dis.* 77: 437-447.

Hwang, I., Cook, D.M., and Farrand, S.K. (1995) A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. *J. Bacteriol.* 177(2): 449-458.

Jackson, R.S., (1994) Wine Science: Principles and Applications. Academic Press, USA.

Jager, J., Lorenz, D.H., and Eichhorn, K.W., (1991) The suppression of *Agrobacterium tumefaciens* biovar 3 populations in grapevine crown gall tumors and tissue of crown gall diseased grapevines by the influence of non-pathogenic Agrobacteria of the biovar 1. *Vitic. Enol. Sci.* 45: 33-40.

Jin, S., Komari, T., Gordon, M.P., and Nester, E.W. (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A218. *J Bacteriol.* 169: 4417-4425.

Jones, J.B., (1988) Systemic movement of *Agrobacterium tumefaciens* in symptomless stem tissue of *Chrysanthemum morifolium. Plant Disease* 72: 51-54.

Keegan, M. (1997) Characterisation of *A. vitis* strains with regard to virulence and DNA transfer. Honours Thesis, The University of Adelaide, Department of Horticulture, Viticulture and Oenology. South Australia, Australia.

Keen, N.T., Tamaki, S., Kobayoshi, D., and Trollinger, D. (1988) Improved broad host range plasmids for DNA cloning in Gram negative bacteria. *Gene* 70: 191-197.

Kerr, A. (1980) Biological control of crown gall through production of agrocin 84. *Plant Disease* 64: 25-30.

Kerr, A., (1989) Commercial release of a genetically engineered bacterium for the control of crown gall. *Agric. Sci.* 2(6): 41-44.

Kerr, A., (1992) The genus *Agrobacterium.* In: The Prokaryotes, 2nd Edition, Vol 3. (Eds. A. Balows, H.G. Truper, M. Dworkin, W. Harder and K-H. Schleifer) pp2214-2235. Springer-Verlag, New York.

Kerr, A., and Ellis, J.G. (1982) Conjugation and transfer of Ti plasmid in *Agrobacterium tumefaciens.* In: Molecular Biology of plant tumors. Eds. G. Kahl and J.S. Schell. Academic Press, New York. pp321-344.

Kerr, A., and Panagopoulos, G.C. (1977) Biotypes of *Agrobacerium radiobacter* var. *tumefaciens* and their biological control. *Phytopathologische Zeitschrift* 90: 172-179.

Kerr, A., and Tate, M.E. (1984) Agrocins and the biological control of crown gall. *Microbiol. Sci..* 1: 1-4.

Kersters, K., and De Ley, J. (1984) Genus 111 Agrobacterium *Conn* 1942, 359. In: Bergeys Manual of Systematic Bacteriology, Volume 1. (Eds. Kreig, N.R., and Hott, J.G.) Williams and Wilkins, USA.

Klapwijk, P., and Schilerperot, R. (1978) Coordination regulation of octopine degradation and conjugative transfer of Ti plasmids in *Agrobacterium tumefaciens*: evidence for a common regulatory gene and separate operons. *J. Bacteriol.* 136: 775-785.

Kloepper, J.W., Schippers, B., and Bakker, P.A.H.M. (1992) Proposed elimination of the term endorhizosphere. *Phytopathol.* 82: 726-727.

Knauf, V.C., Panagopoulos, C.G., and Nester, E.W. (1983) Comparison of Ti plasmids for three different biotypes of *Agrobacterium tumefaciens* isolated from grapevines. *J. Bacteriol.* 153(3): 1535-1542.

Kuc, J. (1990) A case for self defence in plants against disease. *Phyto parasitica* 18: 1.

Leach, J.E., Ramundo, B.A., Pearson, D.L., and Claffin, L.E. (1987) Dot immunobinding assay for detectiong *Xanthomonas campestris* pv. *Holcicola* in sorghum. *Plant Disease* 71: 31-33.

