THE ROLE OF AGROCIN 434 AND OTHER FACTORS IN THE BIOLOGICAL CONTROL OF CROWN GALL DISEASE.

ALI-REZA AHMADI

M.Sc. University of Tarbiat Modarress (Tehran-Iran)

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resource Sciences at the University of Adelaide.

Department of Crop Protection

Waite Campus

University of Adelaide

February 1996

Table of Contents

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other teritary 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.

This thesis may be made available for loan and photocopying provided that an acknowledgment is made in the instance of any reference to this work.

Ali-Reza Ahmadi

February 1996

Acknowledgment

My deepest gratitude to my supervisors Dr Nicholas C McClure and Professor Otto Schmidt for their encouragement, advice and criticism throughout the year, and for their guidance when preparing this manuscript. I wish to thank Dr Bruce G Clare, my former supervisor, for his encouragement and guidance in my work.

I gratefully acknowledge Dr E Scott for allowing me use of her tissue culture room facilities. I wish to thank Dr Ali Vezvaei for providing almond seeds, and also sincere thanks to N Collins for allowing me use of his laboratory equipment in professor Bob Symon's lab.

I wish also thank the former members of Bruce Clare's lab, Natalie Dillon, Tara Dillon and Scott Donner, for their help and friendship. I also thank members of the neighbouring laboratory of Dr Peter Murphy, and Department of Crop Protection in general, for creating an atmosphere of friendliness and cooperation.

I would like to thank Jennie Groom, Emily Shepherd, Zoni, Anna Giuliano and Peter Ladyman for photographic assistance, and Terry Feckner, Gary Taylor and Anke Johnsen for their assistance with departmental matters.

I thank the government of Islamic Republic of Iran, Ministry of Culture and Higher Education who sponsored my scholarship for the PhD studies.

Finally I wish to express my deepest gratitude to my wife and my son for their patience and assistance throughout my studies.

Summary

Crown gall, caused by the soil-borne bacterium *Agrobacterium spp.*, is a common disease of a wide variety of dicotyledonous plants such as peaches, grape vines, almond, cherry, *Rubus* species and various other nut-bearing trees. The biological control agents *A. rhizogenes* strain K84 and its genetically engineered derivative, K1026 have been used successfully for a number of years to control crown gall in stone fruits and ornamentals. Strain K84 produces a potent inhibitory agent agrocin 84. A number of researchers have suggested that other mechanisms of control other than agrocin 84 production may be involved in the biocontrol process of crown gall by strains K84 and K1026. One of these mechanisms may be the effect of another antibiotic, agrocin 434, which is produced by *A. rhizogenes* strains K84, K1026 and K434. This thesis studies the role of agrocin 434 and other factors in the biological control process.

Initial studies have shown that genes involved in the biosynthesis of agrocin 434 are located on a large cryptic plasmid (300-400 kb) of strain K84 and derivatives (Donner *et al.*, 1993). The results of this study indicate that genes involved in the immunity/resistance to agrocin 434 are also carried by pAgK434. This has been demonstrated by transferring the plasmid pAgK434 to the agrocin 434 sensitive strain, K27. The resulting strain became resistant to agrocin 434 and had acquired the ability to produce agrocin 434 as well. Another derivative of K84, strain K1318 with pAgK1318, carries a deleted version of pAgK434. This strain is unable to produce agrocin 434 but produces a modified agrocin 434 (nucleoside 4176) which has no inhibitory activity. The kanamycin resistance transposon Tn*5* was introduced into derivatives of pAgK1318 marked with antibiotic resistance markers and resulting plasmids transferred to an agrocin 434 sensitive strain. The resulting transconjugants

were able to produce modified agrocin 434 as did K1143 pAgK1318. The results of this study show that resistance/immunity functions are also carried on pAgK1318, as transconjugant strains were resistant to agrocin 434.

To understand the role of each of the K84 plasmids, pAgK84, pAtK84b and pAgK434 in the biocontrol process, a range of derivatives of strain K84 harbouring all combinations of these three resident plasmids in the same host background were constructed.

A rapid efficient method for testing pathogenicity and/or the efficacy of biocontrol strains was developed by using a leaf disc tumorigenesis assay. A range of tobacco and tomato cultivars were tested to determine which plant cultivars gave the most rapid and reproducible callus formation with different concentrations of pathogen. The results of these experiments indicated that tobacco cultivar White Burley and *cv.* Virgie are the best host plants for plant transformation because these cultivars produced more calluses than other cultivars. The results of stem inoculation bioassays determined that tomato *cv.* Quick Pick is the best plant for stem inoculation, because gall formation by stem inoculation of tobacco requires a longer incubation period than tomato and this cultivar produced more galls than other tomato cultivars.

The efficacy of all derivatives of strain K84 in controlling the pathogenic biovar 2 strain K27 was assessed using root inoculation of almond seedlings, tomato stem inoculation and leaf disc culture bioassays. Results from stem inoculation and leaf disc culture assays showed that all the derivatives of K84, including the plasmid free strain, K1347, significantly reduced galling by the pathogen *A. rhizogenes* K27. Strains carrying one or more of the three plasmids of K84 showed a significantly greater biocontrol ability than strain K1347. Results from root inoculation assays indicated that strains harbouring pAgK84, pAtK84b or pAgK434 significantly reduced gall formation by the pathogen K27. Gall formation following treatment with strain K1347 was not significantly different from that with the pathogen alone.

The insertion of Tn*5* into pAgK434 stopped agrocin 434 production by strains K1356 and K1357. The biological activity of these new strains was tested using the different bioassays and indicated that these strains can significantly reduce gall formation or callus induction in leaf disc culture and/or stem inoculation bioassays, but they do not produce significant control of the pathogen in almond root bioassays.

The efficacy of agrocin 434 producer strains to control pathogenic *Agrobacterium* strains from different species was assessed using rapid bioassay methods, leaf disc culture and stem inoculation. The results of this study determined that strain K434 is not quite efficient in controlling crown gall by biovar 1 and 3 and *A.rubi* in the stem inoculation and/or leaf disc tumorigenesis bioassays as wel as controlling crown gall induction by a biovar 2 pathogen.

Abbreviations

Amp	Ampicilin
cm	centimeter
cv	cultivar
DIG	digoxigenin
DNA	deoxyribonucleic acid
g	gram
HVPE	high voltage paper electrophoresis
Inc	Incompatibility region of *Agrobacterium* Ti plasmid
kb	kilobase
Km	kanamycin
l	liter
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
nm	nanometer

OD	optical desity
P	probability
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
T-DNA	transferred-DNA
TE	Tris-EDTA buffer
Ti	tumor-inducing
UV	ultraviolet
vir	virulence
w/v	weight per unit volume

Chapter One

General Introduction

1.1 Introduction

Pathogenic soil-borne agrobacteria incite development of crown gall or hairy root on a wide range of dicotyledonous plants such as grape, peach, apricot, nectarine, plum, cherries, almond and ornamental *Prunus* species (Clare & McClure, 1995; Kerr & Brisbane, 1983). More than six hundred and forty dicotyledonous plant species and some gymnosperms have been recorded as being susceptible to crown gall, when artificially inoculated with pathogenic agrobacteria (De Cleene & De Ley, 1979; Morris *et al.*, 1989; Kerr 1992). In some cases *Agrobacterium* can also infect monocotyledonous plants (Hooykaas-van Slogteren *et al.*, 1984; Graves & Goldman, 1986; Bytebier *et al.*, 1987), but not the economically important cereals.

This pathogen is responsible for significant crop losses throughout the world (Panagopoulos *et al.*, 1979; Kerr, 1980). El-Fiki & Giles (1981) estimated that it caused about US$138 million losses of crops annually based on 1974-1975 prices. In Australia alone, it was estimated to cause annual losses of about A$1.5 million dollars (Kerr & Brisbane, 1983).

Crown gall is a plant cancer. Wounding of plant tissue and a suitable environmental temperature are necessary for gall formation. The bacterium enters plants through

wounds and induces unregulated cell division leading to massive gall formation usually at the crown or on the root system (Braun, 1982; Lippincott & Lippincott, 1969).

1.2 *The Genus Agrobacterium*

The bacterium responsible for inducing crown gall was first reported by Smith & Townsend in 1907, and called *Bacterium tumefaciens* (Braun, 1982). Later, the pathogen was classified in the genus *Agrobacterium*, a member of the *Rhizobiaceae* (Conn, 1942). Other species of the *Rhizobiaceae* family are: *Rhizobium, Bradyrhizobium* and *Phyllobacterium* (Jordan, 1984). General characteristics of the genus *Agrobacterium* are: Gram-negative, single-celled, rod shape, motile with a few peritrichous flagella, oxidase positive and oxidative (Kerr & Brisbane, 1983; Kerr, 1992).

Previously, the genus *Agrobacterium* was classified into five different species on the basis of their pathogenicity: *A. tumefaciens,* containing Ti plasmids, induced crown gall on a wide range of host plants, *A. rhizogenes* containing Ri plasmids induced hairy root, *A. rubi* containing Ti plasmids induced cane gall on Rubus species, *A. vitis* with Ti plasmids induced crown gall on grapevine, and *A. radiobacter* represented non-pathogenic strains (Kersters & De Ley, 1984: Ophel & Kerr, 1990). The main problem with the initial classification was that it was based on plasmid-encoded characteristics which could be unstable and transferred between strains of "different" species.

Recently, strains of *Agrobacterium* have been classified into four species on the basis of chromosomal characteristics of the bacterium, carbon source utilisation and other biochemical tests (Ophel & Kerr, 1990). Biovar one and two are equivalent to

pathogenic or non-pathogenic *A. tumefaciens* and *A. rhizogenes*, respectively. Biovar three is equivalent to *A. vitis*, and *A. rubi* refers to pathogens of *Rubus spp*. (Ophel & Kerr, 1990)

The five marine species; *A. meteoris*, *A. ferrugineum*, *A. atlanticum*, *A. gelatinovorum*, *A. stellulatum*, have been classified in a subdivision within the genus *Agrobacterium* (Rüger & Höfle 1992), but these species are not phythopathogenic.

By using the *rrs* sequencing technique, for the 16S rRNA gene, (Reily, 1993; Sawada *et al.*, 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993) the phylogenetic relationship of the members of the genus *Agrobacterium* has been elucidated. According to the phylogenetic classification of Reily (1993), the genus *Agrobacterium* can be divided into two major lineages; one includes: *A. rhizogenes* along with most *Rhizobium spp*; the second includes *A. vitis*, *A. rubi*, *A. tumefaciens* along with *Rhizobium galeae* .

1.3 *The biology of the pathogenic plasmids of Agrobacterium*

Pathogenic strains of *Agrobacterium spp.* usually harbour a single Ti or Ri plasmid. Ti and Ri plasmids are covalently closed, circular, extrachromosomal DNA elements (150 - 250 kb in size) and replicate independently of the chromosome (Clare *et al.*, 1987; Clare, 1993; Clare & McClure, 1995). In 1969, Kerr reported that virulence could be transferred to avirulent strains in a developing crown gall. In 1971 these findings were confirmed and Kerr suggested that the genes responsible for virulence were carried on a transmissible extrachromosomal element (Kerr, 1971). In 1975, Watson and also Van

Larebeke demonstrated that pathogenicity of *A. tumefaciens* depends on the presence of a large plasmid (Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). This large plasmid is now called the Ti plasmid or tumor-inducing plasmid. It was demonstrated that when pathogenic agrobacteria are attracted to plant tissue and attach to the wounded host plant, a part of the Ti or Ri plasmid (T-DNA) is transferred from bacterial cells to the host cells. The T-DNA is transported to the host nucleus and integrated into the host genome (Chilton *et al.*, 1977; Chilton *et al.*, 1980; Thomashow *et al.*, 1980; Chyi *et al.*, 1986; Kerr, 1992). Ti and Ri plasmids are responsible for many of the characteristics of *Agrobacterium spp.* infection. The identified functional regions of Ti or Ri plasmids are: 1) T-DNA: carries oncogenicity genes (*onc*), this region is involved in the synthesis of auxin and cytokinin; and *ops* genes involved in opine synthesis in plant cells (*ops*); 2) Non-transferred DNA region: the virulence region (*vir*) genes code for processing of T-DNA to the plant cell nucleus and integration into the plant genome; *ori/inc* region carries genes for replication control and incompatibility between plasmids; opine catabolic genes (*opc*) encode catabolism of opines by *Agrobacterium*; *tra* region harbours genes controlling conjugal transfer of *Agrobacterium* plasmids (Clare *et al.*, 1987; Binns & Thomashow, 1988; Clare, 1990; Clare, 1993; Clare & McClure, 1995).

The mechanism of pathogenesis by *Agrobacterium spp.* has undergone extensive investigation and has been shown to be a very complex process. Much of this work has been undertaken to develop and extend the use of *Agrobacterium* as a vector for plant transformation. The molecular basis for important stages of the pathogenesis has been elucidated and has been the topic of a number reviews (Day and Lichtenstein, 1991; Ooms, 1991; Grant *et al.*, 1991; Warren, 1991; Hooykaas and Mozo, 1994;), however little is still know about key steps such as the transfer and integration of the T-DNA to the plant nucleus. Both chromosomal genes and Ti-plasmid genes play a role in virulence.

1.3.1 *Chemotaxis*

The early stages of *Agrobacterium* pathogenesis are important as this is a likely stage for interaction between pathogens and potential biological control strains.

As an initial step in the interaction between *Agrobacterium* and potential host cells, bacteria may respond chemotactically to chemicals released into the rhizosphere by wounded plant cells. Many strains of *Agrobacterium* are highly motile (Shaw *et al.*, 1988; Parke *et al.*, 1987) and are attracted towards wounded sites. These wounded sites release plant extracts and exudates such as amino acids, sugars, aromatic and phenolic compounds and aliphatic chemicals (Stachel *et al.*, 1985). Extracts of phenolic compounds released by wounded plant cells have been shown to induce the activity of Ti plasmid *vir* genes that function in the processing and transferring of T-DNA to the plant genome (Stachel *et al.*, 1985; Winans 1992). Acetosyringone and hydroxy acetosyringone, two naturally occurring wound response phenolics in tobacco exudates, have been identified as plant signal molecules that induce the activation of the *vir* genes in *Agrobacterium* (Stachel *et al.*, 1985; Bolten *et al.*, 1986). The nature of attractant compounds may play a role in the host specifity of agrobacteria (Clare & McClure 1995).

Population levels of some soil-borne microorganisms are much higher in the rhizosphere than in bulk soil, because of the stimulating effects of root exudates. Results of recent studies showed that *A. tumefaciens* was chemically attracted to several of the host root phenolic compounds that are necessary for induction of the virulence genes in this bacterium (Ashby *et al.*, 1987,1988; Shaw *et al.*, 1988). Acetosyringone is a selective product of wounded tissue, and wounds are the site of *A. tumefaciens* infection. The pathogenic agrobacteria are able to detect very low concentrations of

this wound specific compound and move towards the wound site (chemotax). Key virulence regulatory genes, *virA* and *virG* are induced when the bacteria get close to the wound sites (Shaw *et al.*, 1988). Gene induction by host phenolics and chemotaxis towards these compounds are highly interrelated processes.

1.3.2 *vir genes*

The part of the Ti and Ri plasmids which is necessary for gall induction is approximately 30-40 kb and is referred to as the *vir* region (Stachel & Nester, 1986; Binns & Thomashow, 1988; Zambryski, 1992; Hooykaas *et al.*, 1994). The *vir* region contains over 25 genes organized into eight operons, *virA*-H. This region is not transferred into the plant cell but is required in *trans* for the processing and transfer of the T-DNA. These genes are induced during infection in response to three different group of stimuli which are thought to be key chemical features of a typical wound site. These stimuli are: guaiacol and syringol derivatives i.e. acetosyringone; sugars i.e. glucuronic acid and glucose; and acidic pH (Winans *et al.*, 1994).

The functions of some of the *vir* region genes have been identified: *virA* and *virG* are regulatory genes; *virB*, *virC*, *virD* and *virE* are responsible for production and processing of the T-DNA copy, and production of the structural machinery which effects T-DNA transfer (Binns & Thomashow, 1988; Winans, 1992; Winans *et al.*, 1994; Endoh *et al.*, 1995; Pan *et al.*, 1993; Roitsch *et al.*,1994; Zambryski, 1992; Hooykaas *et al.*, 1994); the *virH* locus is generally not required for virulence. However, the *virH* gene product may be responsible for detoxification of bactericidal or bacteriostatic plant compounds during the infection process (Kanemoto *et al.*, 1989). The *vir* region of the Ti plasmid of the octopine Ti plasmid contains eight genes, *virA* to

virH. Unlike the octopine Ti plasmid, nopaline Ti plasmids lack the *virF* and *virH* genes but contain another accessory gene named *tzs*. The *virF*, *virH*, *virE* genes play a role in determining plant host range (Hooykaas *et al.*, 1994). The *tzs* gene present in the nopaline Ti or Ri plasmids, and which is involved in the production and secretion of the cytokinin *trans-zeatin* from *Agrobacterium*, may also be a host-range determinant (Zhang *et al.*, 1990). Nopaline strains, which are almost nontumorigenic in *Nicotiana glauca*, become effective tumor-inducers if the *virF* gene is expressed in this plant host (Regensburg-Tunïk & Hooykaas, 1993). Thus, the host range for gall formation was extended by expressing a *vir* gene in the plants. *virF* gene seems not to be essential for tumor formation, "this locus might be the result of genetic exchanges between different *Agrobacterium* strains during evolution (Broer *et al.*, 1995).

1.3.3 *Attachment of Agrobacterium to plant cells*

Agrobacterium spp. are commonly found in agricultural land and non-cultivated soils and have been isolated from roots and the vascular system of plants (Kerr, 1969; Burr & Katz, 1983; Bouzar & Moore, 1987). Both virulent and avirulent strains have been isolated from all of these environments, but the majority of *Agrobacterium* isolates from soils and roots appear to be nonpathogenic (Moore & Warren, 1979).

Initial binding of bacterial cells to plant cells is one of the earliest steps in the tumorigenesis process (Binns & Thomashow, 1988; Matthysse, 1984). The process of attachment of agrobacteria to plant cell has been studied with different plant tissues, such as pinto bean leaves (Lippincott & Lippincott, 1969), tobacco (Smith & Hindley, 1978; Douglas *et al.*, 1982), carrot (Matthysse, 1987, 1994). potato disc (Glogowski &

Glasky, 1978, grape (Pu & Goodman, 1993), and some monocot cells such as corn (Graves *et al.*, 1988, Douglas *et al.*, 1985).

The attachment process was suggested to be required for DNA transfer from the bacteria to the plant host cell. Mutants of *A. rhizogenes* which are unable to bind to carrot cells in culture are avirulent (Robertson *et al.*, 1990). Generally, nonattaching mutants that have been isolated are known to be avirulent (Douglas *et al.*, 1982). Genes identified as playing a role in bacterial attachment are all located on the bacterial chromosome (Matthysse 1987, 1988; Douglas *et al.*, 1982). Ti plasmid-free bacterial strains are able to bind to plant cells. However, there is evidence that Ti plasmids also can contribute to attachment (Smith & Hindley, 1978; Whatley *et al.*, 1978; Matthysse *et al.*, 1978; Tanimoto *et al.*, 1979; Pu & Goodman, 1993).

Three chromosomal genes *chvA*, *chvB* (Douglas *et al.*, 1985) and *pscA* (Thomashow *et al.*, 1987) have been identified which encode products necessary for the attachment process. The *chvA* gene codes for synthesis of a protein that is required for transport of the ß-1,2-glucan into the periplasm and plant cell binding, the *chvB* encodes another protein which converts glucan into the cyclic ß-1,2-glucan. The *pscA* (*exoC*) locus codes for an enzyme which converts glucose 6-phosphate to glucose 1-phosphate, and it has an important role in the production of surface polysaccharides (Thomashow *et al.*, 1987; Nester & Gordon 1991; Wanger and Matthysse, 1992; Romantschuk, 1992). Attachment defective *chvA* and *chvB* mutants are unable to transfer the plasmid pAgK84 by conjugation, which suggests surface alterations (Binns & Thomashow 1988). Matthysse (1988) has isolated avirulent *att* gene mutants that were affected in plant cell binding ability to carrot suspension cells. These avirulent mutants were also characterized by a loss of one or more of the three outer membrane proteins; it is possible that these outer membrane proteins may play a role in the attachment process

(Robertson *et al.*, 1988; Matthysse 1988). Another genetic locus, *picA* has been isolated which affects aggregation and virulence in bacterial attachment process (Rong *et al.*, 1990;).

It has been demonstrated that cellulose fibrils of agrobacteria play an important role in the pathogenicity process and serve to bind the bacteria to the plant cell surface (Matthysse 1983, 1986). The production of these fibrils are stimulated by the presence of plant extracts or plant cells (Matthysse *et al.*, 1981). A part of the chromosome of agrobacteria (11kb) carries genes in two operon groups, *celABC* and *celDE*, for cellulose synthesis. Results from transposon insertion mutagenesis indicated that at least one of *celC* and *celE* were essential for cellulose synthesis (Matthysse *et al.*, 1995 a). The functions of these genes has been studied; it was suggested that *celE* gene produces some sort of lipid intermediate compound which binds to UDP-glucose; the product of *celD* may also be involved in this reaction since*celA B C* are responsible for cellulose production and processing (Matthysse *et al.*, 1995b).

Mutant analysis has been used to test the hypothesis that recognition of cells plays a role in establishment of plant-microbe associations. Tn*5* mutants of *A. tumefaciens* deficient in chemotaxis to the cells exhibit reduced ability to colonize the root zone and to induce crown gall tumorigenesis on pea plants grown in soil (Hawes *et al.*, 1988). In addition, strains both with and without a Ti plasmid expressed attraction to pea root tips and root cap cells, indicating that genes involved in binding and chemotaxis to root cap cells are encoded by chromosomal genes that are present in both pathogenic and nonpathogenic agrobacteria.

Pu and Goodman (1993) reported that the Ti plasmid influences the attachment of *A. vitis* to grape cell. Smith and Hindley (1978) found that agrocin 84 from strain K84

blocks the initial steps of tumour induction in tobacco cell culture, that is the attachment of pathogen to host. Similarly, Pu and Goodman (1993) found that attachment of the virulent strain to grape callus cells is blocked by the avirulent strain HLB-2 in both tissue culture cell suspensions and seedling root systems. In their experiments the nonpathogenic strain K84 failed to suppress grape crown gall infection. They concluded that these two avirulent and Ti plasmidless strains, K84 and HLB-2, have different attachment efficiencies to grape cell and that this may be the reason why strain K84 is unable to control biovar 3 pathogens.

1.3.4 *T-DNA*

Tumor formation is caused by the presence in all virulent strains of *Agrobacterium spp.* of a large Ti plasmid (Van Larebeke, *et al* .. 1975, Watson *et al.*. 1975). A specific segment of the plasmid (the T-DNA of about 24 kb in size) can be transferred into a plant cell and integrated into its genome (Chilton *et al.*, 1980; Nester, *et al.*;.1984). By using a microinjection approach, Escudero *et al.* (1995) demonstrated that the process of T-DNA transfer from the agrobacteria to the plant cell nucleus can occur entirely inside the plant cell. This transfer is strongly dependent on induction of *vir* genes and a functional *virB* gene. They concluded that *Agrobacterium spp.* can function as intracellular infectious agent in plants. The transferred DNA fragment, or T-DNA , encodes genes for the synthesis of the phytohormones auxin and cytokinin (Morris, 1986). The expression of these genes results in unbalanced hormone levels in the plant and leads to undifferentiated cell division at infection sites. Braun (1982) reported that tumor formation was a two-phase phenomenon, the first involves inception and the second phase is the development phase. To complete this process, conditioning of the plant cells is necessary only those cells that have been rendered susceptible to

transformation as a result of irritation accompanying a wound can be transformed into tumor cells. The size of the gall was also determined by this condition.

Among the genes encoded by the T-DNA, two are responsible for high level production of the plant growth regulator, indole-3-acetic acid (IAA). These are: *iaaH* encoding an auxin, tryptophan monoxygenase, and *iaaM* encoding indol acetamide hydrolase (Schröder *et al.*, 1984; Thomashow *et al.*, 1984; Yamada, 1993; Costacurta and Vanderleyden 1995). A gene also present in T-DNA for the biosynthesis of cytokinin, *ipt*, encodes isopentenyl-AMP (Barry *et al.*, 1984). The presence of this gene can affect the host range of *Agrobacterium* strains. In *Agrobacterium* biovar 3 strains the lack of the *ipt* gene was demonstrated to be one of the reasons for their host specifity and efficient tumor induction (Hoekema *et al.*, 1984; Hemstad and Reisch 1985). By inactivation of the *ipt* gene of a wide host range *Agrobacterium* strain it was possible to induce tumors on certain grapevines cultivars (Hemstad & Reisch 1985).

In addition, other genes present on T-DNA appear to be indirectly involved in synthesis of plant growth regulators. The product of gene *6b* modulates the activity of auxin or cytokinin, and gene *5* modulates auxin sensitivity in plant cells (Tinland *et al.*, 1989, 1990; Kober *et al.*, 1991). The product of the *6a* gene is involved in secretion of opines (Messens *et al.*, 1985). Finally, some chromosomal genes, including *acvB* and *virJ*, are required for the transfer of T-DNA from agrobacteria to plant nuclei. *acvB* is necessary for galling by a strain carrying a nopaline-type Ti plasmid, and *virJ* complements this nontumorigenic phenotype in octopine-type Ti plasmids, indicating that the products of these genes have similar functions. *Agrobacterium* strains without *acvB* and *virJ* genes are: proficient for induction of the *vir* functions, able to transfer their Ti plasmids by conjugation, resistant to plant wound extracts and mutations in these genes cannot be complemented extracellularly (Kalogeraki & Winans, 1995).

Opines are synthesized in tumors by enzymes encoded by T-DNA at the expense of the pool of plant metabolites and serve as a carbon and nitrogen source for the pathogenic *Agrobacterium* cells. The T-DNA carries one or more *ops* genes into plant cells. Some of the *ops* genes involved in opine biosynthesis have been identified (Dessaux *et al.*, 1992; Guyon *et al.*, 1993). These genes are: ***nos***, nopaline synthesis; *ocs*, octopine synthesis; *acs,* agrocinopine synthesis; *mas*, involved in mannopine synthesis; *ags*, involved in agropine synthesis. Opines are involved in the following biological activity: opine utilization (*opc*); conjugal transfer of Ti/Ri plasmids (*tra*); opine synthesis (*ops*) and serve as carbon source and possibly nitrogen source for *Agrobacterium* cells (Dessaux *et al.*, 1992;).

Guyon *et al.* (1993) have studied the effect of plant transformation on opine catabolic agrobacteria By cocultivation of bacteria with either transformed or normal Lotus plants, they observed that the carbon and energy sources came from root exudates of the plants, and transformed plants produced more growth substrates for the agrobacteria. They demonstrated that transformed opine producing plants stimulated growth of bacteria which are able to catabolize the opines. They also suggested that "pathogenic strains possess an increased capacity to colonize the rhizosphere of the plants by a mechanism independent of opine catabolism".

To summarize, the basic steps in tumor formation by *Agrobacterium* are: chemotactic response of bacterial cells to low molecular weight chemoattractant compounds from wounded plant cells; attachment of bacterial cells to plant cells; vir gene induction by plant phenolic compounds; processing and transport of T-DNA to host nuclei; integration of T-DNA into the host chromosome; biosynthesis of T-DNA encoded

phytohormones; rapid plant cell division and vegetative proliferation to induce galls. This process is accompanied by the synthesis of opines; growth of agrobacteria utilizing opines as a nutrient source and conjugal transfer of Ti/Ri plasmids to other bacterial cells induced by opines.

1.4 *Biological Control of Crown Gall*

Biological control is one of the most useful approaches for reduction of crop damage caused by plant diseases. The aim of biocontrol is to limit disease by : reduction of the proportion of inoculum of the pathogens, prevention of infection of the host plant by the pathogen, induction of self-defence systems within the host plant and alteration of the pathogen qualitatively. This may be achieved by introducing a biological control agent that employs any of these strategies (Cook & Baker, 1983).

The main reasons for the increasing interest in the use of biocontrol agents include incomplete control by ordinary control methods such as the application of pesticides (chemicals), the development of resistant plants and consumer desire for natural, safe and non-polluted foods.

An effective biocontrol agent effective against root pathogens should do one or more of the following : colonize the root zone rapidly, produce antibiotics or inhibitory compounds to antagonize pathogens, compete for nutrients that are required for growth of the pathogens, compete with the pathogens for infection sites or produce plant growth promoting compound such as IAA or GA (Gould, 1990).

A. rhizogenes strain K84 was originally isolated from soil around a peach gall near Adelaide in 1969 (Kerr, 1989). In soil, the population ratio of pathogens to nonpathogens was high around infected plants and low around healthy plants. Strain K84 was isolated from this large proportion of nonpathogens interacting with the population of pathogens (Kerr, 1980).

Strain K84 is a highly inhibitory non-pathogenic strain and belongs to the species *A, rhizogenes.* New and Kerr (1972) reported that strain K84 completely controlled gall formation by the pathogenic strain *A. rhizogenes* K27 at an inoculum ratio of 1:1. The treatment method to use strain K84 as a biological control agent is simple: planting material such as seeds, seedlings roots or cuttings are immersed in a non-chlorinated tapwater suspension of strain K84 cells immediately before planting. The strain K84 bacteria should not be suspended in chlorinated water or exposed to high temperatures or direct sunlight which may damage their effectiveness (New & Kerr, 1972; Kerr, 1980; Clare, 1990).

Strain K84 acts as a preventative agent which means that if T-DNA is transferred into plant cells it is too late to control crown gall induction. However, by applying a large population of strain K84 to roots, seeds or cuttings in advance of infection, by immersing the plant material at the time of transplanting, K84-sensitive pathogens are inhibited before infection and the biological control process comes into play (Kerr, 1980; Farrand, 1990; Ryder & Jones, 1990). Strain K84 not only prevented gall formation by the pathogen but also reduced the population of tumorigenic bacteria around the roots and crowns of plants growing in the infested soil (Farrand, 1990). Strain K84 competes with pathogenic agrobacteria for the opines produced by the transformed plant cells. Competition by strain K84 is facilitated by its potential to

produce an antibiotic-like compound, agrocin 84, to which many pathogenic strains are sensitive (Ellis *et al.*, 1979; Kerr & Tate 1984; Kerr 1989).

The non-pathogenic *Agrobacterium* strain K84 has been successfully used in many parts of the world for the past 20 years in the biological control of certain crown gall pathogens. Strain K84 and its derivatives are not effective against all crown gall pathogens (Donner, *et al* 1993; Panagopoulos *et al.*, 1979; Burr *et al.*, 1993; Lopez *et al.*, 1989; Vicedo *et al.*, 1993; Penalver *et al.*, 1994). Production of the antibiotic like compound , agrocin 84, was thought to be the major reason for the success of strain K84. The continued success of this strain has been jeopardised by the possibility of transfer of the agrocin 84 plasmid to pathogenic agrobacteria.

Breakdown of biological control by K84 was demonstrated to be possible via the transfer of the plasmid responsible for agrocin production, pAgK84, into pathogenic agrobacteria (Ellis *et al.*, 1979; Panagopoulos *et al.*, 1979; Stockwell *et al.*, 1990). Transconjugant strains were resistant to agrocin 84 and no longer subject to control by strain K84 (Ellis *et al.*, 1979). Through genetic engineering Jones *et al* (1988) constructed a transfer deficient deletion derivative of K84 which they designated strain K1026. A part of the transfer region (Tra) from pAgK84 was removed by deleting 5.9 kb from the plasmid. This achievement is significant because the agrocin 84 producer plasmid (pAgK1026) can no longer be transferred to other agrobacteria. Jones and Kerr (1989) found that strain K1026 was as effective as K84 in controlling crown gall on the seedlings of almond planted in soil infested with a *A. rhizogenes* pathogen. Strain K1026 has been approved for commercial release as a pesticide in Australia with the trade name No Gall™ (Kerr, 1989). The efficacy of strain K1026 against agrocin 84 sensitive and resistant strains of *A. tumefaciens* has been tested by Vicedo *et al*

(1993). They observed that strain K1026 is able to control both agrocin 84 sensitive and some resistant strains.

A. rhizogenes strain K84 contains three plasmids, pAgK84, pAgK84b and pAgk434 (Donner *et al.*, 1993). pAgK84 and pAgK434 encode agrocin 84 and 434 respectively, pAtK84b encodes nopaline and agrocinopine catabolism (Ryder *et al.*, 1987; Clare *et al.*, 1990). This plasmid also causes incompatibility to tumor inducing Ti plasmids of the Inc Rh1 incompatibility group of *Agrobacterium* pathogens (Clare *et al.*, 1990).

1.4.1 *Agrocins*

Agrocins are low molecular weight antibiotic compounds that are produced by certain *Agrobacterium* strains and inhibitory to other agrobacteria (Kerr and Tate 1984; Clare 1995). *A. rhizogenes* strain K84 and its genetically engineered derivative, strain K1026 produce agrocin 84 and 434 (Clare, 1995). Agrocin production also has been reported in other *Agrobacterium* strains, K108 (Kerr and Htay 1974), H100, T37 (Stonier, 1960), D286 (Hendson *et al.*, 1983), J73 (Webster *et al.*, 1986) HLB-2 (Chen and Xiang 1986) and K434 and K1143 (Donner *et al.*, 1993; McClure *et al.*, 1994). More information is available about agrocin 84 than the other agrocins. One of the important mechanisms for the biocontrol of soil-borne bacterial disease is the production of antibacterial compounds by the biocontrol agent. Antibiosis may be facilitated by production of toxic secondary metabolites or specific antibiotics such as agrocins. The factors which affect antibiotic effectiveness in the rhizosphere are inactivation of the antibiotic by binding with soil colloids, biodegradation by other soil microorganisms, chemical degradation, induction of resistance by the target pathogen and inadequate production

of inhibitory compound by the biocontrol agent (Alconero, 1980; Moore and Warren, 1979; Gould 1990).

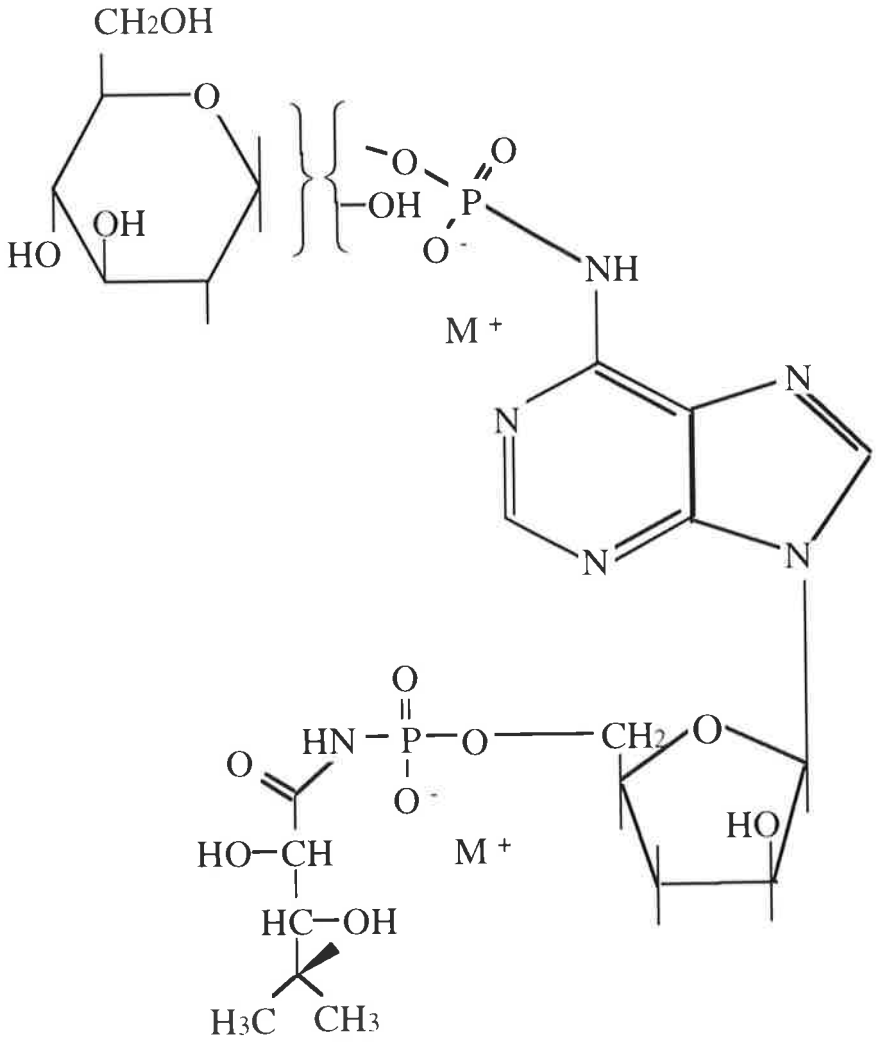
1.4.2 *Agrocin 84*

Production of agrocin 84 by strain K84 is encoded by a 47.7 kb conjugal plasmid, designated pAgK84 (Ellis *et al.*, 1979; Slota and Farrand 1982, Farrand *et al.*, 1985; Clare *et al.*, 1990). Agrocin 84 is a disubstituted adenine nucleotide analogue (Ellis and Murphy, 1981) (Fig 1.1). It contains 3'-deoxy-D-arabinose in place of ribose and thus lacks a 3' OH group. Agrocin 84 may act as a DNA replication inhibitor by causing premature chain termination (Murphy *et al.*, 1981). Agrocin 84 is ethanol-soluble, heat stable, and has biological activity in a wide range of pH from 4 to 9 (Moore & Warren 1979).

Results from transposon (Tn*5*) insertion mutagenesis indicated that agrocin 84 synthesis was encoded by a 21 kb region of the plasmid pAgK84 (Farrand *et al.*, 1985; Ryder *et al.*, 1987). Wang *et al.* (1994), used a combination of mutagenesis with Tn3HoHo1 and complementation analysis to show that the 21 kb segment of pAgK84 encoding agrocin 84 biosynthesis genes is organized into five identifiable complementation operons, which they have called *agnA* through *agnE.* They also found that the expression level of each *agn* locus was not affected by the presence of the other *agn* genes or by the presence or absence of the large nopaline plasmid, pAtK84b, present in *Agrobacterium* strain K84. The expression levels were not influenced by the addition of opines or root exudates to the culture medium. They have also demonstrated that the *agn* loci are expressed *in planta*, and presence of pAtK84b does not affect *agn* expression *in planta.* These results support the conclusion that

Fig 1.1 Structure of agrocin 84, a 3' -deoxyarabinofuranosyladenine bisphosphoramidate.

AGROCIN 84: A 3'-DEOXY-ARABINOFURANOSYL ADENINE BIS-PHOSPHORAMIDATE NUCLEOTIDE



agrocin 84 production in soil or on plant roots by strain K84 plays an important role in the biocontrol process of crown gall disease, but do not provide direct evidence for this. Immunity/resistance to inhibition by agrocin 84 is encoded independently by two regions of pAgK84 (Ryder *et al.*, 1987; Wang *et al.*, 1994).

Agrocin 84 sensitivity is correlated with the Ti or Ri plasmid characteristics and ability to utilize opines. On the basis of opine synthesis by the transformed plant tissue and the catabolic system of the pathogenic agrobacteria, *Agrobacterium* strains harbouring Ti or Ri plasmids can be classified into three major groups: nopaline, octopine and agropine (Kerr & Tate 1984; Dessaux *et al.*, 1992). A glucofuranoside substituent at the N^6 position is required for specific, high affinity uptake of the agrocin 84 by the Ti plasmid encoded metabolic system. The *acc* genes on Ti plasmids are responsible for the transport and catabolism of the opines agrocinopineA and B. Agrocin 84 inhibits only strains carrying a Ti plasmid with *acc* genes and nopaline/agrocinopine A catabolic functions (Ellis and Murphy, 1981, Hayman and Farrand 1988; Hayman *et al.*, 1993). Strain K84 does not control crown gall under all situations. Particularly, octopine strains of *A. tumefaciens* and strains that cause crown gall in grapevine, *A. vitis*, are resistant to agrocin 84 and therefore not subject to control. In some cases, agrocin 84-resistant strains of *A, tumefaciens* and*A. rhizogenes* have been partially controlled by strain K84. This may be due to its ability to colonize roots effectively and/or involvement of other inhibitors produced by strain K84 (Moore 1977; Lopez *et al.*, 1989; Vicedo *et al.*, 1993). Genetic factors influencing agrocin 84 production have been studied by Ryder *et al.* (1987) who observed that agrocin 84 production was unaffected by nopaline, agrocinopine A, acetosyringone, or low or high level of ferric iron. Formica (1990) studied a range of sugars and sugar alcohols and observed that D-fructose to be the best sugar for agrocin 84 production. Dickie and Bell (1995) observed that factors such as pH, temperature, medium, strain of pathogen and strain of antagonist influenced the antagonistic action of endophytic bacterial isolates from grapevines against virulent *A. vitis*. The mechanism of action of agrocin 84 to inhibit

gall formation by pathogenic strains has been studied by Smith and Hindley (1978). They found that agrocin 84 blocks attachment of the pathogen to tobacco cells by interfering with the synthesis of the outer cell envelope of the bacteria, thus affecting binding of the pathogen to the plant cell and transfer of T-DNA into the plant genome.

1.4.3 *Agrocin 434*

Production of agrocin 434 by strain K84 is encoded by a large plasmid (300-400 kb in size), designated pAgK434 (Donner *et al.*, 1993). Agrocin 434 is synthesized by strain K84 and its derivatives K1026, K434, K1143 (Fajardo *et al.*, 1994; Clare, 1995). The chemical structure of agrocin 434 (Fig 1.2) is a disubstituted cytidine (Fajardo *et al.*, 1994; Clare, 1995; Tate *et al.*, 1995). Preliminary results have shown that agrocin 434 is less inhibitory than agrocin 84 but inhibits a wider range of *A. rhizogenes* strains of *Agrobacterium in vitro* (Donner *et al.*, 1993; Fajardo, 1995; McClure *et al.*, 1994). Only *A. rhizogenes* strains that produced agrocin 434 were resistant to agrocin 434. However, no *A. tumefaciens* , *A. vitis* or *A. rubi* strains were sensitive to agrocin 434 on Stonier's medium.

1.4.4 *Other biocontrol agents*

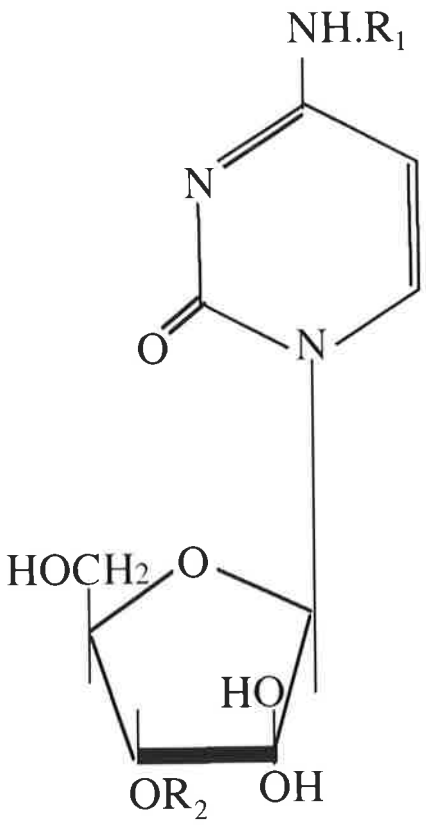
Cooksey and Moore (1980) reported that isolates of *Penicilium* and *Bacillus* reduced gall formation by virulent *Agrobacterium* strains but were not as efficient as K84. A non-pathogenic *Agrobacterium* strain, D286, produces an agrocin with a wider host

Fig 1.2 The chemical structure of agrocin 434. Agrocin 434 is a disubstituted cytidine nucleoside.

AGROCIN 434

R_1 = Disaccharide (β-1,4- glucuronopyranosyl-α-1,N^4-glucopyranosyl) (Fajardo, 1995)

R_2 = Small Acid (enol ether of pyruvic acid amide) (Tate *et al.*, 1995)



range than agrocin 84. It inhibited strains carrying octopine, agropine and nopaline Ti plasmids (Hendson *et al.*, 1983), but it was not as effective as K84 in preventing tumor formation (Van Zyle *et al.*, 1986). Bakanchkova *et al.* (1993) found that the nitrogen-fixing bacterium *Azospirillum brasilense* effectively inhibited the development of crown gall by *A. tumefaciens* strains W15955 or 460. Other fungal bacterial antagonists and some marine algal species have been tested against several of the pathogenic agrobacteria and reduced the incidence of galling by these pathogens. The antagonists were: *Penicillium*, *Trichoderma* (Moore & Warren, 1979); *Bacillus* (Moore & Warren, 1979; Utkhede & Smith, 1993); *Pseudomonas* (Moore & Warren, 1979; Dowling & O'Gara, 1994; Khmel *et al.*,1995) and three marine algal species: *Codium tomentosum*; *Jania ruben* and *Padina pavonia* (El-Masry *et al.*, 1995).