Lehoczky, J. (1968) Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine, after natural infection. *Phytopath. Z.*, 63: 239-246.

Lehoczky, J., (1978) Root system of the grapevine as a reservoir of *Agrobacterium tumefaciens* cells. *Proc. 4th. Int. Conf. Plant Path. Bact.* - Angers. pp239-243.

Leong, J. (1986) Siderophores: Their biochemistry and possible role in the biological control of plant pathogens. *Ann. Rev. Phytopathol.* 24: 187-209.

Leroux, B., Yanofsky, M.F., Winans, S.C., Ward, J.E., Zeigler, S.F., and Nester, E.W. (1987) Characterisation of the *virA* locus of *Agrobacterium tumefaciens*: a transcriptional regulator and host range determinant. *EMBO. J.* 6: 849-856.

Liang, Y-J., Di, Y.B., Zhao, J-Y., and Ma, D-Q. (1990) Strain E26 of *Agrobacterium radiobacter* efficiently inhibits crown gall formation on grapevines. *Chinese Science Bulletin* 12: 1055-1056.

Lippincott, B.B., and Lippincott, J.A. (1969) Bacterial Attachment to a specific wound site as an essential stage in tumour initiation by *Agrobacterium tumefaciens. J. Bacteriol.* 97: 620-628.

Lippincott, J.A., and Lippincott, B.B. (1980) Microbial Adherence in Plants. In: Bacterial Adherence Series B. Volume 6. (Ed: E. Beachey) Chapman and Hall, London.

Loper, J.E., and Kado C.I. (1979) Host range conferred by the virulence specifying plasmid of *Agrobacterium tumefaciens. J. Bacteriol.* 139: 591-596.

Lopez, M.M., Gorris, M. T., Salcedo, C.I., Montojo, A.M. and Miro, M. (1989) Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistance to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. *Appl. Environ. Microbiol.* 55(3): 741-746.

Ma, D-Q., Yanofsky, M.F., Gordon, M.P., and Nester, E. (1987). Characterization *of Agrobacterium tumefaciens* strains isolated from grapevine tumors in China. *Appl. Environ. Microbiol.* 53(6): 1338-1343.

Macrae, S., Thomson, J.A., and Van Staden, J. (1988) Colonisation of tomato plants by two agrocin producing strains of *Agrobacterium tumefaciens. Appl. Environ. Microbiol.* 54:3133-3137.

Matsumoto, S., Ophel, K., Skeene, K.G.M., and Scott, N.S. (1992) Partial characterisation of *Agrobacterium vitis. Vitis* 31: 195-203.

Matthysse, A.G., (1987) Characterisation of non-attaching mutants of *Agrobacterium tumefaciens. J. Bacteriol.* 169: 313-323.

Matthysse, A.G., (1996) Adhesion in the rhizosphere. In: Molecular and Ecological Diversity of bacterial adhesion. (Eds. M. Fletcher and D. Savage) John Wiley and Sons Inc., New York, N.Y. pp123-153.

Matthysse, A.G., and McMahan S. (1998) Root colonisation by *Agrobacterium tumefaciens* is reduced in *cel, attB, attD* and *attR* mutants. *Appl. Environ. Micro.* 64(7): 2341-2345.

Matthysse, A.G., Holmes, K., and Gurlitz, R. (1981) Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. *Journal of Bacteriology* 145(1): 583-595.

Matthysse, A.G., Lightfoot, R., and White, G. (1995) Genes required for cellulose synthesis in *Agrobacterium tumefaciens. J. Bacteriol.* 177:1069-1075.

Matthysse, A.G., Stretton, S., Dandie, C., McClure, N.C., and Goodman, A.E. (1996) Construction of GFP vectors for use in Gram negative bacteria other than *E. coli. FEMS Microb. Lett.* 145(1): 87-94.