Kerr and Panagopoulos (1977) reported that strain K84 was ineffective as a biocontrol agent for grapevine crown gall caused by *A. vitis*. Gall formation by *A. vitis* strains is host specific and most pathogenic isolates have a narrow host range Ti plasmid (Inze *et al.*, 1984). For this reason there is a close association between the pathogen and host plant. An effective biological control agent for *A. vitis* needs to colonise grapevine as well as the pathogens. *Agrobacterium* strain K84 is unable to attach to grapevine cells and is not effective in controlling crown gall disease in grapevines. Recently, Burr and Reid (1994) found that *A. vitis* strain F2/5 effectively controlled tumor formation on grapevines by pathogenic *A. vitis* strains. The efficacy of the non-pathogenic strain HLB2 in controlling grapevine crown gall disease was assessed by Pu and Goodman (1993) and it was shown to reduce gall formation when inoculated before the pathogen or at a higher concentration.

1.5 *Objectives of this study*

The biological control agents *A. rhizogenes* strain K84 and its genetically engineered derivative, K1026, have been used successfully for a number of years to control crown gall in stone fruits and ornamentals. Strain K84 produces a potent inhibitory agent agrocin 84. A number of researchers have suggested that factors of control other than agrocin 84 production may contribute to the biocontrol of crown gall by strains K84 and K1026 against agrocin 84 resistant pathogens (Cooksey & Moore, 1982; Lopez *et al.*, 1989; Wang *et al.*, 1994; Vicedo *et al.*, 1993; Penalver *et al.*, 1994). One of these factors may be the effect of another antibiotic, agrocin 434, produced by *A. rhizogenes* strains K84, K1026 and K434 (Donner *et al.*, 1993).

The aims of the present study were:

1- To study the role of agrocin 434 and 84 and also other possible contributing factors in the biological control of *Agrobacterium* pathogens.

2- To isolate derivatives of K84 carrying different combinations of plasmids pAgK84, pAgK434 and pAtK84b and complete the range of the K84 derivatives available. These derivatives were tested in bioassays against pathogenic strains to provide further information of the role of each plasmid in the biocontrol process.

3- To develop a rapid plant bioassay to assess the efficacy of *Agrobacterium* biocontrol strains using leaf disc tumorigenesis in comparison to whole plant and stem inoculation bioassays.

4- To characterise agrocin 434 synthesis and immunity genes. The largest of the K84 plasmids, pAgK434 (300-400kb), has been shown to encode functions involved in the

synthesis of agrocin 434 (Donner *et al.*, 1991; 1993). The biosynthetic genes need to be localized on this large plasmid.

Genetic characterization of agrocin 434 biosynthetic genes may improve understanding of the factors regulating production and the role this compound plays in the biocontrol process.

Chapter Two

Construction of a range of derivatives of strain K84

2.1 Introduction

Biological control of pathogenic strains of *Agrobacterium* has been achieved using the non-pathogenic strain, *A. rhizogenes* K84, and, more recently, a genetically engineered derivative of K84 designated K1026 (Jones *et al.*, 1988; Jones and Kerr, 1988; Kerr, 1989). *A. rhizogenes* strain K84 contains three plasmids pAgK84, pAtK84b and pAgK434 (Donner *et al.*, 1993). Both strains K84 and K1026 produce an antibiotic, agrocin 84, which specifically inhibits *Agrobacterium* pathogen activity (Jones and Kerr, 1989). A number of researchers have suggested that other mechanisms of control may be involved (Cooksey and Moore, 1982; Lopez *et al.*, 1989). One of these mechanisms may be the effect of another antibiotic, agrocin 434, which is produced by *A. rhizogenes* strains K84, K1026, K434 and K1143, a K434 derivative strain lacking the pAtK84b plasmid (Donner *et al.*, 1993). Other factors thought to play a role in the biocontrol process include production of additional inhibitory compounds and competition for nutrients/ infection sites (Penalver *et al.*, 1994; Farrand and Wang, 1992). The objectives of this study were to construct a range of derivatives of strain K84, with every combination of the three resident plasmids, to assess their efficacy in controlling pathogens. These strains can then be tested by root inoculation of almond seedlings and tomato stem inoculation to evaluate the role of each plasmid in the biological control process.

Previously, strain K434, a spontaneous mutant of K84 lacking pAgK84, and strain K1143, K434 cured of pAtK84b, have been constructed. These two strains produce agrocin 434 (Donner *et al.*, 1993; Clare 1995; Fajardo *et al.*, 1994). Essential steps in the biosynthesis of agrocin 434 are encoded on pAgK434, the largest of the reported strain K84 plasmids (Donner *et al.*, 1993).

pAtK84b encodes nopaline and agrocinopine catabolism (Ryder *et al.*, 1987, Clare *et al.*, 1990). pAtK84b also causes incompatibility to tumour inducing (Ti) plasmids of pathogenic *Agrobacterium* strains in the Inc Rh1 group and may play an important role in biological control by preventing transfer of Ti plasmids to the biological control strain (Clare *et al.*, 1990)

Strain K84 has been used successfully for the biological control of crown gall disease since 1974. Most workers have investigated the ability of strain K84, and particularly the role of agrocin 84, in the biocontrol process (Ryder & Jones 1990, Clare 1993, Jones *et al.*, 1991, Smith, 1978, Moore 1977, Moore 1979, Formica, 1990, Farrand, 1990, Htay and Kerr 1974). Therefore more information is available about strain K84 than derivatives. There is little information available about the biological activity of the pAtK84b and pAgK434 plasmids. Production of agrocin 84 by strain K84 is encoded by a 48 kb conjugal plasmid, designated pAgK84 (Ellis*et al.*, 1979; Slota and Farrand, 1982). It is thought to play an important role in the control of crown gall. In some cases, agrocin 84-resistant strains of *A, tumefaciens* and *A. rhizogenes* have been partially controlled by strain K84. This may be due to its ability to colonize roots effectively and/or involvement of other inhibitory compound produced by strain K84 (Moore 1977; Lopez *et al.*, 1989; Vicedo *et al.*, 1993).

Previously, strains K84, K434, K1143 (Donner *et al.*, 1993) K1347 (McClure *et al.*, 1994) K1356 and K1357 (NC McClure, unpublished) have been isolated (table 1).

The agrocin 434$^-$ derivatives of K1143 with Tn*5* insertions had been isolated previously by NC McClure as follows: transposon Tn*5*:Mob was transferred from *E.coli* strain S17.1 (pSup5011, Simon *et al.*, 1984) to K1143. Recipients all carried Tn*5* in either the plasmid, pAgK434 or chromosome. About ten thousand transconjugants were patch mated with *E.coli* C600pNJ5000 as a donor to introduce moblization functions. Transconjugants carrying pNJ5000 were then used as donors to mobilise pAgK434::Tn*5*:Mob to the bv1 plasmidless strain K749. This was done to distinguish between insertions of Tn*5*:Mob into plasmid pAgK434, and insertions into the chromosome. Only plasmid insertions would result in strains capable of transferring the Tn*5*-encoded kanamycin resistance to the K749 recipient. The K749 transconjugants were then tested for ability to produce agrocin 434 by bioassay and any negative strains confirmed by HVPE. From this test, K1356 and K1357 were identified and picked from the original mating plate (without pNJ5000). These strains are therefore derivatives of K1143 with Tn*5*:Mob insert in pAgK434 and are agrocin 434$^-$.

The plasmid free strain K1347, was isolated by NC McClure from a derivative of K1143 carrying Tn*5* in pAgK434 isolated using the same method described above. Plasmid pAgK434::Tn*5* was cured by growth at 34 °C for three days and testing for loss of kanamycin resistance. Kanamycin sensitive derivatives were readily isolated and tested for agrocin production and plasmid content. One derivative, K1347 was confirmed agrocin 434$^-$, kanamycin sensitive and plasmid free.

A transposon is a discrete element of DNA which can transpose from one site within a bacterial cell to another. Insertion sites may be random or in some cases there are preferred sites of integration. As a result of insertion a gene may be physically disrupted, resulting in a mutation. Transposons often carry genes for antibiotic resistance which can be used as selective agents for mutagenesis experiments and can also be used to introduce additional selective markers into plasmids for use in transfer experiments. Plasmids which are not self-transmissible (i.e..non-conjugative) may be mobilized to recipients if they carry a suitable origin of transfer (oriT) and the necessary transfer functions are supplied *in trans*. The IncP-type specific recognition site (Mob site) has been cloned from RP4 and introduced into transposon Tn*5* (Simon,R., 1984). As a result, plasmids carrying Tn*5*:Mob can be mobilized to suitable recipients if transfer functions are supplied by a co-resident plasmid such as RP4 or derivatives. The Mob site can also be introduced into plasmids by homologous recombination between the target plasmid and a plasmid carrying the Mob site and a cloned fragment from the target plasmid which acts as the region of homology.

As a result of a previous attempt to isolate Tn*5* inserts into pAgK434 an agrocin 434$^-$ derivative, K1318 was isolated (Jones & Donner, personal comm.). K1318 carries a single copy of Tn*5* in the chromosome and its inability to produce agrocin 434 is probably the result of a large deletion from pAgK434 giving rise to pAgK434Δ (pAgK1318, N. McClure, unpublished). K1318 produces a modified agrocin (nucleoside 4176), presumably a precursor of agrocin 434 which has no inhibitory activity and can be distinguished from agrocin 434 by HVPE. The Mob region of RP4 and a chloramphenicol resistance marker were introduced into pAgK1318 by recombination with a derivative of pBR325 carrying the Mob region and a cloned fragment of pAgK1318. Two independently isolated cointegrate plasmids, designated pAgK1318:Mob4 and pAgK1318:Mob5 were obtained. These plasmids were transferred into a spontaneous streptomycin resistant derivative of strain K1143 for future transfer experiments.

The objective of the work described here was to complete the range of the K84 derivatives available carrying different combinations of plasmids pAgK84, pAgK434 and pAtK84b. These derivatives could be tested in bioassays against pathogenic strains to provide further information about the role of each plasmid in the biocontrol process. To allow standardisation of inoculum levels with the various K84 derivatives, growth curves for key strains were also prepared.

The second major objective was to mobilize pAgK434 to a strain sensitive to agrocin 434 and determine whether resulting transconjugants aquired both the ability to produce agrocin 434 and resistance/immunity to this compound, and to determine if the deleted pAgK434 derivative, pAgK1318 also carried resistance/immunity to agrocin 434. This required the mobilization of pAgK1318:Mob4/5 to the susceptible pathogenic strain K27. As chloramphenicol resistance proved to be a poor selective agent in transfer experiments with strain K27 as recipient, it was necessary to introduce an additional marker, the kanamycin resistance gene of Tn*5* into pAgK1318:Mob4/5.

2.2 Materials and Methods

2.2.1 *Media and* strains

The *Agrobacterium* strains used in this study and their relevant characteristics are shown in table 2.1. Details of media preparation are given in Appendix A.

2.2.2 *Transfer of nopaline catabolic plasmid by conjugation*

The nopaline catabolic plasmid, pAtK84b, is a conjugative plasmid and conjugal transfer is induced when the host strain is grown with nopaline or agrocinopine A (Ellis *et al.*, 1979), the following patch mating method was used :

The patches of donor strain K815 with pAtK84b were replica plated on the recipient lawn strain K1347 on TY agar medium with nopaline (125 mg/ml). Transconjugants were selected on bv2 medium with nopaline as the sole selective carbon source.

2.2.3 *Conjugal transfer of pAgK84:Tn5 and pAgK84*

The pAgK84 plasmid has the ability to be transferred by conjugation to *Agrobacterium* hosts (Farrand *et al.*, 1985). To transfer pAgK84 and

Table No.2.1 *Agrobacterium* strains and plasmids used in experiments described in this chapter.

Strain	Plasmids	Agrocin	Description/Source
A. rhizogenes K27	pTiK27 pAtK27	none	Pathogenic strain sensitive to agrocin 84 & 434. A.Kerr; Collection (New & Kerr, 1972)
A. rhizogenes K84	pAgK84 pAtK84b pAgK434	84, 434	Peach gall, S. Australia A. Kerr (New & Kerr, 1972).
A. rhizogenes K434	pAtK84b pAgK434	434	Spontaneous mutant of K84 lacking pAgK84 (Shim *et al.*, 1987)
A. rhizogenes K1143	pAgK434	434	K434 cured of pAtK84b (Donner *et al.*, 1993).
A. rhizogenes K1352	pAgK84	84	Transconjugant of mating between K1347xK325 this study.
A. rhizogenes K1353	pAgK84 pAtK84b	84	Transconjugant of K1347 and K325; this study.
A. rhizogenes K1355	pAgK84::Tn*5* pAgK434	84,434	Transconjugants of mating between K1143xK1295; this study.
A. rhizogenes K1351	pAtK84b	none	Transconjugants of K1347 and K815; this study.
A. rhizogenes K1347	-	none	K1143 cured of pAgK434; NC McClure.
A. tumefaciens K325	pAgK84 pAtK84b NocCTraC	84	Rif, Str, Spe, Ery resistant, constitutive for octopine and nopaline catabolism and transfer; A. Kerr collection.
A. tumefaciens K815	pAtK84b	none	Rif, Str resistant A. Kerr ; collection.
A. tumefaciens K1295	pAgK84::Tn*5*	84	Km resistant; S. Farrand.
A. rhizogenes K1356	pAgK434::Tn*5*	none	Km resistant; NC McClure
A. rhizogenes K1357	pAgK434::Tn*5*	none	Km resistant; NC McClure

pAgK84::Tn*5* the patch mating method on TY medium was used as described above. Transconjugants carrying pAgK84::Tn*5* were selected on bv2 medium with kanamycin 200(mg/ml), and transconjugants carrying pAgK84 were selected on bv2 medium. As this medium did not select against the recipient strain, all putative transconjugants were tested for production of agrocin 84 by HVPE and bioassay.

2.2.4 *3-Ketolactose production test*

To test 3-ketolactose production by *Agrobacterium* strains, the method of Bernaerts and De Ley (1963) was used. The *Agrobacterium* strains were grown on lactose agar containing 20 g agar, 10 g lactose, and 1 g yeast extract per litre water at 28 °C for 2 days. The plates were then flooded with 10 ml of Benedict's reagent. The reagent used was: Solution A: sodium citrate (173 g), sodium carbonate (100 g) dissolved in 600 ml water by heating and made up to 850 ml; Solution B: copper sulphate (18 g) dissolved in 100 ml water, made up to 150 ml and then slowly added to Solution A. On flooding plates with reagent the positive strains (bv 1) produced a bright yellow-orange zone around colonies which produce 3-ketolactose.

2.2.5 *Growth on 2% NaCl*

Oxoid nutrient broth media with 2% Na Cl was used for this test. The *Agrobacterium* strains were grown on this media for 10 days at 28°C.

2.2.6 *Growth at 37°C*

Slopes of Oxoid nutrient agar were inoculated with each strain and incubated at 37 °C up to 10 days.

2.2.7 *Plasmid isolation and electrophoresis*

Plasmid isolations from *Agrobacterium* strains were based on the Farrand *et al* ., (1985) miniprep procedure. The bacterial cultures were grown in 5 ml of nutrient broth (NB) for 2 days at 28 °C. Cells were collected by 2 min centrifugation in 1.5 ml micro-centrifuge test tubes, and washed in 1 ml of TE buffer. Then the pellet was resuspended in 100 μl 5M NaCl and 10 μl of 10% Na Sarkosyl and centrifuged. Next the supernatant was removed by aspiration, cells were resuspended in 100 μl of Solution 1: 50 mM Glucose, 10 mM Na_2 EDTA 25 mM Tris, HCl base, pH 8.0 with 2 mg/ml of Lysozyme and incubated on ice for 5 min. Bacterial cells were lysed by adding 200 μl of Solution 2: 0.2 N NaOH, 1% SDS and the tube contents were mixed by inversion. Clear lysates resulted after 10 to 15 min incubation at room temprature. 50 μl of 2 M TrisHCl, pH7.0 was added and the contents of the tubes mixed well by gentle inversion, and left to stand for 30 min at room temprature. NaCl (50 μl of 5M stock) was added, mixed by gentle inversion, then the mixture was extracted with an equal volume of phenol saturated with 3% NaCl.

The phases were mixed by gentle inversion maintaining an emulsion for 5 min. The aqueous phase was separated by centrifugation for 10 min at 4°C and removed using a wide tipped pipette to avoid shearing. DNA in the aqueous phase was precipitated

with 1/10 volume of 3 M sodium acetate and two volumes of cold absolute ethanol. After 15 min incubation at room temperature the DNA was collected by centrifugation for 10 min at 4°C, washed with 1 ml of 70% ethanol and the pellet dried under vacuum . The DNA was then dissolved in 20-40 μl of TE buffer and stored at 4°C.

Gel electrophoresis was performed in a horizontal submerged gel apparatus using 0.7% SeaKem ME Agarose (Sigma) in TEB buffer, (89 mM Tris, 2.5 mM EDTA, 89 mM Boric acid), pH 8.0 at 100 mA constant current for 2 hr. DNA was stained with 0.5 μg/ml ethidium bromide solution in TEB buffer.

2.2.8 *Agrocin 84 bioassay*

The agrocin producing strains were subcultured onto YM slopes for 3 days and then a loopful of inoculum was spot-inoculated onto a plate of Stonier's agar medium (Stonier, 1960). After two days of incubation at 28°C , the plates were inverted and a piece of filter paper was placed in the centre of each lid, moistened with several drops of chloroform. After 10 minutes, the lids were opened for another 10 minutes to allow the chloroform to evaporate.

The indicator strain (K27) and transconjugant strains were suspended in 5ml of sterile buffer saline. One ml of the suspension was added to 3ml of buffered soft agar at 45°C and poured on to the plate. After 1 or 2 days of incubation at 28°C the plates were examined. Sensitive strains showed a clear zone of inhibition around the K84 colony.

2.2.9 Agrocin purification

Agrobacterium strains were grown in a glucose-glutamate broth medium modified to contain 1.74 g of K_2HPO_4 and 5.44 g of KH_2PO_4 per litre. After incubation for 3 days at 28°C on a rotary shaker, the cells were removed by centrifugation and 1 ml of supernatant was mixed with 100 μl portions of an aqueous suspension of activated charcoal (15mg/ml). The charcoal was washed three times by suspension and centrifugation in 1 ml volumes of double distilled water. Then the charcoal was suspended in 200 μl of 70% ethanol, and the supernatant was collected afer centrifugation. This procedure was repeated twice more and the 600 μl eluants were combined and evaporated to dryness for 2-3 hr under vacuum. These crude extracts were each dissolved in de-ionized water to bioassay agrocin activity or were purified further by high paper electrophoresis (HVPE) (Donner ***et al.***, 1993).

2.2.10 *High Voltage Paper Electrophoresis (HVPE)*

Ten μl samples of crude extracts of agrocin 434 as well as a standard of agrocinopine A and orange G were applied to the centre of Whatman 3 mm paper (57 cm x 15 cm). The filter paper was then immersed in either 1.03 M acetic/0.75 M formic acid, pH 1.75 or 0.05 M citrate, pH 5-6 to advance the buffer slowly to the load origin and excess buffer was blotted off. The paper was placed in a percleen-coolea high voltage electrophoresis apparatus (Tate, 1968) and electrophoresed at 3000 volts for 10 min (formic acetic) or at 1500 volts for 30 min (citrate).

2.2.11 *Production of growth curves for Agrobacterium strains using TY broth*

Growth curves were produced for the *Agrobacterium* strains K27, K84, K1143 and K1347 in TY. Bacteria from 48 hr TY broth cultures were added to 150 ml TY broth to give initial concentrations of $5.75x10^6$, $3.8x10^6$, $8x10^6$ and $5.48x10^6$ CFU/ml for strains K27 , K84 , K1143 and K1347, respectively. Cells were grown on a rotary shaker at 28°C and 5 ml of culture were removed and the optical density (600 nm) measured every 2 hours for 30 hours , by which time all cultures had reached maximum OD. All isolates were tested at least in duplicate. Cell numbers were determined using plate counts and a standard curve of optical density versus cell numbers was prepared.

2.2.12 *Evidence that agrocin 434 resistance /immunity genes are on pAgK434*

Plasmid pAgK434::Tn*5* Mob was transferred from K1143 pAgK434::Tn*5* Mob pNJ5000 as donor to K27 Str^r in a patch mating on TY agar at 28°C overnight. Transconjugants were selected on TY agar containing 250 μg/ml streptomycin and 200 μg/ml kanamycin. After purifying transconjugants on the same medium, colonies with a single plasmid, pAgK434::Tn*5* Mob were tested for agrocin 434 synthesis capacity by bioassay and by HVPE. Eight transconjugants and the parent strains were tested for sensitivity to agrocin 434 and/or agrocin 84. Plasmid DNA from the putative transconjugants was gel electrophoresed to verify transconjugants as described by Eckhardt (1987), using 0.65% Seakem ME agarose in TEB buffer, at 7 mA for 20 min, then at 100 mA constant current for 2 hr. Gels were then stained with 0.5 μg/ml ethidium bromide in TEB buffer.

2.2.13 *Evidence that agrocin resistance/immunity functions are located on pAgK434Δ (pAgK1318)*

Tn*5* :*tdnC* was transferred from E.coli strain S17.1 (pNMB3) (McClure *et al.*, 1990; Saint & McClure, 1993) as a donor to K1143 Str^r pAgK1318:Mob4/5 in a filter paper disc mating on TY agar at 28°C overnight. Then K1143 Km^r, Cm^r colonies were selected on selective medium, Bergensen's Medium (BM) with kanamycin (200 μg/ml) and chloramphenicol (30 μg/m). Four hundred transconjugants were patch-mated with E.coli strain C600 pNJ5000 as a donor on TY agar at 28°C. Transconjugants were selected on BM with kanamycin (200 μg/ml), chloramphenicol (30 μg/ml) and tetracycline (10 μg/ml). After purifying on the same media, transconjugants were multiple patch mated on TY agar with K27 Rif^r, Str^r. This was done to distinguish between insertions of Tn*5* into pAgK1318:Mob4/5 and insertions into the chromsome. Only plasmid insertions would result in strains capable of transferring the Tn*5*-encoded kanamycin resistance to the K27 recipient. Three transconjugants were selected on TY agar with rifampicin (25 μg/ml), kanamycin (200 μg/ml) and streptomycin (250 μg/ml). These strains produced modified agrocin 434, which was confirmed by HVPE.

2.3 Results

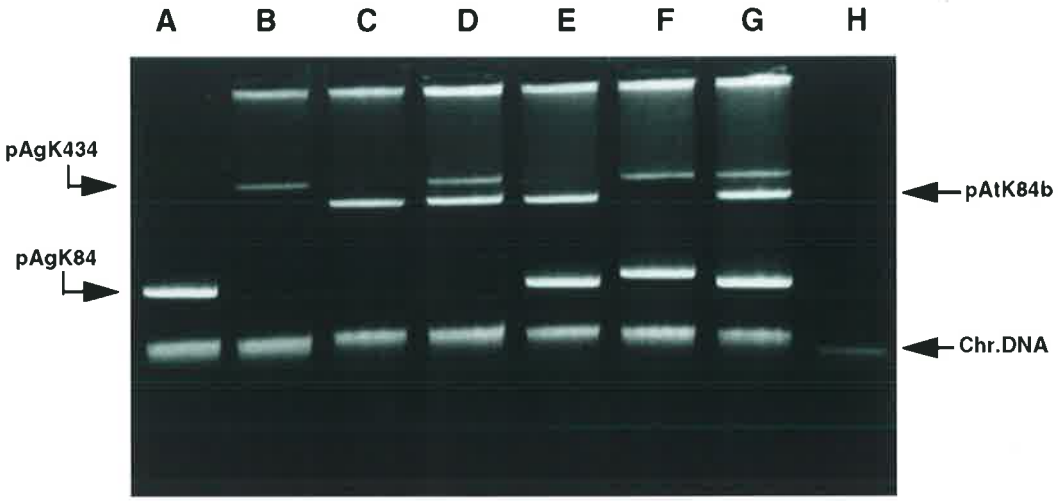
2.3.1 *Construction of a derivative of K84 carrying only plasmid pAtK84b*

Plasmid AtK84b was transferred from K815 as donor to the plasmidless strain, K1347, in a patch mating on TY agar medium with nopaline (125 mg/l) at 28°C overnight. Transconjugants were selected on bv2 medium with nopaline as sole carbon source. After purifying transconjugants on the same medium, transconjugants were tested for 3-ketolactose production, growth on 2% NaCl medium and maximum growth temperature. Presence of the pAtK84b plasmid was verified by agarose gel electrophoresis (Plate 2.1). One resultant product was strain K1351.

2.3.2 *Construction of K84 derivatives carrying pAgK84 +/- pAtK84b*

Plasmid pAgK84 was transferred from K325 as donor to K1347 in a patch mating on TY agar at 28°C. Putative transconjugants were tested for agrocin 84 production, 3-ketolactose production, growth on 2% NaCl and growth at 37°C. The identity of transconjugants was verified by gel electrophoresis. The resultant product of this conjugation was strain K1352. Plasmids pAtK84b and pAgK84 were transferred from K325 to K1347 in a patch-mating on TY agar medium with nopaline (125 mg/l) at 28°C for 24 hr. Transconjugants of this mating were selected on bv2 medium with nopaline (125 mg/l). Transfer frequencies of pAgK84 from donor to recipient was very low an average 2.5×10^{-3} per recipient and transfer frequencies of pAgK84 and pAtK84b from *A. rhizogenes* donor to *A. tumefaciens* recipient was approximately 6.6

Plate 2.1 Biocontrol strain K84 and its derivatives. Strain K84 carries 3 plasmids: pAgK84, encoding agrocin 84 synthesis and immunity to agrocin 84; pAtK84b, the nopaline catabolizing plasmid; and pAgK434, encodes functions involved in the biosynthesis of agrocin 434.



A - K1352; B - K1143; C - K1351; D - K434;
E - K1353; F - K1355; G - K84; H - K1347.

x 10^{-3} per recipient. Transconjugants were then purified on the same medium. The medium was not selective against the recipient, therefore transconjugants were identified by screening resulting colonies for agrocin 84 production, 3-ketolactose production, growth on medium with 2% NaCl and growth at 37°C. The identity of transconjugants were verified by gel electrophoresis. The resultant product of this conjugation was strain K1353.

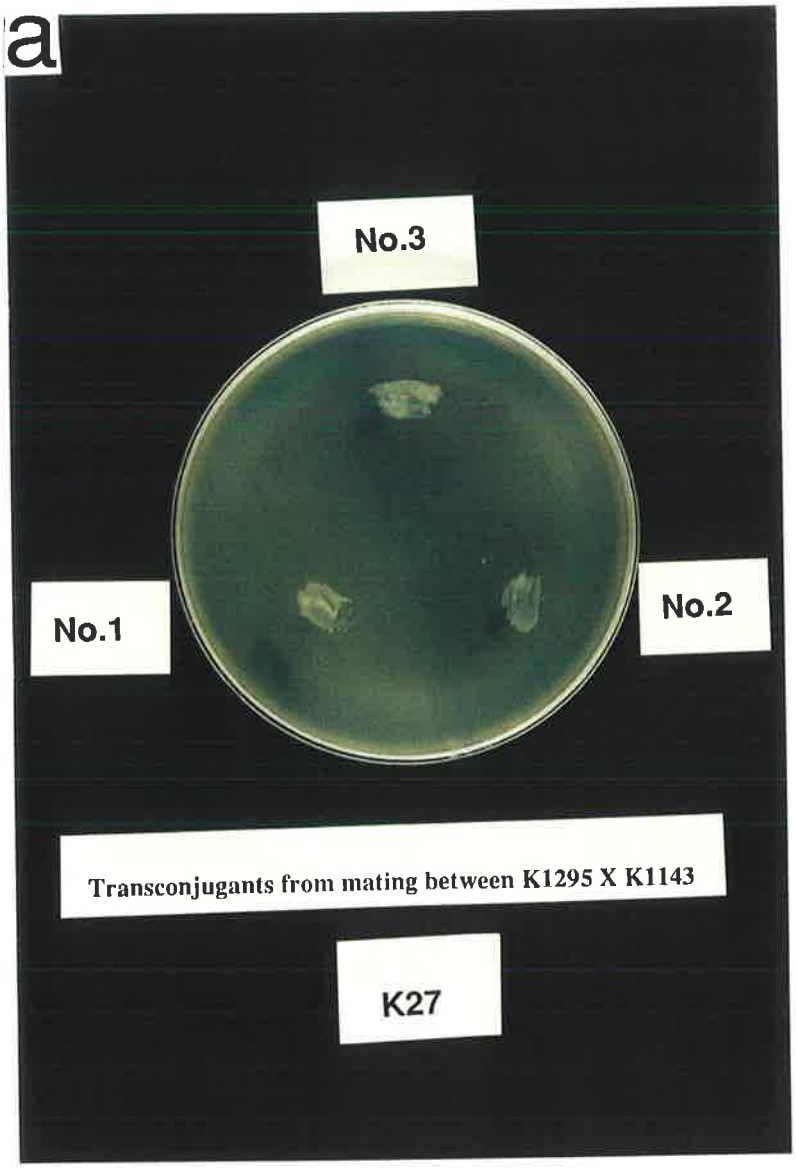
2.3.3 *Construction of K84 derivative carrying plasmid pAgK434 and pAgK84::Tn5*

Plasmid pAgK84b::Tn5 was transferred from strain K1295 (bv1) as donor to strain K1143 (bv2) in a patch mating on TY agar at 28°C. Transconjugants were selected on selective medium for bv2 with kanamycin (200 mg/l) at 28°C. The resulting transconjugants were tested for agrocin 84 and 434 production, 3-ketolactose production, growth on 2% NaCl, growth at 37 °C. The resultant product of this conjugation was strain K1355 (Plate 2.1, 2 and 3).

2.3.4 *Production of growth curves for Agrobacterium strains using TY broth*

Growth curves were produced for *Agrobacterium* strains, K1143, K1347, K84 and K27 in TY broth. The results from the growth curves of the *Agrobacterium* strains tested in TY broth indicate that there were no obvious differences in growth rate on this medium (fig 2.1,2.2). Bacterial populations reached maximum values of about 6.3 X 10^9 CFU/ml.

Plate 2.2 Inhibition of biovar 2 strain K27 by the donor biovar 1 strain K1295, which contains pAgK84:Tn5, the biovar 2 recipient strain K1143, and the resultant biovar 2 transconjugant K1355, which also contains pAgK84:Tn5 and pAgK434.


a
No.3
No.1
No.2
Transconjugants from mating between K1295 X K1143
K27

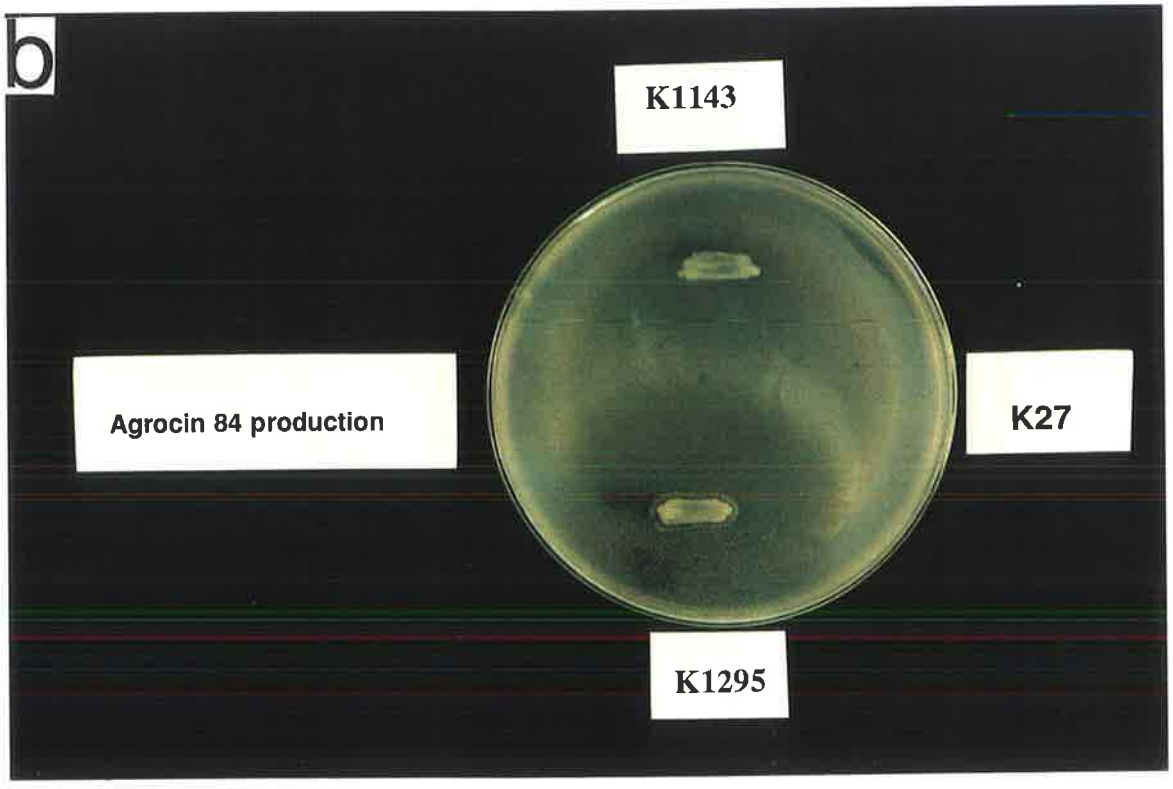
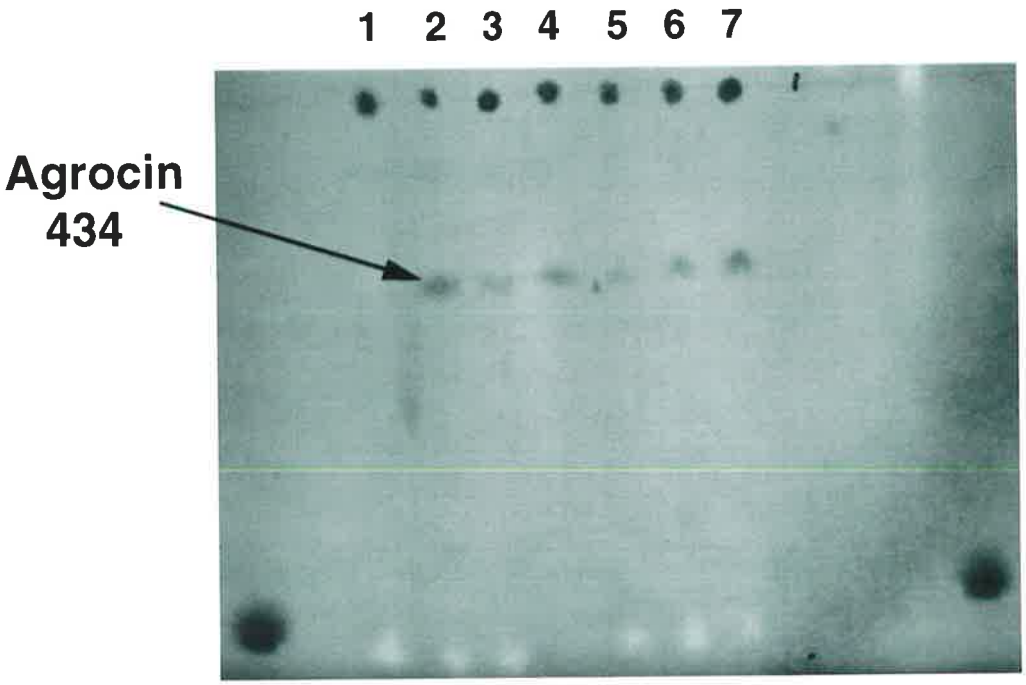

b
K1143
Agrocin 84 production
K27
K1295

Plate 2.3 High voltage paper electrophoresis of charcoal adsorbed fractions from liquid culture media. Electrophoresis was in 0.05 M citrate, pH 6.0 at 2 kV for 30 min: photographed using transmitted UV light.



1) K1295 pAgK84:Tn*5* ;
2,3,4,5,6) K1355 pAgK84, pAgK434
7) K1143 pAgK434

Fig No.2.1

Growth curve of Agrobacterium Strains

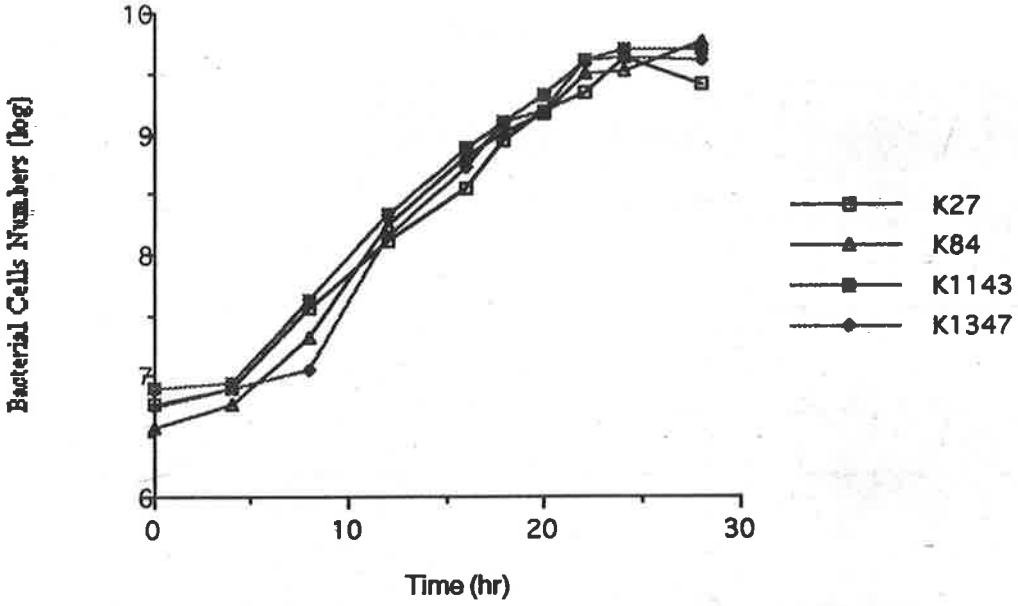
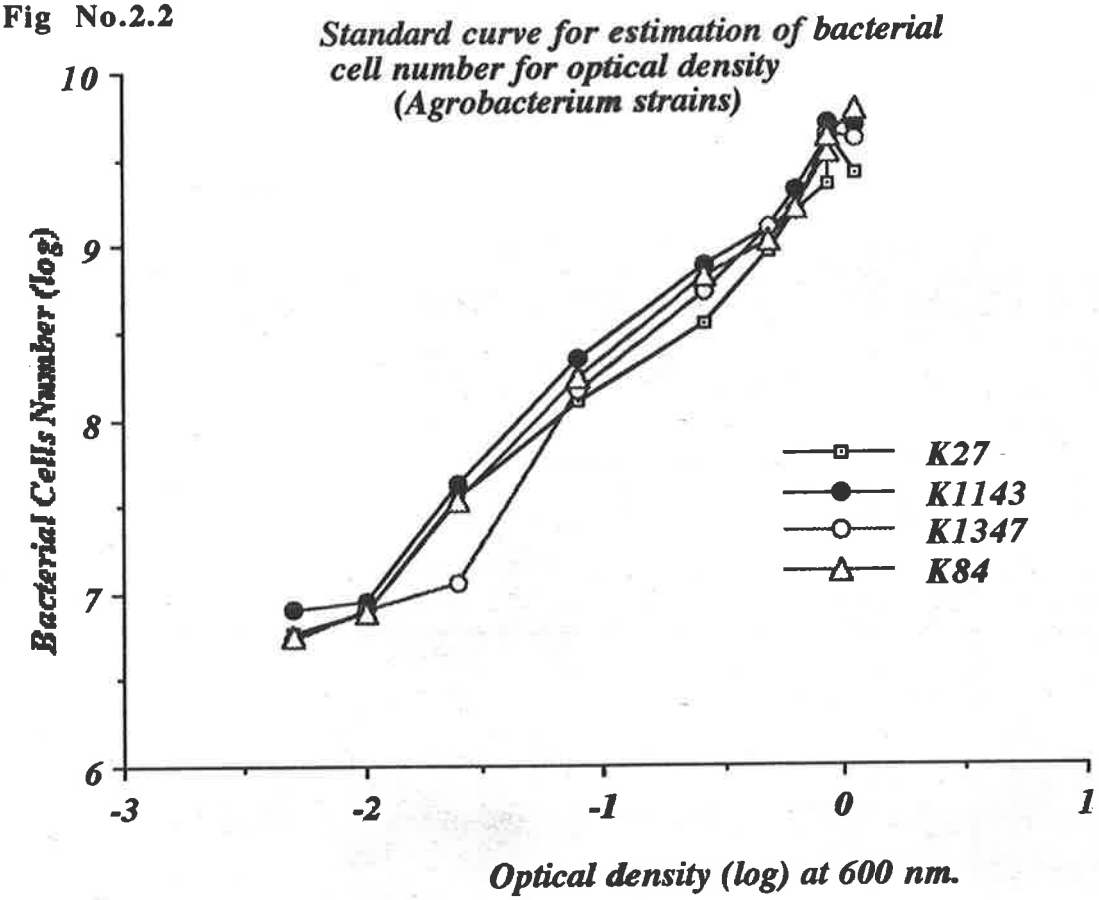


Fig No.2.2


Standard curve for estimation of bacterial cell number for optical density (Agrobacterium strains)
Bacterial Cells Number (log)
10
9
8
7
6
-3
-2
-1
0
1
K27
K1143
K1347
K84
Optical density (log) at 600 nm.

2.3.5 *Evidence that agrocin 434 resistance/immunity genes are on pAgK434*

The agrocin 434 plasmid, pAgK434::Tn*5* Mob, from biocontrol strain, K1143 carrying a Tn*5*:Mob insert was mobilized into the pathogenic strain K27, an *A. rhizogenes* recipient unable to produce agrocin 434. Putative transconjugants were obtained from the overnight mating and control plates exhibited no growth. When eight transconjugants were tested against *A.tumefaciens* strain K27 all these transconjugants had acquired the ability to produce agrocin 434. Using purified agrocin 434 all transconjugants produced an inhibition zone against the indicator strain, K27, as did K1143 (pAgK434) and K1143 (pAgK434::Tn*5*:Mob pNJ5000), Km^r. In contrast, all transconjugants were insensitive to agrocin 434 (Plate 2.4). All transconjugants tested were inhibited by agrocin 84, from strain K84, as was the K27 indicator strain. Plate 2.5 shows the plasmids of the parent strains K1143, K1143::Tn*5*:Mob pNJ5000,Km^r, K27 and transconjugants products of this mating. Plate 2.6 shows the corresponding HVPE electrophoretogram demonstrating agrocin 434 presence in K1143 and K1143 Tn*5* Mob pNJ5000,Km^r , transconjugants and its absence in K27.

The agrocin 434 plasmid pAgK434 of the biocontrol strain, K1143, was mobilized into a pathogenic strain K27, an *A. rhizogenes* recipient unable to produce agrocin 434. Resulting strains were recovered which were *A. rhizogenes*, contained the agrocin 434 plasmid and synthesized agrocin 434. The results of this study indicate that agrocin 434 resistance/immunity functions are located on pAgK434.

Plate 2.4 Bioassay of charcoal adsorbed fractions from liqiud media of cultures of the donor biovar 2 strain K1143, which contains pAgK434, the pathogenic biovar 2 strain K27, and the resultant transconjugant K27 pAgK434, pTiK27 and pAtK27. Preparations from K1143 and K27 pAgK434, pAtK27 and pTiK27 both produced zones of inhibition but the K27 pAgK434, pTiK27 and pAtK27 was not inhibited by K1143. K27 used as the indicator strain.