McClure, N.C., Ahmadi, A.R., and Clare, B.G. (1994) The role of agrocin 434 produced by *Agrobacterium* strain K84 and derivatives in the biological control of *Agrobacterium* biovar 2 pathogens. In: Improving plant productivity with rhizosphere bacteria. International workshop on plant growth-promoting rhizobacteria. (Eds. Ryder, M.H., P.M. Stephens and G.D. Bowen) CSIRO Division of Soils Adelaide, Australia. pp125-127.

McGuire, R.G., Rodriguez-Palenzuela P., Colmer, A., and Burr, T.J. (1991) Polygalacturonase production by *Agrobacterium tumefaciens* Biovar 3. *Appl. Environ. Microbiol.* 57(3): 660-664.

Merlo, D., and Nester, E. (1977) Plasmids in avirulent strains of *Agrobacterium. J. Bacteriol.* 129: 76-80.

Momol, E.A., Herlache, T.C., and Burr, T.J. (1998) Detection of *Agrobacterium vitis* in grape by PCR. *Phytopathology* 88 (Suppl.) S64.

Moore, L.W. (1977) Prevention of crown gall on *Prunus* roots by bacterial antagonists. *Phytopath.* 67: 139-144.

Mudge, S.R., Lewis-Henderson, W., and R.G. Birch (1996) Comparison of *Vibrio* and firefly luciferases as reporter systems for use in bacteria and plants. *Aust. J. Plant Physiol.* 23:75-83.

Neff, N.T., and Binns, A.N. (1985) *Agrobacterium tumefaciens* interaction with suspension cultured tomato cells. *Plant Physiol.* 75: 35-42.

Neilan, B.A., Gurvitz, A., Leigh, D.A., Lai, L.Y.C., and McDonald, B. (1993) Rapid preparation of limited biological samples for small volume PCR. *PCR Rapid Methods Applic.* 2: 261-262.

Nester, E.W., and Gordon, M.P. (1991) Molecular strategies in the interaction between *Agrobacterium* and its hosts. In: Molecular strategies of pathogens and host

plants. (Eds. Patil, S., S. Ouchi, D. Mills and C. Vance) Springer-Verlag, USA, pp3-14.

Ophel, K., (1988a) Crown gall disease of grapevines: status in Australia. *The Australian Grapegrower and Winemaker.* April: 15-16.

Ophel, K., and Kerr, A. (1990) *Agrobacterium vitis* sp. New strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systemic Bacteriology* 40: 236-241.

Ophel, K., Burr, T.J., Magarey, P.A., and Kerr, A. (1988b) Detection of *Agrobacterium tumefaciens* biovar 3 in South Australian grapevine propagation material. *Australasian Plant Pathol.* 17(3): 61-66.

Ophel, K., Nicholas, P.R., Magarey, P.A., and Bass, A.W. (1990) Hot water treatment of dormant grape cuttings reduces crown gall incidence in a field nursery. *Am. J. Enol. Vitic.* 41(4): 325-329.

Orffer, C.J. (1977) Hot-water treatment of vine propagating material - Urgently necessary in RSA. *The Deciduous Fruit Grower.* July: 224-230.

Orffer, C.J., Goussard, P.G., Bosman, D.C., Morkel, D.C., and Weise, J.J. (1979) The effects of hot water treatment and growth regulators on budburst and rooting of cuttings of grapevine rootstock cultivar USVIT 2-1. *The Deciduous Fruit Grower.* October: 340-344.

Panagopoulos, C.G., and Psallidas, P.G. (1973) Characteristics of Greek Isolates of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. *J. Appl. Bacteriol.* 36: 233-240.

Panagopoulos, C.G., Psallidis, P.G., and Alivizatos, A.S. (1978) Studies on biotype *3 Agrobacterium radiobacter var. tumefaciens. Proc. 4th Int. Conf. Plant Path. Bact.*-Angers. 221-228.

Pavlica, D.A., Hora, T.S., Bradshaw, J.J., Skogerboe, R.K., and Baker, R. (1978) Volatiles from soil influencing activities of soil fungi. *Phytopathol.* 68: 758-765.

Penalver, R., Vicedo, B., Salcedo, C.I., and Lopez, M.M. (1994) *Agrobacterium radiobacter* strains K84, K1026 and K84agr$^-$ produce an antibiotic like substance, active *in vitro* against *Agrobacterium tumefaciens* and phytopathogenic *Erwinia* and *Pseudomonas* species. *Biocontrol Science and Technology* 4: 259-267.

Perry, K.L., and Kado, C.I. (1982) Characteristics of Ti-plasmids from broad-host-range and ecologically specific biotype 2 and 3 strains of *Agrobacterium tumefaciens. J. Bacteriol.* 151(1): 343-350.

Prosser, J., (1994) Molecular marker systems for the detection of genetically engineered microorganisms in the environment. *Microbiol.* 140: 5-17.

Pu, X-A., and Goodman, R. N. (1993a) Tumor formation by *Agrobacterium tumefaciens* is suppressed by *Agrobacterium radiobacter* HLB-2 on grape plants. *Am. J. Enol. Vitic.* 44(3): 249-254.

Pu, X-A., and Goodman, R.N. (1993b) Attachment of Agrobacteria to grape cells. *Appl. Environ. Microbiol.* 59(6): 2572-2577.

Pu, X-A., and Goodman, R.N. (1993c) Effects of fumigation and biological control on infection of indexed crown gall free grape plants. *Am. J. Enol. Vitic.* 44(3): 241-248.

Raskie, D.J., Gaheen, A.C., Lider, L.A., and Meredith C.P. (1983) Strategies against grapevine fanleaf virus and its nematode vector. *Plant Disease* 67:335-337.

Ream, W., (1989) *Agrobacterium tumefaciens* and the interkingdom genetic exchange. *Annu. Rev. Phytopathol.* 27:583-618.

Rezaian, M.A., and Krake, LR. (1987) Nucleic acid extraction and virus detection in grapevines. *J. Virol. Methods.* 17: 272-285.

Robinson, J., (ed.) (1994) The Oxford Companion to Wine. Oxford University Press, Oxford, United Kingdom. pp821.

Roy, M.A., and Sasser, M. (1983) A medium selective for *Agrobacterium tumefaciens* biotype 3 (Abstract) *Phytopathology* 73: 810

Ruffner, H.P., (1982) Metabolism of tartaric and malic acids in *Vitis*: A review Part A. *Vitis* 21: 247-259.

Ryder, M.H., and Jones, D.A. (1991) Biological control of crown gall using *Agrobacterium* strains K84 and K1026. *Australian J. Plant Physiol.* 18: 571-579.

Salomone, J-Y., Crouzet, P., De Ruffray, P., and Otten, L. (1996) Characterisation and distribution of tartrate utilisation genes in the grapevine pathogen *Agrobacterium vitis. MPMI* 9(5): 401-408.

Sawada, H., Oyaizu, H., Matsumoto, S., and Ieki, H. (1992) Differentiation of Agrobacteria taxa based on partial sequences of 16s rRNA. *Ann. Phytopathol. Soc. Jpn.* 58: 761-765.

Schoenbeck, F., Dehne, H.W., and Balder, H. (1982) The efficiency of induced resistance under practical cultural conditions I. Powdery mildew of grape, cucumber and wheat. *J. Plant Dis. Prot.* 89: 177.

Schoenbeck, F., Dehne, H.W., and Beicht, W. (1980) Activation of unspecific resistance mechanisms in plants. *J. Plant Dis. Prot.* 87: 177.

Schroth, M.N., McCain, A.H., Foott, J.H., and Huisman, O.C. (1988) Reduction in the yield and vigour of grapevine caused by crown gall disease. *Plant Disease* 72: 241-246.

Schroth, M.N., Weinhold, A.R., McCain, A.H., Hildebrand, D.C., and Ross, N. (1971) Biology and control of *Agrobacterium tumefaciens. Hilgardia* 40: 537-552.