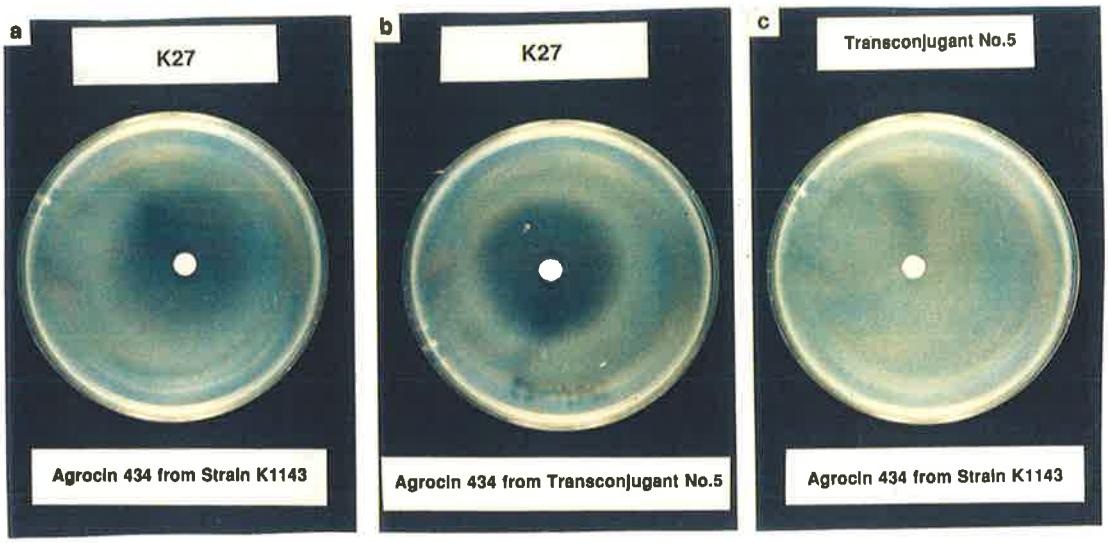
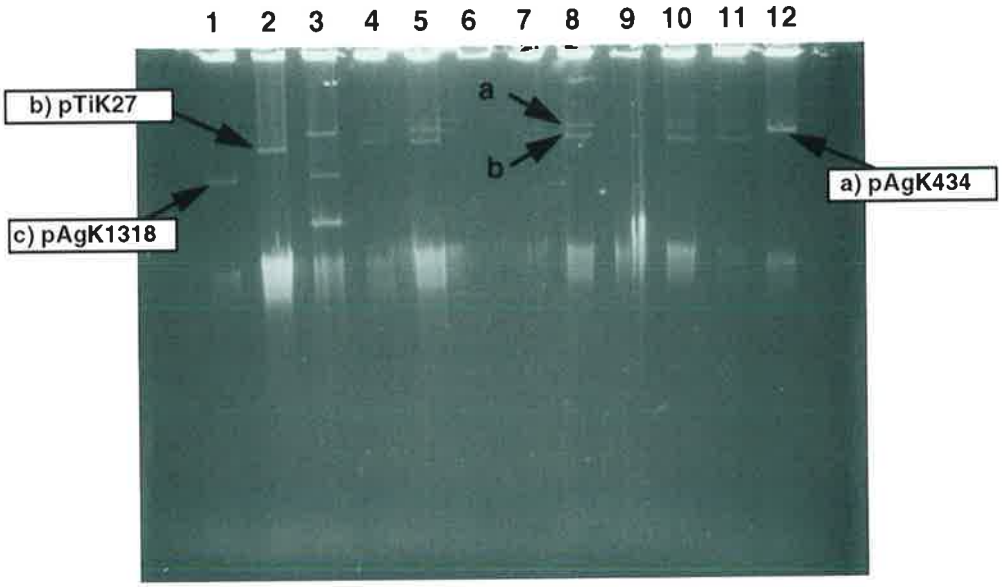
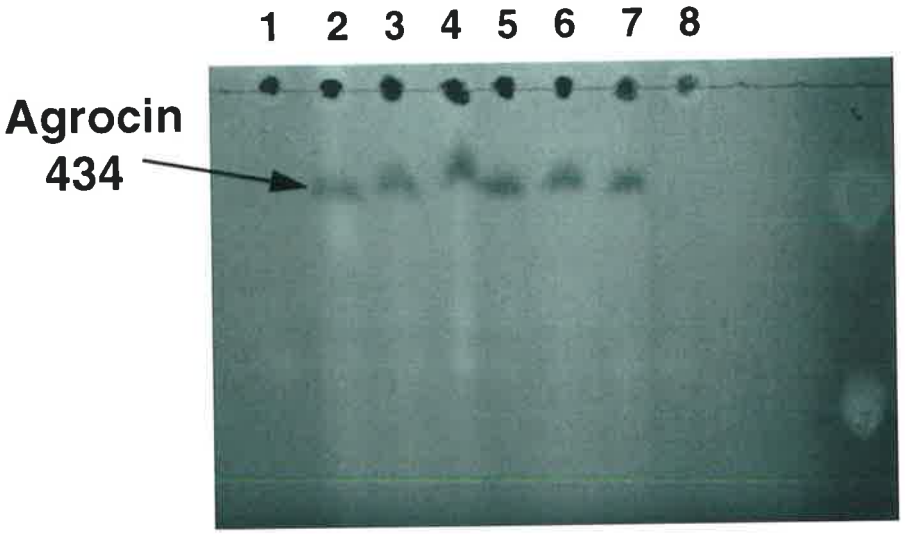

a
K27
Agrocin 434 from Strain K1143
b
K27
Agrocin 434 from Transconjugant No.5
c
Transconjugant No.5
Agrocin 434 from Strain K1143

Plate 2.5 Plasmids of *Agrobacterium* strains separated by electrophoresis in 0.7% agarose gel.



1) K1318; 2) K27; 3) K1143 Tn*5* :mob pNJ5000;
4,5,6,7,8,9,10,11) K27 pAgK434, pTiK27 (Transconjugants)
12) K1143 pAgK434.

Plate 2.6 High voltage paper electrophoresis of charcoal adsorbed fractions from liquid culture media. Electrophoresis was in 0.05 M citrate, pH 6.0 at 2 kV for 30 min: photographed using transmitted UV light.



1) K1318; 2)K1143;
3,4,5,6) K27 pAgK434 pTiK27 (Transconjugants)
7) K1143 pAgK434 Tn*5* :mob pNJ5000; 8) K27

2.3.6 *Evidence that agrocin resistance/immunity functions are located on pAgK434Δ (pAgK1318)*

The kanamycin resistance transposon Tn*5* was introduced into pAgK1318:Mob4/5 as described in material and methods. Plate 2.7 shows corresponding HVPE results demonstrating agrocin 434 presence in K1143. Two of the transconjugants chosen produced modified agrocin as did K1143 (pAgK1318). One potential transconjugant 5AxK27 did not produce the modified agrocin.

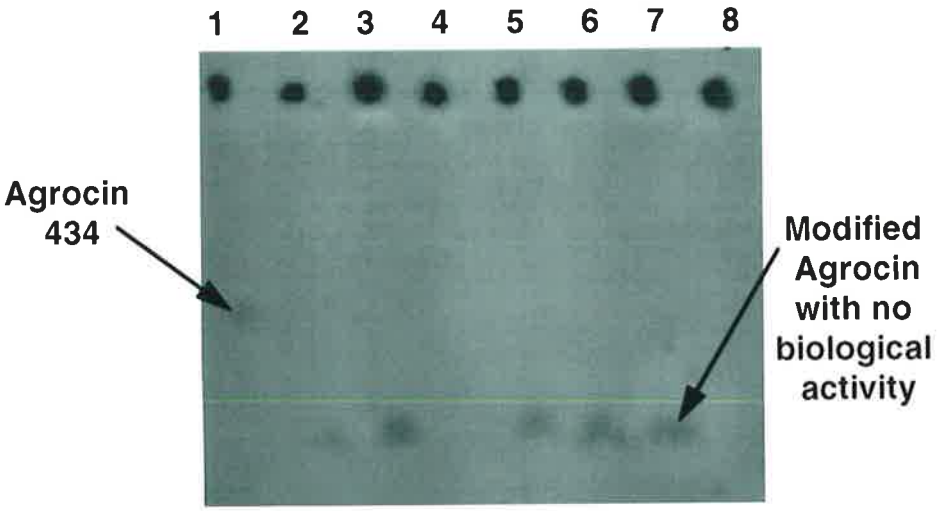
Transconjugants were verified by agarose gel electrophoresis and sensitivity to agrocin 434 in bioassay. Resulting strains were recovered which were *A. rhizogenes*, contained pAgK1318, synthesized modified agrocin 434 and were resistance to agrocin 434 as well. This result indicates that resistance/immunity functions are also carried on pAgK1318.

2.4 *Conclusion*

The main object of this study was to understand the role of each of the K84 plasmids, pAgK84, pAtK84b and pAgK434 in the biocontrol process. For this reason, the successful construction of a range of derivatives of strain K84 harbouring all combinations of the three residential plasmids in the same host background has been described.

Plate 2.7 High voltage paper electrophoresis of charcoal adsorbed fractions from liquid culture media. Electrophoresis was in 0.05 M citrate, pH 6.0 at 2 kV for 30 min: photographed using transmitted UV light.

High Voltage Paper Electrophresis



1) K1143; 2,3,4,5,6) K27 pK1318; 8) K27

Previously, strains K84, K434, K1143 (Donner *et al.*, 1993), K1347 (McClure *et al.*, 1994), K1356 and K1357 (NC McClure, unpublished) had been isolated. The plasmid-free strain, K1347 was resistant to agrocin 434 and 84.

Because the nopaline catabolic plasmid, pAtK84b could influence ecological competence, it was necessary to introduce this plasmid into the plasmid-free strain K1347. One of the resultant transconjugants of this test was designated K1351. Other derivatives of strain K84 have been constructed including strain K1352 with pAgK84, K1353 with pAgK84 and pAtK84 and K1355 with pAgK84::Tn5 and pAgK434. Tn5 insertions into pAgK434 in strains K1356 and K1357 are very important because these inserts can be use to locate the position of agrocin 434 synthesis genes. Other factors carried by pAgK434 should not be affected.

These strains were unable to produce agrocin 434 and were resistant to agrocin 434 and 84. Although strains K1356 and K1357 had lost agrocin 434 production their biological activity needs to be verified by bioassays and more information about these strains is available from studies described in chapters 4.

By constructing all combinations of the three resident plasmids of K84 and also derivatives of strain K1143 with transposon insertions in pAgK434, we could assess the efficiency of these potential biocontrol strains using three methods of testing as described in chapters 3, 4 and 5.

There were no obvious differences in growth rate of *Agrobacterium* strains on TY medium and doubling time during the exponential growth phase was approximately 2.4 hr.

The studies investigating immunity/resistance to agrocin 434 were made possible by the introduction of a selectable marker onto the plasmids pAgK434 and pAgK1318. Plasmid pAgK434 can be mobilized to the agrocin-sensitive tumorigenic strain K27. The results of this study indicate that genes involved in the immunity/resistance to agrocin 434 by agrocin 434 producer strains are carried by pAgK434. This has been demonstrated by transferring the plasmid pAgK434 to an agrocin 434 sensitive strain. The resulting strain became resistant to agrocin 434 and acquired the ability to produce agrocin 434 as well. Similarly, Donner *et al.*, 1993, reported that genes involved in the synthesis of agrocin 434 were located on the cryptic plasmid pAtK84, which was designated pAgK434.

This situation with pAgK434 resembles pAgK84, which carries both agrocin 84 immunity and synthesis genes (Ryder, *et al.* 1987; Ellis *et al.*, 1979; Farrand *et al.*, 1985; Wang *et al.*, 1994). Experiments with cured derivatives of K84 indicate that additional resistance/immunity functions to agrocin 434 are chromosomally-borne in this strain (N.McClure, unpublished).

The kanamycin resistance transposon Tn*5* was introduced into pAgK1318:Mob4/5, and resulting strains were able to produce modified agrocin 434 as did K1143 pAgK1318. Resistance/immunity functions are also carried on pAgK1318, as transconjugant strains were resistant to agrocin 434.

Chapter Three

Development of rapid bioassay method

3.1 Introduction

Rapid efficient methods for testing pathogenicity and/or the efficacy of biocontrol strains are very important for studying the factors involved in the control process. Currently, root inoculation bioassays and stem inoculation are used to test the pathogenicity and/or efficacy of biocontrol strains in outdoor conditions . Root inoculation assays need long cultivation period to demonstrate the results. The purpose of this study is to compare methods that can be used to evaluate the efficacy of biocontrol strains. The techniques are: (1) leaf disc tumorigenesis assay; (2) stem inoculation bioassay and tobacco root inoculation.

Leaf discs and stem segments (McCormick, *et al.*,1986, Chyi & Philips, 1987) are two in-vitro *Agrobacterium* - based transformation systems reported for tobacco and tomato plants (Horsch, *et al.*, 1988). There have been numerous plant tissue studies with many dicotyledonous species documented as transformable by *Agrobacterium* (Smith, & Hindley, 1978; Chyi & Philips, 1987; Eapen-Kohler *et al.*, 1987; Pang & Sanford, 1988). However, there has been little research on the effect of specific plant host factors such as plant cultivars and host plant response to various bacterial inoculum densities. Therefore, the aim of this work was to study tumor induction in tobacco and tomato cultivars and to evaluate the effect of bacterial inoculum density and bacterial strain, using leaf discs and stem inoculation bioassays. Major aims were to determine which plant cultivars gave the most rapid and reproducible tumor formation with the pathogen tested and develop rapid bioassays for assessing the efficacy of biocontrol strains. To find a fast growing test plant for root inoculation assays, tobacco plants

were tested. Previously, most investigator used almond seedlings as test plants but these plants need at least 9 months to demonstrate results.

3.2 Materials and Methods

3.2.1 *Media and Strains*

Agrobacterium strains K27 and K84 were tested by leaf disc tumorigenesis assay. Bacterial cultures were grown overnight in nutrient broth (Oxoid, NB) at 28°C on a gyratory shaker at 125r min^{-1}.

3.2.2 *Leaf disc tumorigenesis assay*

Leaf disc infection protocols for tobacco and tomato were devised for plant transformation using uniform culture conditions with maximum consideration for utilisation of space. The following cultivars were chosen for these experiments on the basis of their reaction with K27, K84 and K27+K84 : tobacco cultivars Virgie, White Burly, Samson and Ti68, and tomato cultivars Gross Lisse, Quick Pick, Yellow Pear, Day Dream and Burley Bounty. Plants of each cultivar were grown in 7 inch pots in UC potting mix and were maintained under greenhouse conditions. Leaf discs were prepared and inoculated according to the protocol described by Horsch *et al* (1988) and Bush &Pueppke (1991).

Healthy leaves were washed in soapy water, surface sterilised by submersion in 75% ethanol for 30 sec, rinsed in autoclaved, double distilled water, submerged in 1% sodium hypochlorite for 5 min and then rinsed three times in sterile double distilled water. Leaves were cut with a hand held paper punch, avoiding the main vein, but

including branch veins at random, the discs were 6.5mm in size. Discs were incubated abaxial side up at 25°C on MSO⁻ media (hormone free media) (Bush & Puppke, 1991) and illuminated for 16hr.

After pre-incubation for 2 days, discs were immersed for 5 min in MSO⁻ liquid media containing K27, K84 or a mixture of both . The bacterial concentrations are listed in tables 2 and 3. Bacterial concentrations were calculated from a standard curve for estimation of bacterial cell numbers from optical density, as described in chapter2. The negative control consisted of discs immersed in MSO⁻ liquid lacking bacteria. Discs were blotted dry on autoclaved Whatman No.1 filter paper. Five discs were placed in each petri dish containing MSO⁻ media. Explants were incubated for 3 weeks at 25°C under a 16 hour light : 8 hour dark photo period using cool white fluorescent light. Transformation was demonstrated by tumor induction on the hormone free medium at least one week after inoculation. Tumors were scored 7, 15 and 21 days after inoculation. For all experiments, controls consisted of non-inoculated plant tissue placed on hormone-free MSO medium. To confirm no growth of tumor tissue without *Agrobacterium spp.* controls were dipped into liquid MSO⁻ medium.

3.2.3 *Stem inoculation bioassays*

The efficiency of biological control strains K84, K1143 and K1347 was assessed using tomato and tobacco seedling assays. First, using a flame sterilized blade, stems were wounded then inoculated with a mixture of pathogen and non-pathogen at different ratios and concentrations. Inocula were prepared from 3-day cultures on YMA slopes. Bacteria were removed and suspended in sterile, double distilled water and pathogen and non-pathogen mixed in various ratios. A flame-sterilised blade was dipped into the suspensions and stabbed into the stems of the tomato and/or tobacco plants, four times

for each treatment. The stems were assessed for gall formation after 5 and 10 weeks for tomato and tobacco, respectively. Six wound sites were used for each treatment.

3.2.4 *Tobacco seedling assay for biological control of crown gall (root inoculation)*

The method of Htay and Kerr (1974) was used for root inoculation of tobacco *cv.*Wb. Unsterilised soil was placed in 24 six inch pots with 6 replicates for each treatment, and inoculated with 3 day old cultures of K27, about 10^7 cells/ml to give approximately 10^6 cells per gram of soil. The actual distribution of K27 in the soil was not examined. The soil was kept for 2 days prior to planting. The 3-day old cultures of K84, K1143 and K1347 were suspended in 3 litre of non-chlorinated water. The suspensions were estimated by optical density measurements to contain about 10^7 cells/ml. The six week old tobacco seedlings were removed from their pots, the soil was rinsed gently from their roots with water, and the taproots were trimmed to a length of approximately 10 cm. The plants were dipped in either water or a suspension of the biocontrol strains. They were then replanted in the K27 infested soil. After 3 months, plants were removed and washed in running water.

3.3 Results

3.3.1 *Leaf disc bioassay treatment with K27.*

Four tobacco cultivars and five tomato cultivars were selected for leaf disc tumorigenesis. To determine the sensitivity of leaf discs of these cultivars to various dilutions of *A. tumefaciens* strain K27, the effect of disc pre-incubation on medium

lacking hormone (MSO^-), was examined. Photographs of discs from experiments are shown (Plate 3.1).

The number of tumors was estimated on the leaf discs of tobacco and/or tomato which were treated with different concentrations of K27 (Fig. 3.1&3.2). Genstat 5, Factorial Experiment in Radomized Complete Block Design was used to compare the five treatments of bacterial concentration (bac-conc) and plant cultivars. A significant difference was found between the number of tumors formed with different bacterial concentrations and between the tobacco cultivars tested with $p<0.001$ in both cases and also between the number of tumors formed with different bacterial concentrations and between the tomato cultivars tested, $p<0.001$ and $p<0.0856$, respectively.

Virgie and White Burley (tobacco cultivars) and Yellow Pear and Quick Pick (tomato cultivars) produced the highest number of tumors with 10E 8.09 cfu/ml and 10E 7.7 cfu/ml of the pathogen for tobacco cultivars and tomato cultivars, respectively (Fig. 3.1,3.2,3.3 and 3.4).

Tumor induction on leaf discs of tomato by strain K27 was low at all bacterial concentrations regardless of tomato cultivars. Tumor formation was low at 10E 8.4 cfu/ml, increased significantly to its maximum at 10E 7.7 cfu/ml and decreased at higher concentrations. When compared to QP and YP, a lower number of Dd, Bb and Gl leaf discs produced tumors at all levels of K27, but the difference was not significant. More necrosis of tomato explants was seen at the higher concentrations of bacteria for all cultivars. Dd and Bb were more susceptible to necrosis than other tomato cultivars at higher concentrations of bacteria.

Fig. No.3.1

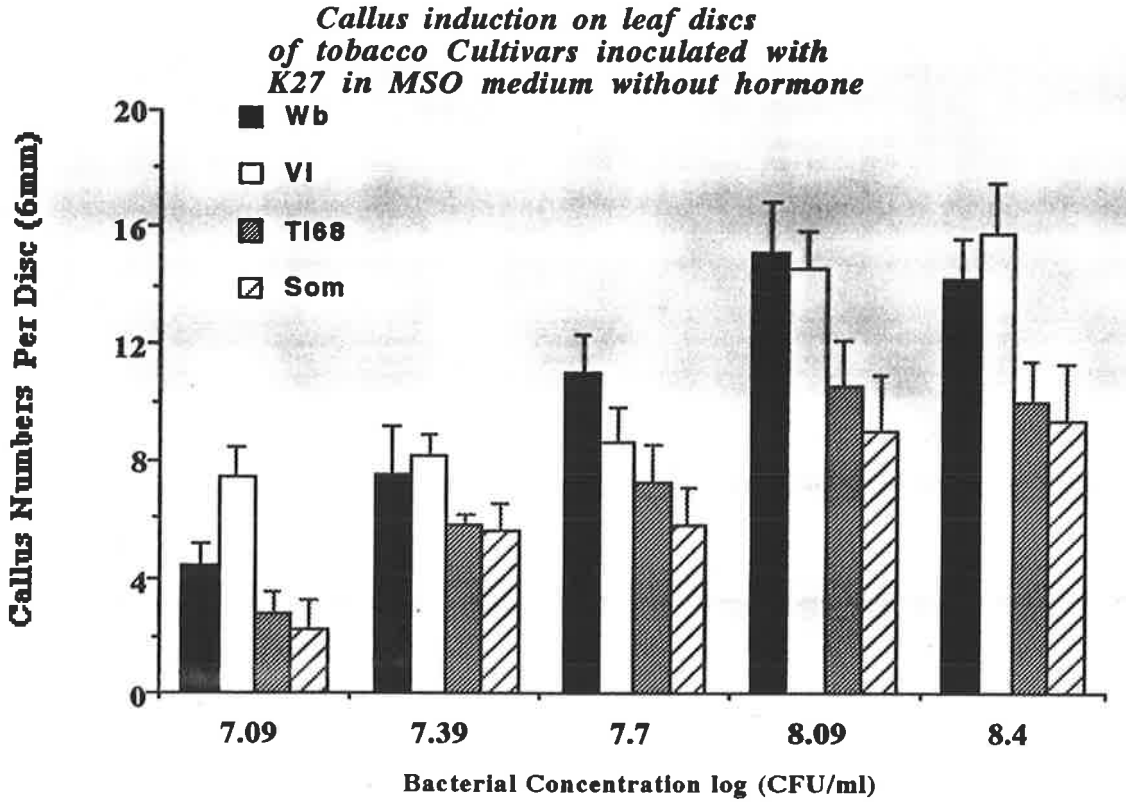

Callus induction on leaf discs of tobacco Cultivars inoculated with K27 in MSO medium without hormone
Wb
VI
TI68
Som
Callus Numbers Per Disc (6mm)
20
16
12
8
4
0
7.09
7.39
7.7
8.09
8.4
Bacterial Concentration log (CFU/ml)