Sheng, J., and Citovsky, V. (1996). *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *The Plant Cell* 8: 1699-1710.

Shim, J.S., Farrand, S.K. and Kerr, A. (1987) Biological control of crown gall: Construction and testing of new biocontrol agents. *Phytopath.* 77: 463-466.

Simon, R., (1984) High frequency mobilisation of Gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-mob transposon. *Mol. Gen. Genet.* 196:413-420.

Skerman, V.B.D., McGowan, V., and Sneath, P.H.A. (1980) Approved lists of bacterial names. *International Journal of Systematic Bacteriology* 30:225-420.

Slota, J.E., and Farrand, S.K. (1982) Genetic isolation and physical characterisation of pAgK84, the plasmid responsible for agrocin 84 production. *Plasmid* 8: 175-186.

Solovova, G.K., Krivopalov, Y.V., Velikov, V.A., and Chumakov, M.I. (1995) Attachment of *Agrobacterium radiobacter* to the roots of wheat. *Microbiology* 64(4):446-450.

Spencer, P.A., Tanaka, A., and Towers, G.H.N. (1990) An *Agrobacterium* signal compound from grapevine cultivars. *Phytochemistry* 29: 3785-3788.

Stachel, S.E., Messens, E., van Montagu, M., and Zambryski, P. (1985) 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., and Zambryski, P. (1986) *virA* and *virG* control the plant-induces activation of the T-DNA transfer process of *A. tumefaciens. Cell* 46: 325-333.

Staphorst, J.L., van Zyl, F.G., Strijdom, B.W., and Groenewold, Z.E. (1985) Agrocin-producing pathogenic and non-pathogenic biotype 3 strains of *Agrobacterium tumefaciens* active against biotype 3 pathogens. *Current Microbiol.* 12: 45-52.

Stockwell, V.O., Kawalek, M.D., Moore, L.W., and Loper, J.E. (1996) Transfer of pAgK84 from the biocontrol agent *Agrobacterium radiobacter* K84 to *A. tumefaciens* under field conditions. *Phytopathology* 86: 31-37.

Stockwell, V.O., Moore, L.W., and Loper, J.E. (1993) Fate of *Agrobacterium radiobacter* K84 in the environment. *Appl. Environ. Microbiol.* 59(7): 2112-2120.

Stonier, T., (1960) *Agrobacterium tumefaciens* II. Production of an antibiotic substance. *J. Bacteriol.* 79: 889-898.

Stover, E.W., Burr, T.J., and Swartz, H.J. (1996) Transformation of crown gall resistant and susceptible *Vitis* genotypes by *Agrobacterium vitis. Vitis* 35(1): 29-33.

Sule, S. (1978) Biotypes of Agrobacterium tumefaciens in Hungary. *Journal of Applied Bacteriology* 44: 207-213.

Sule, S. (1983) Survival of *Agrobacterium tumefaciens* in Berlandieri X Riparia grapevine rootstock. *Acta Phytopathol. Acad. Sci. Hung.* 21: 203-206.

Sule, S., Mozsar, J., and Burr, T.J. (1994) Crown gall resistance of *Vitis* spp. and grapevine rootstocks. *Phytopath.* 84(6):607-611.

Szegedi, E., (1985). Host range and specific L (+) tartrate utilisation of biotype 3 of *Agrobacterium tumefaciens. Acta Phytopath. Academiae Scientiarum Hungaricae* 20(1-2): 17-22.

Szegedi, E., Czako M., and Otten, L. (1992) Further evidence that the vitopine-type pTi's of *Agrobacterium vitis* represents a novel group of Ti plasmids. *MPMI* 9(2): 139-143.

Szegedi, E., Czako, M., Otten, L., and Koncz, C.S. (1988) Opines in crown gall tumors induced by biotype 3 isolates of *Agrobacterium tumefaciens. Physiol. Molec. Plant Pathol.* 32: 237-247.

Szegedi, E., Korbulv, J., and Otten, L. (1989) Types of resistance of grapevine varieties to isolates of *Agrobacterium tumefaciens* biotype 3. *Physiol. Molec. Plant Pathol.* 35:35-43.

Szegedi, E., Otten, L., and Czako, M. (1992) Diverse types of tartrate plasmids *in Agrobacterium tumefaciens* biotype 3 strains. *MPMI* 5(5): 435-438.

Szegedi, E., Sule, S., and Burr, T.J. (1999) *Agrobacterium vitis* strain F2/5 contains tartrate and octopine utilisation plasmids which do not encode functions of tumour inhibition on grapevine. *J. Phytopathol.* 147: 665-669.

Tarbah, F.A., and Goodman, R.N. (1986) Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. *Plant Disease* 70(6): 566-568.

Tempe, J., and Petit, A. (1983) La piste des opines. In Molecular Genetics of the bacteria - Plant Interaction (Ed. A. Puhler), pp 14-32. Springer, Berlin.

Thomashow, L.S., and Weller, D.M. (1988) Role of phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. tritici. *J. Bacteriol.* 170: 3499-3508.

Thomashow, L.S., Weller, D.M., Bonsall, R.F., and Pierson, L.S. (1990) Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* spp. in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 56: 908-912.

Thomson, J. A., (1986) The potential for biological control of Crown gall disease on grapevines. *TIBTECH*: August 219-224.

Tuzan, S., and Klopper, J. (1994) Induced resistance by plant growth promoting rhizobacteria. In: Improving plant productivity with rhizosphere bacteria. International workshop on plant growth-promoting rhizobacteria. (Eds. Ryder, M. H., P.M. Stephens and G. D. Bowen) CSIRO Division of Soils Adelaide, Australia.

Vande Broek, A., and Vanderleyden, J. (1995) The role of bacterial motility, chemotaxis and attachment in plant-bacteria interactions. *MPMI* 8(6): 800-810.

Vicedo, B., Lopez, M.J., Asins, M.J., and Lopez, M.M. (1996) Spontaneous transfer of the Ti plasmid of *Agrobacterium tumefaciens* and the nopaline catabolism plasmid of *A. radiobacter* strain K84 in crown gall tissue. *Phytopath.* 86(5): 528-534.

Wample, R.L., (1992) Influence of pre- and post- treatment storage on bud break of hot water treated cuttings of Cabernet Sauvignon. *Am. J. Enol. Vitic.* 44(2): 153-158.

Weller, D.M., (1988) Colonisation of wheat roots by a fluorescent pseudomonad suppressive to take-all. *Phytopathol.* 73: 1548.

Yanofsky, M., Lowe, B., Montoya, A., Rubin, R., Krud, W., Gordon, M., and Nester, E. (1985) Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* 201: 237-246.

Yanofsky, M., Montoya, A., Knauf, V., Lowe, B., Gordon, M., and Nester, E. (1985) Limited host range plasmid of *Agrobacterium tumefaciens*: Molecular and genetic analyses of transferred DNA. *J. Bacteriol.* 163(1): 341-348.

Zambryski, P.C., (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:465-490.

PUBLICATIONS AND PRESENTATIONS

Publications:

A.R. Keegan, M.H. Ryder, N.C. McClure, P.J. Murphy and K. Ophel Keller Biological control of crown gall disease in grapevine nurseries. Proceedings of the Australasian Soilborne Diseases Symposium. Lorne, Vic. 5-8 March 2001. Porter, I.J. *et* al (eds.). (Second Soilborne Diseases Symposium, Victoria, Australia), pp157-158.

Presentations:

A.R. Backhouse, K.M Ophel Keller, N.C. McClure, P.J. Murphy and M.H. Ryder Factors involved in the biological control of crown gall disease of grapevine. Inaugural and International Conference of the Federation of Asia-Pacific Microbiology Societies (FAPMS) Suntec City, Singapore. May 1998.