Fig No.3.2

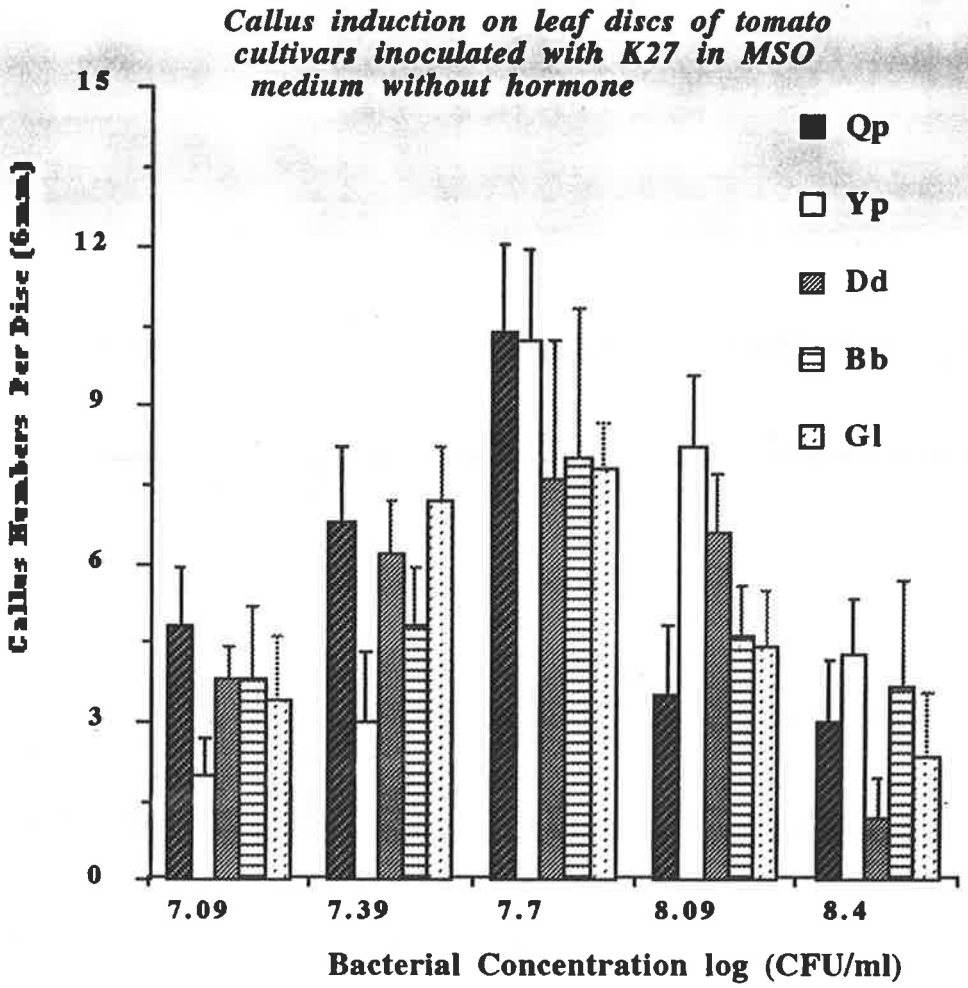

Callus induction on leaf discs of tomato cultivars inoculated with K27 in MSO medium without hormone
Callus Numbers Per Disc (6mm)
15
12
9
6
3
0
Qp
Yp
Dd
Bb
Gl
7.09
7.39
7.7
8.09
8.4
Bacterial Concentration log (CFU/ml)

Fig No.3.3

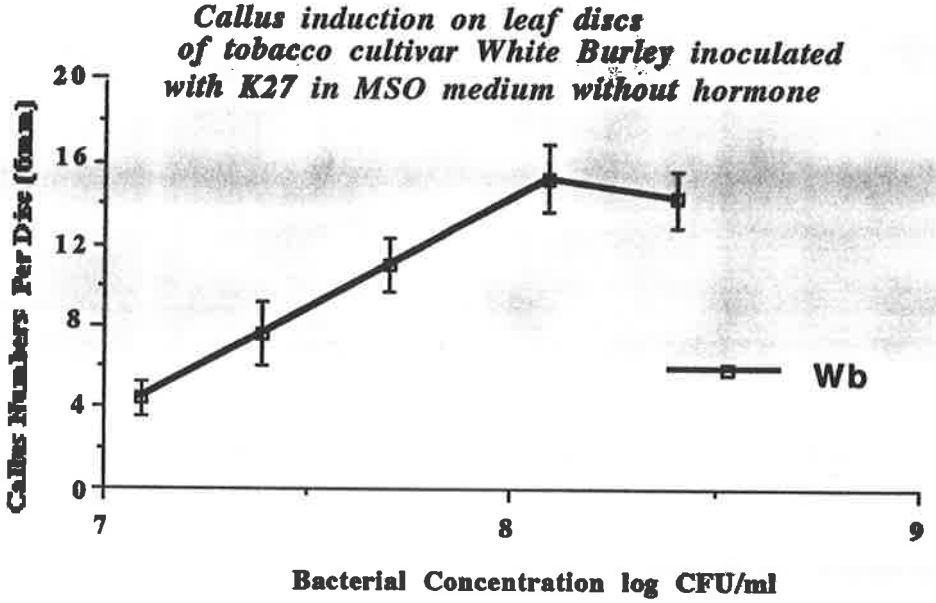

Callus induction on leaf discs
of tobacco cultivar White Burley inoculated
with K27 in MSO medium without hormone
Callus Numbers Per Disc (6mm)
20
16
12
8
4
0
7
8
9
Wb
Bacterial Concentration log CFU/ml

Fig No.3.4

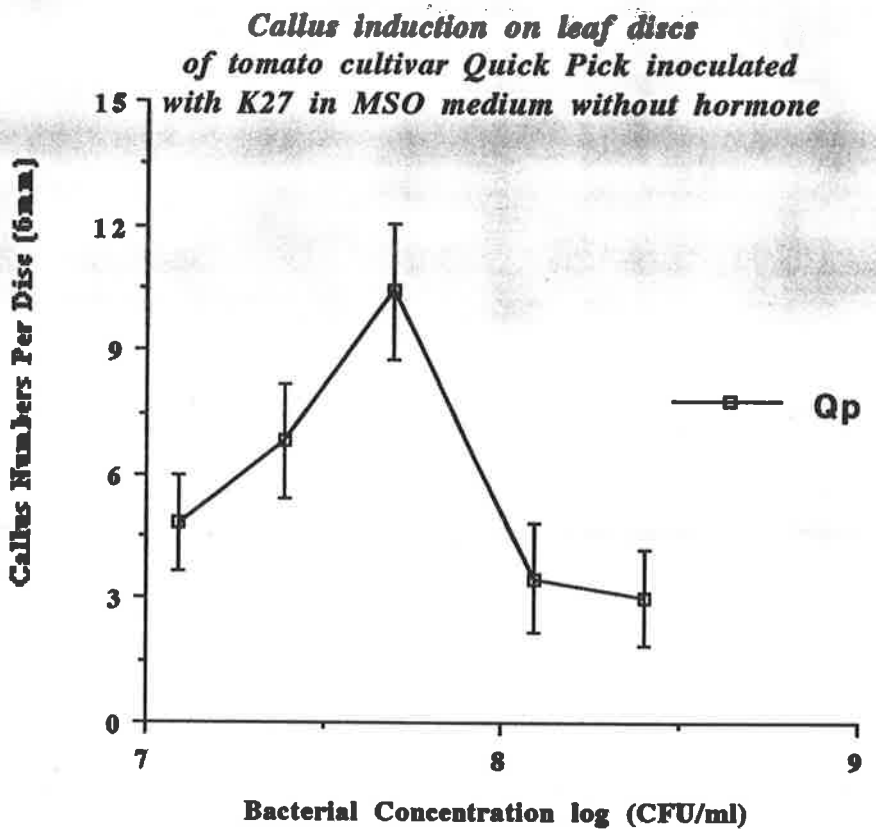
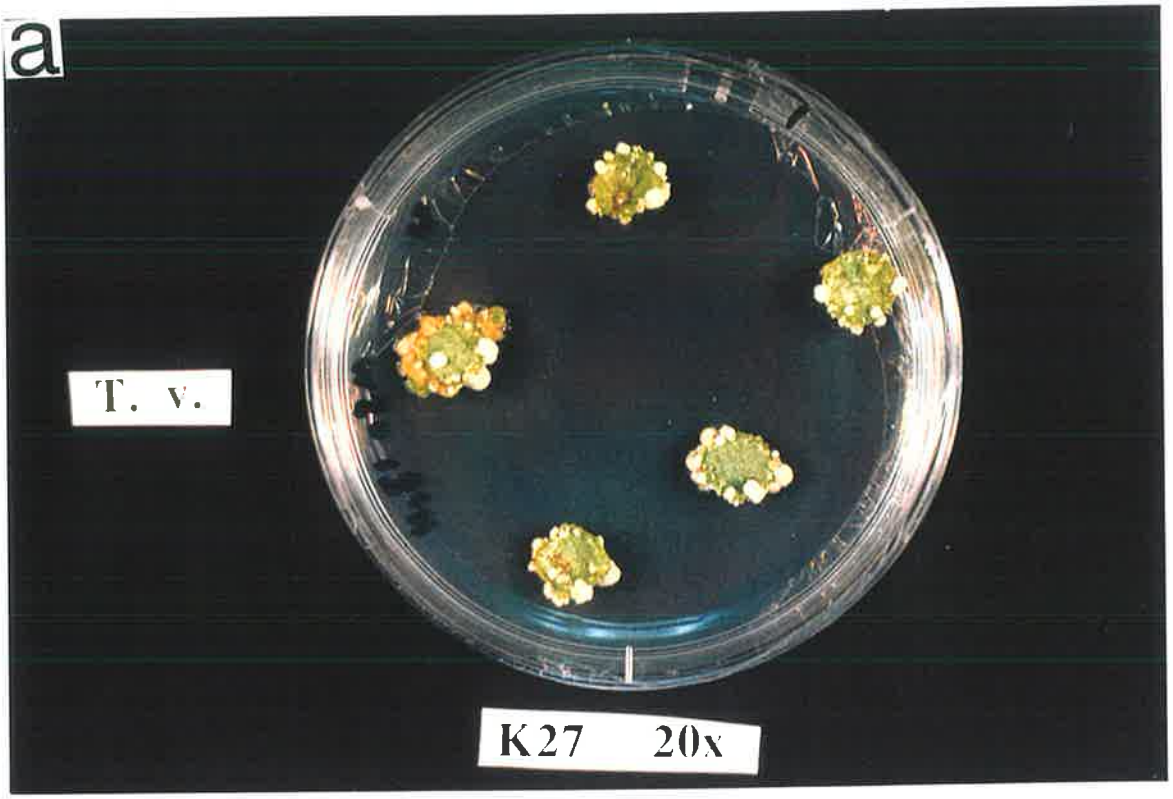
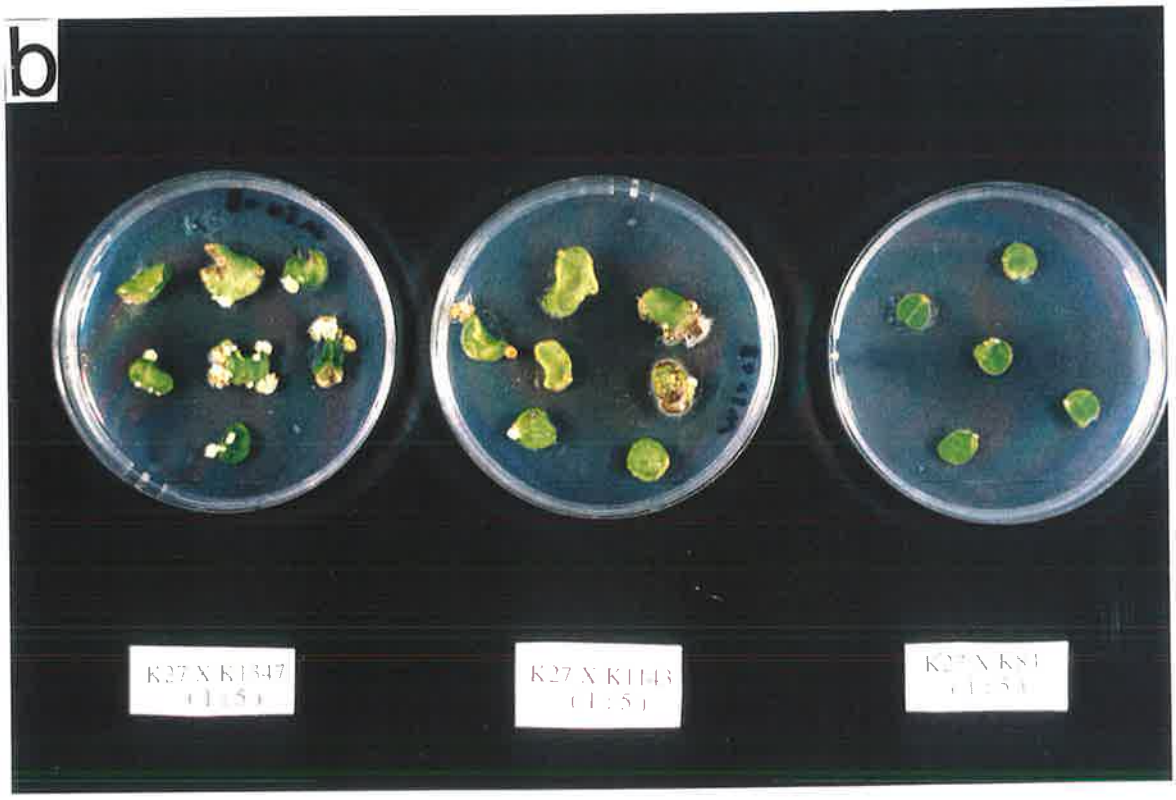

Callus induction on leaf discs
of tomato cultivar Quick Pick inoculated
with K27 in MSO medium without hormone
Callus Numbers Per Disc (6mm)
15
12
9
6
3
0
7
8
9
Qp
Bacterial Concentration log (CFU/ml)

Plate 3.1 Pathogenicity of biovar 2 strain K27 on on leaf disc of tobacco *cv* Virgie in MSO media without hormone. Photographed 3 weeks after inoculation (a).

Callus induction on leaf disc of tobacco *cv* White Burley inoculated with the pathogen K27 ± biocontrol strains (K84 with pAgK84, pAgK434 and pAtK84b; and K1143 with pAgK434; K1347 plasmidless derivative of K84) at raio 1:5 (pathogen to biocontrol) in MSO media without hormone (b)


a
T. v.
K27 20x


b

Generally, the number of tumors per leaf disc of tomato cultivars ranged between 1 and 12. Bacterial concentrations of 10E 7.7 cfu/ml resulted in the largest tumors for all tomato cultivars. Tumor induction of tobacco leaf discs by *A. rhizogenes* strain K27 was high at all bacterial concentration levels compared to tomato plants. Transformation was high at 10E 8.09 cfu/ml and decreased significantly at lower concentrations. The necrosis of explants was not observed at any level of bacterial concentration . The number of tumors per tobacco leaf disc ranged between 2 and 16. bacterial concentrations of 10E 8.09 cfu/ml resulted in the highest tumor number for all tobacco cultivars, and Wb tumor numbers were higher than those on other cultivars.

3.3.2 *Assessment of biological control capacities of non-pathogenic strains by leaf disc culture*

The strain K84 was tested for ability to control tissue transformation by strain K27 on hormone free medium (MSO^-). The pathogenic strain K27 was mixed with K84 at ratios of 1:1, 1:5 and 1:10. There were five replicates of each treatment. The number of tumors was determined on the leaf discs of tobacco cultivars and tomato cultivars which were treated with a mixture of pathogen and non pathogen strains at different ratios and concentrations (Fig No.3.5,3.6). Factorial Experiment in Randomized Complete Block Design in the Genstat 5 program was used for statistical analysis. There were significant differences between the mean numbers of tumors formed with concentration of the biocontrol strain and between the tobacco cultivars tested with $p<0.001$ in both cases. Similarly there were significant differences between the mean number of tumors formed with different concentration of the biocontrol strain and between the tomato cultivars tested, $p<0.001$ and $p<0.012$, respectively.

Fig No.3.5

Callus induction on leaf discs of tobacco cultivars inoculated with K27±K84 in MSO medium without hormone

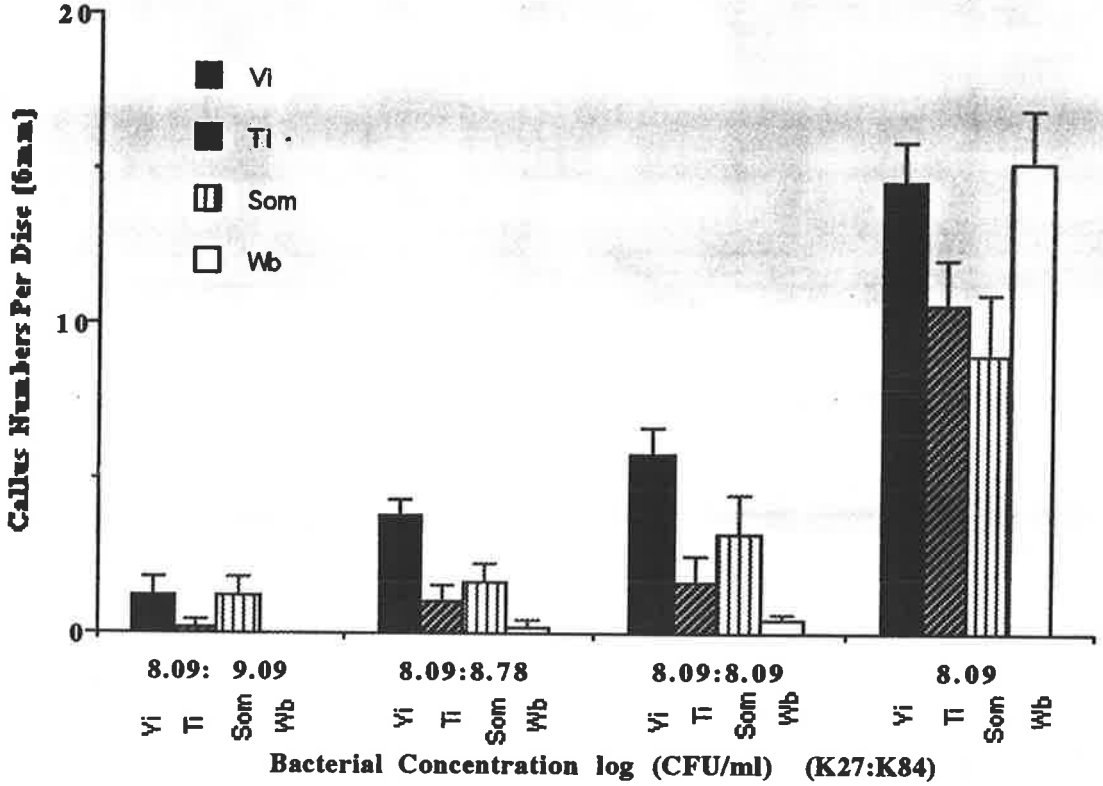
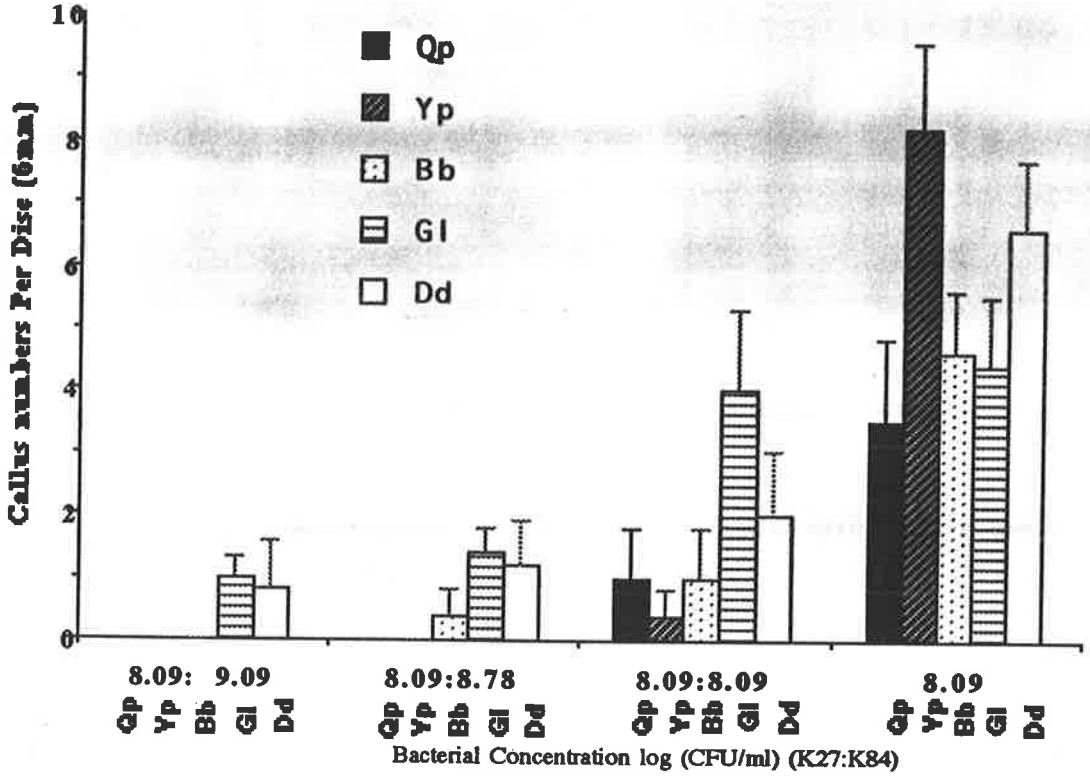


Fig No3.6

Callus induction on leaf discs of tomato cultivars with K27±K84 in MSO medium without hormone



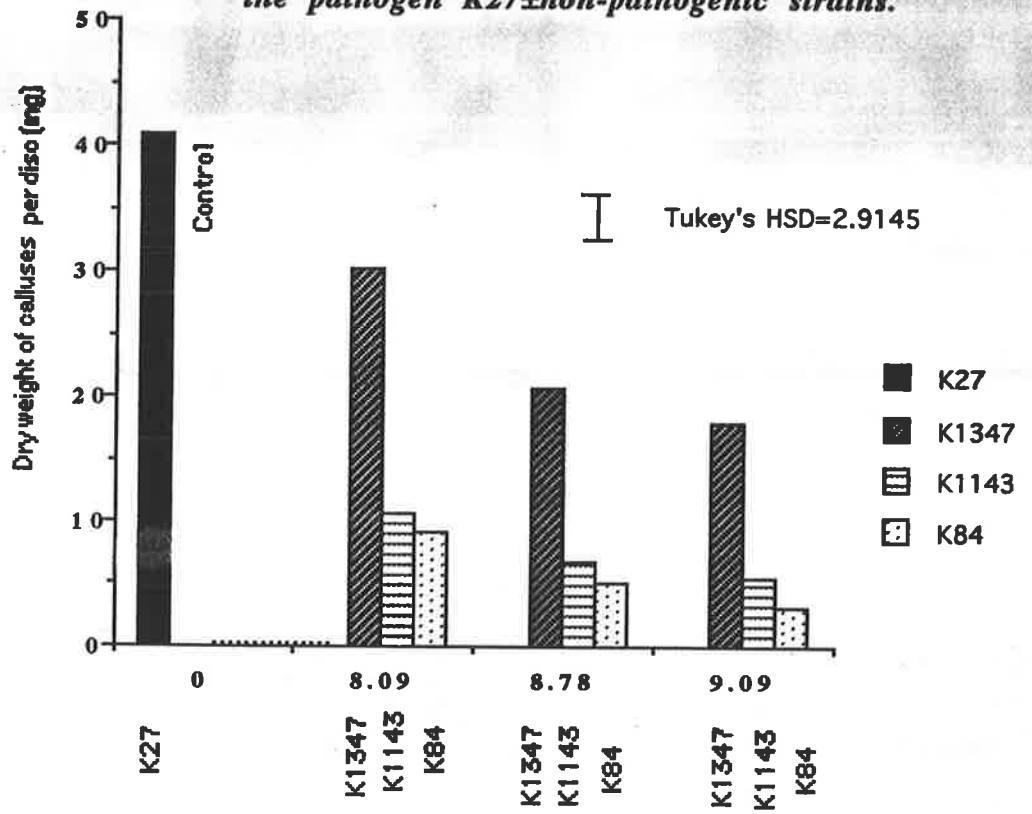
2-The strains K84, K1143 and K1347 were tested for ability to control crown gall induction or tumor induction by strain K27 on hormone free medium (MSO^-). The test strains were mixed with K27 at ratios of 1:1, 5:1 and 10:1. There were seven replicates of each treatment. In this experiment the tobacco plant cultivar White Burley was used for the leaf disc culture. Three weeks after inoculation the discs were removed from the plates, dried for 3 days at 65 °C, and weighed individually. Results are presented in figure 3.7. Randomized Complete Block Design in the Genstat 5 program was used for statistical analysis. There was a significant difference between K84 and K1143 in biocontrol of pathogen K27 with $p<0.003$. From these results it is concluded that the ability of strain K1143 with pAgK434 to control the pathogenic strain *Agrobacterium* (K27) was slightly but significantly lower than strain K84. Results indicated that the ability of the cured strain K1347 to reduce tumor formation by strain K27 was significantly lower than strain K1143 with pAgK434. This ability is probably related to agrocin 434 production. The results of the treatment with mixtures of K1347 and K27 showed a significant reduction of tumor induction by the pathogen, compared with the pathogen alone.

3.3.3 *Stem inoculation bioassays*

The stems of tomato and tobacco plants were inoculated with *A.tumefaciens* strain K27 at different concentrations, and strains K1143, K1347 and K84 were tested for ability to control crown gall induction by strain K27. The test strains were mixed with strain K27 at ratios of 1:1, 5:1 and 10:1. There were four replicates for each treatment. After incubation, the galls from each wound site were removed from the stems, dried for 3 days at 65°C, and weighed individually. Results are presented in figures 3.8 and 3.9. Randomized Complete Block Design in the Genstat 5 program was used for

Fig No.3.7

Callus weights on leaf discs of the tobacco cv. White Burley inoculated with the pathogen K27±non-pathogenic strains.



Concentration of non-pathogenic strains log (CFU/ml), concentraion of pathogen(K27)=8.09

statistical analysis. Significant differences were found between the concentrations of the pathogen, K27 and gall formation on tobacco and/or tomato plants with $p<0.001$. Tomato plant cv.Quick Pick was the best plant for stem inoculation bioassay, because gall formation by stem inoculation of tobacco required a longer incubation period than tomato. This cultivar (Qp) produced more galls than other cultivars at all bacterial concentrations and was the quickest test plant for gall formation (plate 3.2 and table 3.1).

In the tobacco stem inoculation test (Fig 3.9) there was a significant difference between K84 and K1143 in biocontrol of the pathogenic strain K27, $p<0.020$. The results indicated significant differences between K1143 and K1347 in biocontrol of the pathogenic strain K27, $p<0.001$. These results indicated that strain K1143 has more ability to control the pathogen than the cured strain, K1347 and that there is a significant difference between treatment with K1347 compared to the pathogenic strain K27 alone, $p<0.001$.

When inoculated to tomato stems (Fig 3.9) there was no significant difference between K84 and K1143 in biocontrol of the pathogenic strain K27, $p<0.117$. The results indicated significant differences between K1143 and K1347 in biocontrol of pathogenic strain K27, $p<0.001$, indicating that strain K1143 has more ability to control the pathogen than the cured strain, K1347. Treatment with mixtures of the cured strain, K1347, and K27 significantly reduced tumor induction by the pathogen, as compared with the pathogen alone, $p<0.001$.

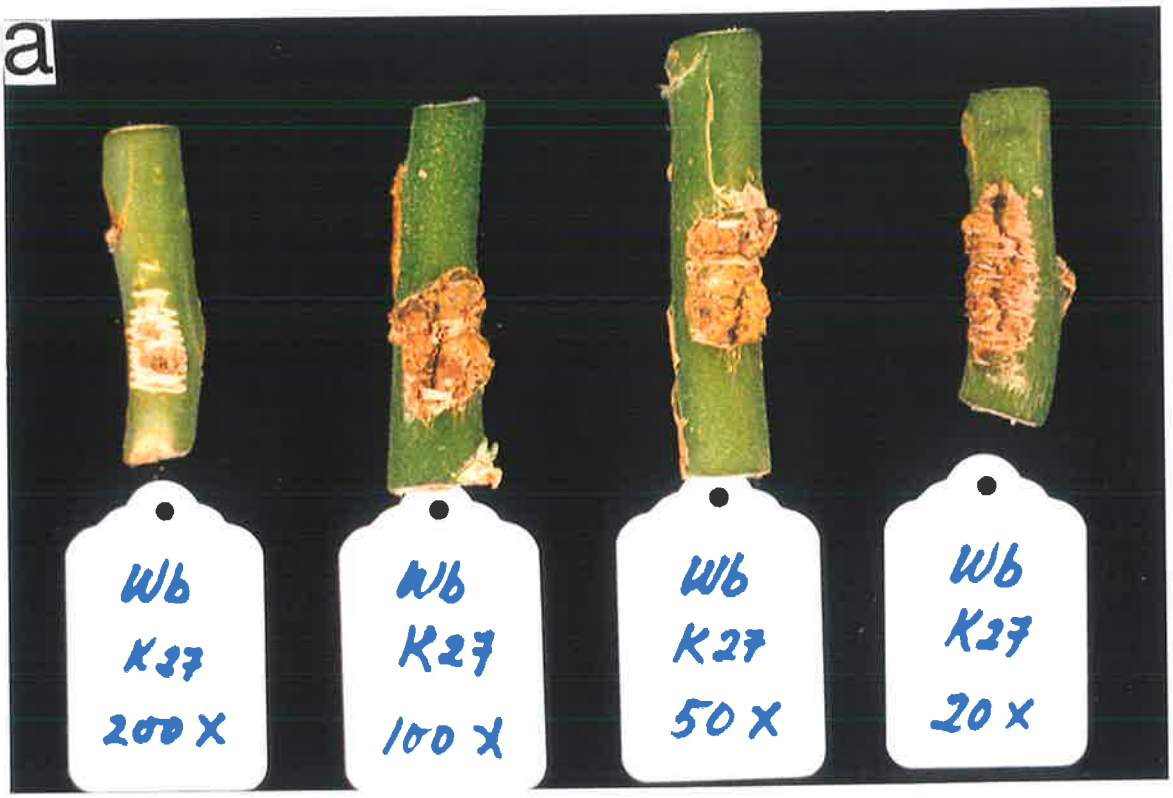
Table 3.1. Response of tomato and tobacco plants to stem inoculation with *Agrobacterium tumefaciens* strain K27

Means of Dry weight of galls in Stem Inoculation of Tomato and Tobacco Plants with K27.

Bacterial concentration log (CFU/ml) or (dilution factor)		tobacco cultivar Wb	tomato Cultivars QP	YP	DD	BB	GL
7.090	(200)	82.8	47.5	15	25	25	42.5
7.390	(100)	72.8	72.5	32.5	27.5	27.5	35
7.700	(50)	215	130	47.5	25	86.2	37.5
8.090	(20)	240	237.5	42.5	62.5	57.5	15
8.400	(10)	164.5	115	97.5	80	47.5	55

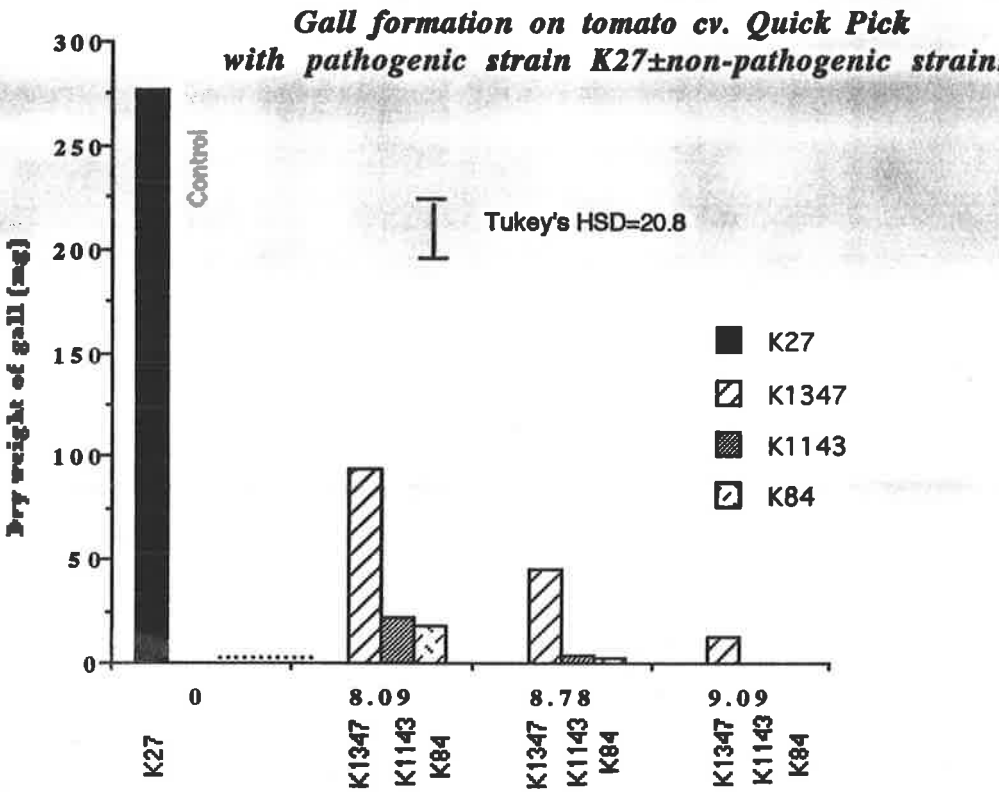
Plate 3.2 Pathogenicity of biovar 2 strain K27 on tobacco cv White Burley at different bacterial concentrations (a).

Pathogenicity of biovar 2 strain K27 on tomato cv Quick Pick at different bacterial concentrations (b).


a
Wb
K27
200 X
Wb
K27
100 X
Wb
K27
50 X
Wb
K27
20 X


b
K27
200X
QP
K27
100X
QP
K27
50X
QP
K27
20X
QP
K27
10X
QP
K27
neat
QP

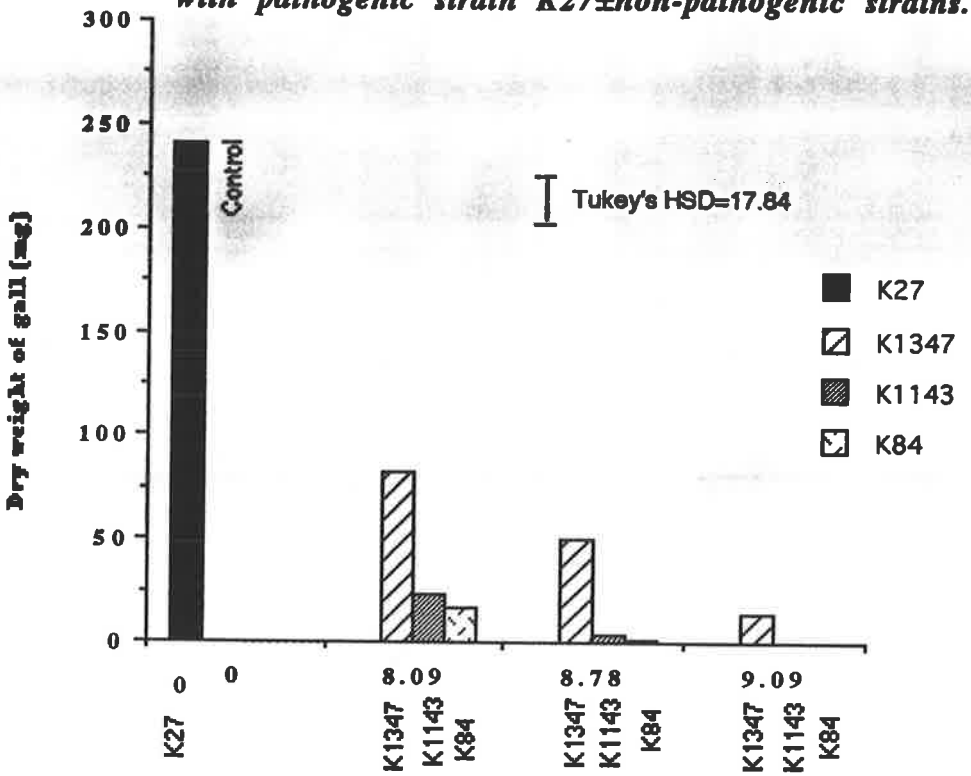
Fig No.3.8



Concentraion of non-pathogenic strains log (CFU/ml), concentraion of pathogen (K27)=8.09

Fig No.3.9

Gall formation on tobacco cv. White Burley inoculated with pathogenic strain K27±non-pathogenic strains.



Concentration of non-pathogenic strains log (CFU/ml), concentraion of pathogen (K27)=8.09

3.3.4 *Tobacco seedlings assays (root inoculation)*

Tobacco were sensitive to inoculation by *Agrobacterium* strains. Root rot of the tobacco plant was observed in control plants, which were treated with the pathogenic strain K27. There was no gall formation on the roots in the time period examined, but there were some differences between biocontrol strains in the colour of the roots of plants compared with the control (Plate 3.3). Comparison between strains indicated that the length of roots which were treated with K84+K27 and K1143+K27 were longer than K1347. The length of roots which were treated with K1347+K27 were longer than the pathogen alone, K27.

3.4 Conclusions

Major aims of this work were to determine which plant cultivars gave most rapid and reproducible tumor formation when inoculated with a pathogen and to develop a rapid bioassay.

Transformation of leaf discs was tested by tumor formation in leaf disc tumorigenesis assays. It was possible that transformation occurred without visible tumors, but this possibility was not tested. Tobacco cultivars affected transformation although the differences between the four cultivars examined in this study were generally small. White Burley tissue was transformed more readily and produced tumors more rapidly than other cultivars as seen by a higher tumor induction for all bacterial concentrations for both tomato and tobacco cultivars. Plant cultivar was also reported to be important in determining tumor formation frequencies in moth bean *Vigna aconitifolia,* in which one cultivar was found to have a higher transformation rate than another (Eapen-Kohler *et al.*, 1987).

Plate 3.3 Biological control of crown gall on roots of tobacco *cv.*White Burley inoculated with pathogenic strain K27±non-pathogenic strains (K84 with pAgK84, pAgK434 and pAtK84b; and K1143 with pAgK434; K1347 plasmidless derivative of K84).


Wb
K1347
x
K27
Wb
K1143
x
K27
Wb
K84
x
K27
Wb
Water
x
K27

Comparison between pathogen concentrations indicated that 10E 8.09 (CFU/ml) of inoculum is the optimum concentration for tumor induction with tobacco and 10E 7.70 (CFU/ml) of inoculum is the optimum concentrations for tumor induction with tomato. The necrotic response was high in tomato cultivars. It was greater in Dd and Bb than other cultivars. This necrosis was probably due to a hypersensitivity response of the tomato tissue. Necrotic effects resulting from a plant hypersensitive response to *Agrobacterium* strains were recently documented in thin cell lyre explants of *Brassica napus* L. and were partially overcome by replacing agar with agarose in the plant medium. A hypersensitive response was more likely to occur when small or tender leaf tissue was used (Charest *et al.*, 1988). In contrast there was no necrotic response in tobacco cultivars.

Comparison between the plasmidless strain, K1347, and K1143 with pAgK434, indicated that strain K1143 significantly reduced gall formation and/or tumor induction by the pathogenic strain, and that this strain had a greater ability to control the pathogen than the cured strain, K1347. This ability is probably related to agrocin 434 production. One important consideration is that there may be additional agrocins or inhibitory compounds encoded by pAgK434 as well as agrocin 434 and it has not been confirmed that the only difference between K1347 and K1143 is agrocin 434 production. The biological control ability of these strains is described in chapter 4.

Comparison between the cured strain, K1347, and the pathogenic strain, K27, indicated that strain K1347 significantly reduced gall formation and/or tumor induction by the pathogenic strain. This capacity may be related to competition for site and/or nutrients between the pathogenic strain and the cured strain, K1347. One important point is the possibility of other chromosomally encoded inhibitory compounds being produced by the cured strain K1347.

Comparison between plant tests showed that the tobacco cv.White Burley and cv. Virgie are the best hosts for plant transformation because these cultivars produced more tumors than other cultivars.

Tomato cv. Quick Pick is the best plant for stem inoculation, because gall formation by stem inoculation of tobacco requires a longer incubation period than tomato and this cultivar also produced more galls than other tomato cultivars and was the quickest test plant for gall formation.

Comparison between leaf disc culture and stem inoculation indicated that leaf disc culture is a rapid method for assessment of the efficacy of biocontrol strains. This method produced results after 15-21 days compared with 4-5 weeks for tomato and 10 weeks for the tobacco stem inoculation assay method.

Comparison between three difference bioassays methods used in this chapter indicated that each method has some advantages and also disadvantages as well. Advantages of each method are: 1) leaf disc tumorigenesis assay: this method is rapid and needs a shorter time period to demonstrate results of treatment, 2) stem inoculation assays: this method is easier to do than other methods, very simple and needs shorter time compared to root inoculation bioassay method 3) root inoculation bioassays: results of this methods of infection simulate the real situation in soil conditions where other factors such as competition for nutrients, motility of bacterial strains and attachment to wound sites may play a major role in the pathogenic process.

Disadvantages of each method are as follows: 1) Leaf disc culturemay result in a hypersensitivity response and contamination by fungi and bacteria. This method needs special equipment such as a tissue culture room and materials are very expensive. 2) Leaf disc culture and stem inoculation assays do not really mimic the real situation as compared to root inoculation where other factors involved in the biocontrol process

such as soil structure, pH humidity, presence of other microorganisms in soil and competition for sites and nutrients. 3) Root inoculation bioassays take longer to yield results, a minimum of 6-9 months and require more space than other methods. Tobacco roots were very sensitive to bacterial inoculation and gall formation was not observed.

Use of tobacco seedling root inoculation bioassays indicated that there was no gall formation on the roots of seedlings treated with pathogen and/or with mixture of pathogen and biocontrol strains. Root rot of the tobacco plants was found in control plants, which were treated with the pathogen alone. The absence of galls on tobacco roots and root rot may be due to hypersensitivity of tobacco roots to *Agrobacterium* strains. However, there were some differences between treatments with a mixture of biocontrol strains and pathogen in the colour of the roots of plants compared with pathogen alone and biocontrol strains prevented root rot of tobacco by pathogen. From these results it was concluded that tobacco seedlings are not suitable as a test plant for root inoculation bioassays. Almond seedlings were used in futher experiments.

Chapter Four

The Role Of Agrobacterium strain K84 Derivatives In The Biological Control Of Crown Gall Disease Of Plants

4.1 Introduction

Many factors may affect the antagonistic action of strain K84 towards *Agrobacterium* pathogens. Soil factors include: the presence of other competitor rhizosphere microbes, soil salts, moisture, gasses, pH, temperature, planting site, root exudates and motility. In addition the ratio of the population of biocontrol agent to pathogens may be important. The population of agrobacteria present in soil is high and most of the agrobacteria in soil are nonpathogenic (Moore & Warren, 1979).

Colonization of the host plant rhizosphere by bacteria is generally believed to be necessary for disease suppression (Parke, 1991). Two important factors in biological control by a bacterial agent are the synthesis of sufficient quantities of an antibiotic agent or inhibitory compound and the effective colonization of the target plant. In addition to the effectiveness of agrocin 84, strains K84 and K1026 colonize almond roots efficiently (Jones and Kerr, 1989). Shim *et al.* (1987) found that the chromosomal background in which pAgK84 is present has an influence on the ability of the biocontrol strain to control crown gall disease. Competition for nutrients may also play an important role in biological control process. In the rhizosphere most nutrients come from root exudates. Some compounds serve not only as food but also

act as signals that initiate interactions between plants and microbes. Biological control agents can provide plant protection by efficient removal of such signals from the rhizosphere (Elad & Chet, 1987; Nelson & Craft, 1991).

As described earlier in chapter 2, a range of derivatives of *A. rhizogenes* strain K84 has been constructed. These strains are K1143, K434, K1347, K1351, K1352, K1353, K1355, K1356 and K1357.

The objective this study were to assess the efficacy of these new potential biocontrol strains compared to strain K84 to control the pathogenic strain *A. rhizogenes* K27 using root inoculation of almond seedlings, tomato stem inoculation and leaf disc culture bioassay.

4.2 Material and Methods

4.2.1 *Bacterial strains*

The *Agrobacterium* strains used in this study and their characteristics are shown in table 2.1.

4.2.2 *Leaf disc culture bioassay for biological control of crown gall*

Leaf disc infection protocols for tobacco were devised for plant transformation using uniform culture conditions with maximum consideration for utilisation of space. Leaf discs were prepared and inoculated as described previously in chapter 3.

The strains K434,K1143, K1351, K1352, K1353, K1355 and K1347 were tested as biocontrol agents and compared with the commercial strain K84 for ability to control

crown gall induction or tumor induction by strain K27 on hormone free medium (MSO^-). Also in another experiment the following agrocin 434$^-$ strains with Tn*5* insertion were used, K1356, K1357 and compared with strains K1347 and K1143. The test strains were mixed with K27 at ratios of 1:1, 5:1 and 10:1. The bacterial concentrations are listed in Fig. 4.1. The bacterial concentrations were calculated from a standard curve for estimation of bacterial cell numbers from optical density, as described in chapter 3. The negative control consisted of discs immersed in MSO^- liquid lacking bacteria. Discs were blotted dry on autoclaved Whatman No.1 filter paper. Five discs were placed in each petri dish containing MSO^- media. Explants were incubated for 3 weeks at 25°C under a 16 hour light : 8 hour dark photoperiod from cool white flourescent light. To confirm no growth of tumor tissue without *Agrobacterium* spp. controls were dipped into liquid MSO^- medium. In this experiment the tobacco plant cultivar White Burley was used for the leaf disc culture.

4.2.3 *Stem inoculation bioassays.*

The efficiency of biological control strains K84, K434, K1352, K1353, K1355, K1351,K1143 and K1347 (S1) were assessed using tomato seedling assays. Also in a different experiment (S2) the following strains were tested K1143, K1356, K1357 and K1347. First, using a flame sterilized blade stems were wounded (at six locations), then inoculated with a mixture of pathogen and non-pathogen at different ratios and concentrations. Inocula were prepared from 3-day cultures on YMA slopes. Bacteria were removed and suspended in sterile double distilled water with pathogen and non-pathogen mixed in various ratios . The stems were assessed for gall formation after 5 weeks .

4.2.4 *Preparation of almond seedlings*

Fresh almond seeds cultivar Fritz (gift from Dr.Ali Vezvaei) were soaked in distilled water containing (1 g/l) of the fungicide Benlate and incubated for 3 days in the dark at 4°C to initiate germination. The seeds were then planted, one per pot, in 7" pots in UC mix, and were maintained moist under outdoor conditions for 5 months. The seedlings were then used for almond seedling assays.

4.2.5 *Root inoculation bioassays*

The method of Htay and Kerr (1974) was used for root inoculation of Almond cv.Fritz. Unsterilized soil was placed in 72 pots, 9 inch, 8 replicates for each treatment, and inoculated with 3 day old cultures of K27, about 10^7 cells/ml, to give approximately 10^6 cells per gram of soil. The inoculated soil was mixed thoroughly but the actual distribution of K27 in the soil was not examined. The soil was kept for 2 days prior to planting. Three-day old cultures of K84, K434, K1352, K1353, K1351, K1143 and K1347 were suspended in 3 l of nonchlorinated water. The suspensions were estimated by optical density measurements to contain about 10^7 cells/ml. The 5 month old Almond seedlings were removed from their pots, the soil was shaken gently from their roots with water, and the taproots were trimmed to a length of approximately 10 cm. The plant roots were dipped in either water or a suspension of the biocontrol strains. They were then replanted in the K27 infested soil. After 6 months, plants were removed and roots washed in running water.

4.2.5.1 *Inhibion of tumor formation by A. rhizogenes strain K1143 and its derivatives on almond seedlings roots.*

The efficacy of strains K1356 and K1357 with a Tn*5* insertion in pAgK434, and also a cured derivative of *Agrobacterium* strain K84 were compared with the agrocin 434 producer strain, K1143, for ability to control the gall formation of almond seedlings.

The genetically altered strains with Tn*5* insertion were tested seperately and incubated in a containment glasshouse to avoid environmental contamination. The method of Htay and Kerr (1974) was used for root inoculation of almond cv.Fritz. The plants were inoculated either with the potential biocontrol strain or water and were then replanted in the K27 infested soil. Untreated seedlings were replanted in uninfested soil as control. Inoculated plants were incubated in a growth chamber at 25°C with a 16-hour photoperiod. The results were assessed after 6 months.

4.2.6 *Statistical Analysis.*

Statistical analysis was preformed using the Genstat 5 program and significant diferences were determined using Tukey's wholly significant difference (HSD)(Groebner & Shannon, 1990).

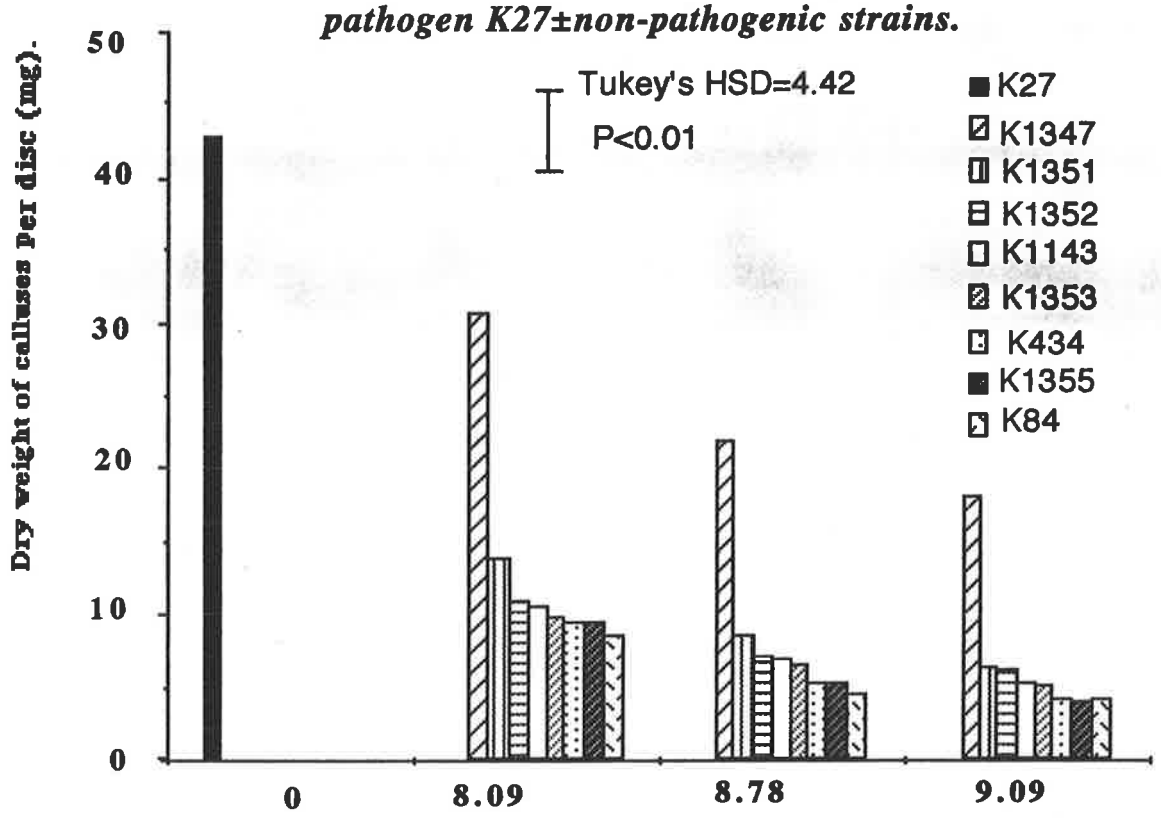
4.3 *Result and Discussion*

4.3.1 *Assessment of biological control efficacy of non pathogenic strains by leaf disc culture.*

The strains listed in Fig 4.1 were tested for ability to control crown gall induction or tumor formation by strain K27 on hormone free media (MSO^-). In these experiments the tobacco plant cultivar White Burley was used for the leaf disc culture. Transformation experiments could be scored as early as 3 weeks after inoculation. Tumors could be seen (plate 3.1) at the cut edges of the leaf disc and also at wound sites where tissue was damaged during the inoculation process. Often the leaf edge gave rise to green structures that developed directly into shoots. Three weeks after inoculation the discs were removed from the plates, dried for 3 days at 65°C, and weighed individually. Result are presented in figures 4.1 and 4.2. Randomized Complete Block Design in the Genstat 5 program was used for statistical analysis. The results indicated all biocontrol agents used in these experiments significantly reduced tumor formation by pathogenic strain K27 at all ratios with $P<0.01$. From these results it is concluded that there is no significant differences between strains harbouring pAgK84, pAtK84b and pAgK434 in reduction of tumor formation by pathogenic strain K27. The results indicate a significant difference between K1347 with other biocontrol agent with $P<0.01$. From these results it is concluded that the ability of strains K1357, K1356 with pAgK434::Tn*5* (Fig. 4.2) to control the pathogenic strain *Agrobacterium* (K27) was slightly but significantly lower than strain K1143 with pAgK434, this may be due to Tn*5* insertion and lack of agrocin 434 production.

Fig No.4.1

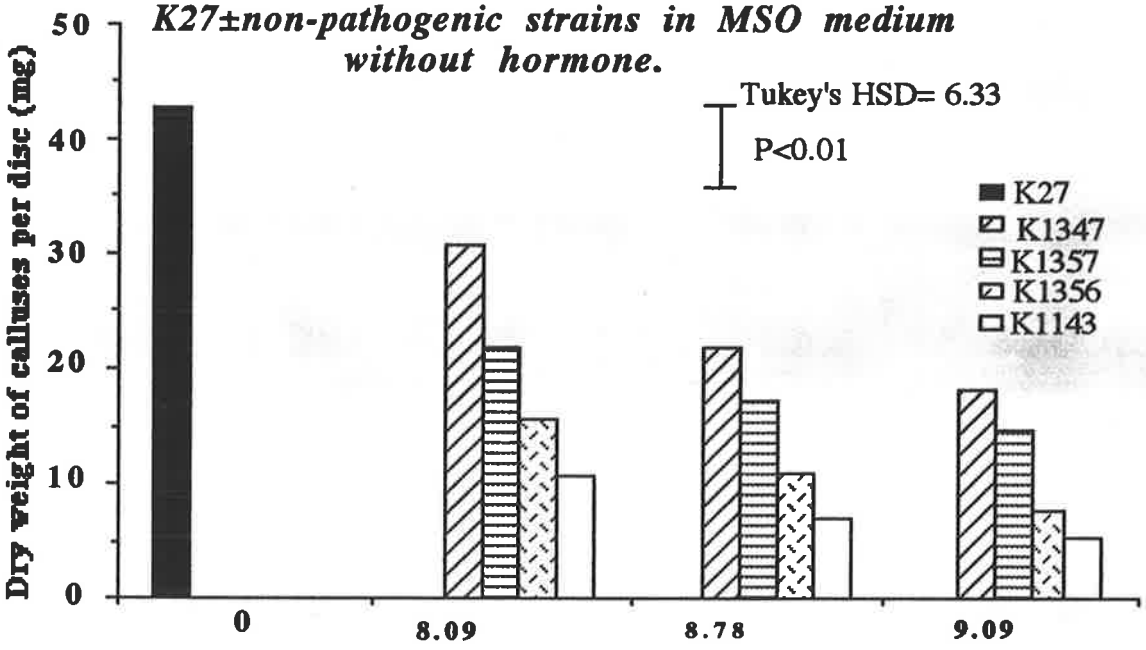
Callus induction on leaf disc of tobacco cultivar White Burley inoculated with pathogen K27±non-pathogenic strains.



Concentration of non-pathogenic strains log (CFU/ml), concentration of pathogen (K27)=8.09

Fig No.4.2

Callus induction on leaf disc of tobacco cultivar White Burley with the pathogen K27±non-pathogenic strains in MSO medium without hormone.



Concentration of non-pathogenic strains log (CFU/ml),

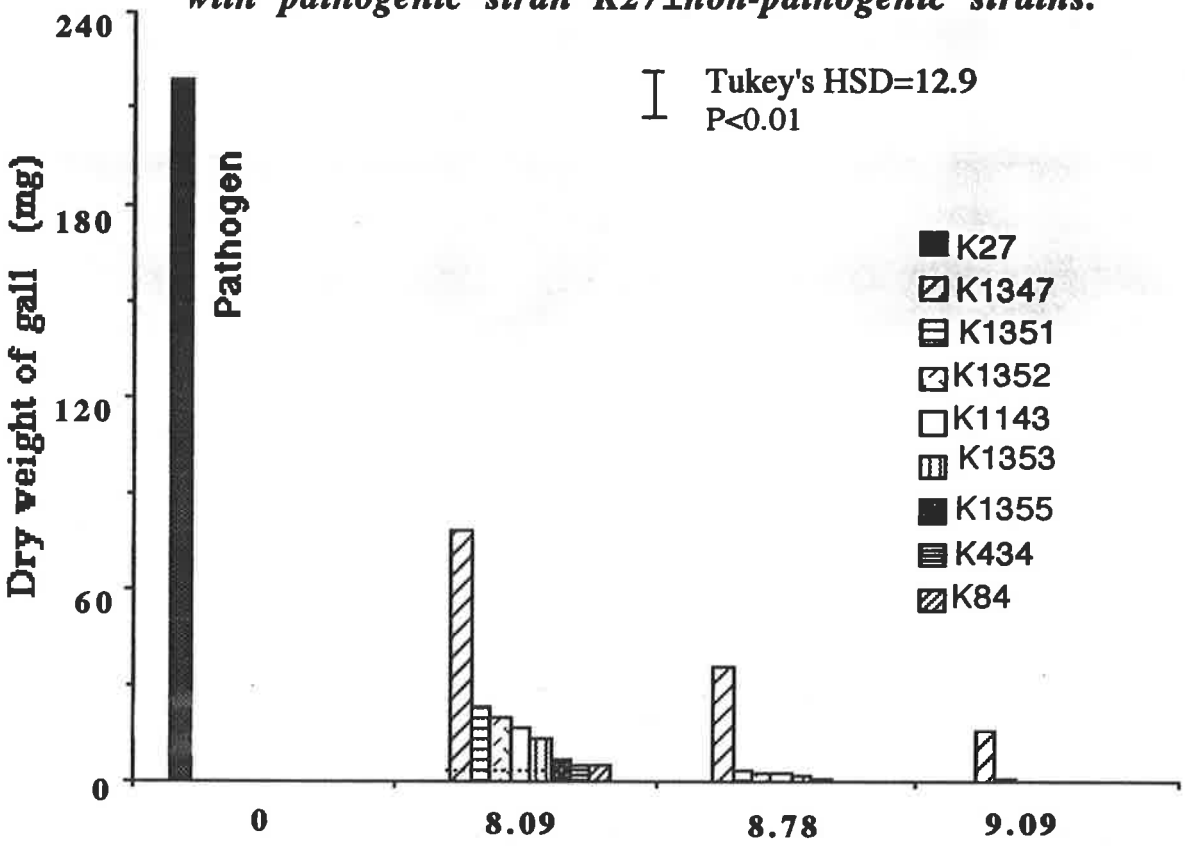
concentration of pathogen (K27)=8.09

4.3.2 *Stem Inoculation*

Stems of tomato *cv* Quick Pick plants were inoculated with *Agrobacterium* strains. Strains were tested for their ability to control crown gall induction by strain K27. The test strains were mixed with strain K27 at ratios of 1:1, 1:5 and 1:10. After incubation, the galls were removed from the stems, dried (3 days at 65°C) and weighed individually. Results are presented in fig 4.3. The Genstat 5 program was used for statistical analysis. Analysis of the tomato stem inoculation data showed that there was no significant difference in biological control ability between the strains K84, K434, K1355 and K1353 at all ratios, and also between K1143, K1352 and K1351 at all ratios. There is a significant difference between K1347 and other non-pathogenic strains in biocontrol of the pathogenic strain K27 with $P<0.001$ (plate 4.1). The treatment with mixture of cured strain, K1347 and strain K27 significantly reduced gall formation by the pathogen, compared with the pathogen alone with $P<0.001$.

Fig No.4.3

Gall formation on Tomato cv. Quick Pick with pathogenic stran K27±non-pathogenic strains.



Concentration of non-pathogenic strains log (CFU/ml),
concentration of pathogen (K27=log 8.09 CFU/ml)

Plate 4.1 Biological control of pathogenic strain K27 by non-pathogenic strains on tomato cv.Quick Pick stems at ratios of 1:1, 1:5 and 1:10 (pathogen to biocontrol).


K27*
K84
1:1 QP
K27*
K1143
1:1 QP
K27*
K1347
1:1 QP
K27
20X
QP
K27*
K84
1:5
QP
K27*
K1143
1:5 QP
K27*
K1347
1:5 QP
K27
20X
QP
K27*
K84
1:10
QP
K27*
K1143
1:10 QP
K27*
K1347
1:10
QP
K27
20X
QP

4.3.2.1 *Tomato seedling bioassays for biological control of crown gall with K1143 derivatives.*

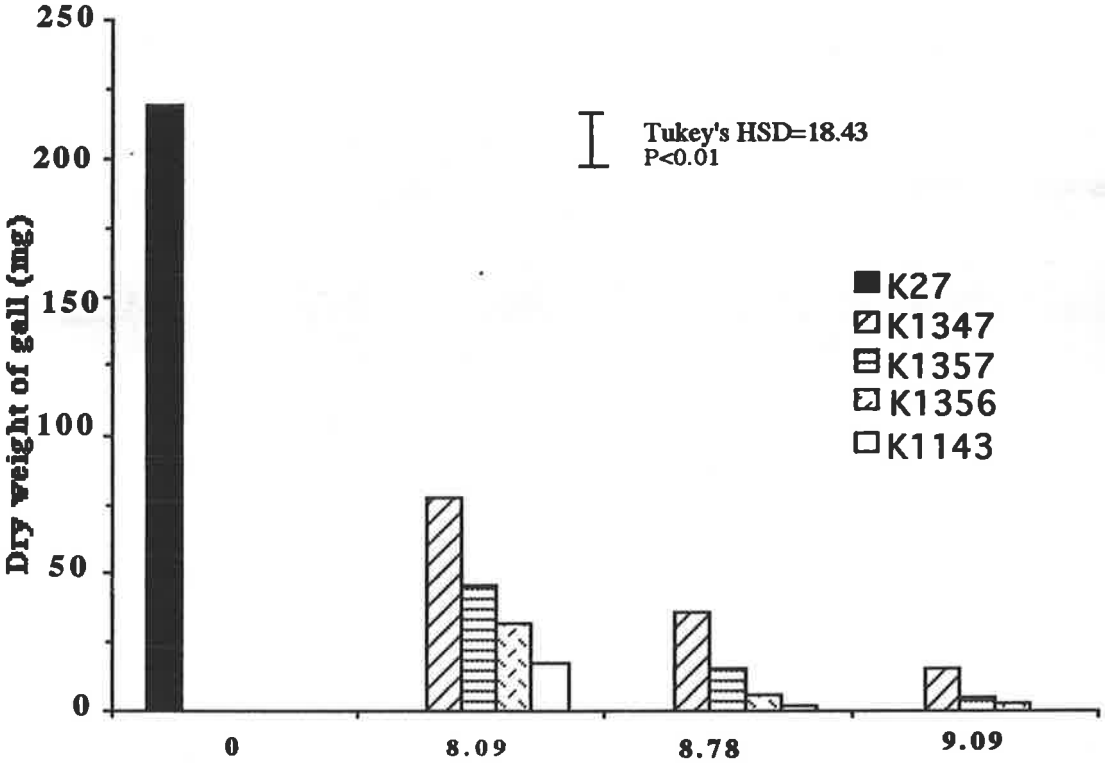
The strains K1143, K1356, K1357 and K1347 were tested for ability to control crown gall induction by indicator strain K27. The test strains were mixed with pathogen at ratios 1:1, 1:5, 1:10. There were six replicates of each treatment. At all ratios all strains were effective biocontrol agents. Results are presented in Fig. 4.4. The Genstat 5 program was used for statistical analysis . Analysis of the tomato stem inoculation data showed that there was no significant difference between strains K1356 and K1357 at all ratios and all potential biocontrol agents significantly reduced gall formation by the pathogen. There was a significant difference between the strain K1357, K1347 with the agrocin 434 producer strain, K1143, at ratios of 1:1, 1:5 with $P<0.01$. Strains K1356 and K1357 with the Tn*5* insertions have less ability to control the pathogen compared with the agrocin 434 producer strain, K1143. The strains with Tn5 insertions in pAgK434 were more effective in controlling gall formation by the pathogen K27 compared with the plasmid free strain K1347, although at higher concentartions of biocontrol strain these differences were not significant.

4.3.3 *Root inoculation*

The roots of Almond *cv.*Fritz plants were inoculated with *Agrobacterium* strains. Strains were tested for their ability to control crown gall induction by strain K27. The test strains were mixed with strain K27 . The experimental design was Randomized Complete Block Design. There were eight replicates for each

Fig No.4.4

Gall formation on tomato cv. Quick Pick with pathogenic strain K27±non-pathogenic strains



treatment. After six month, the galls were removed from the roots, scored, dried (3 days at 65°C) and weighed individually. Results are presented in fig 4.5 and 4.6. The Genstat 5 program was used for statistical analysis.

Analysis of the almond root inoculation data showed that there was no significant difference between the strains K84, K434 and K1353, and also between K1143, K1352 and K1351. Large galls were observed on treatment with pathogen alone, and also with the mixture of cured strain, K1347, and pathogen, K27. Galls were rare or absent on the roots of seedlings treated with K84, K434 and K1353, but frequent on those treated with pathogen alone (Plate 4.2). Gall formation was reduced significantly by treatment with K1351, compared with pathogen alone (Fig. 4.6), The results suggest that pAtK84b which does not encode any known agrocins or antibiotic products may play a role in the biological control process.

No significant differences could be detected between dry weight of galls of plants treated by *Agrobacterium* strain K84 derivatives, except between plasmidless strain K1347 and other derivatives.

A higher incidence of crown gall was found on treated plants with pathogen alone than in plants treated with a mixture of potential biocontrol strains. No significant difference in gall formation was observed between treatment with pathogen alone and mixture of K1347 with K27. Inoculation of almond seedlings with a mixture of pathogen with K1143, K1351, K1352, K1353, K434, K84 seperately, produced a significant (P= 0.01) reduction in number of galls per plants (Fig. 4.6 and Plate 4.2, 4.3).

The inhibitory effect of plasmidless strain K1347 on biocontrol of K27 in stem inoculation and leaf disc culture bioassays (McClure *et al.*, 1994) could not be

Fig No.4.5

Gall formation on the root of the almond cv. Fritz with pathogenic strain K27±non-pathogenic strains.

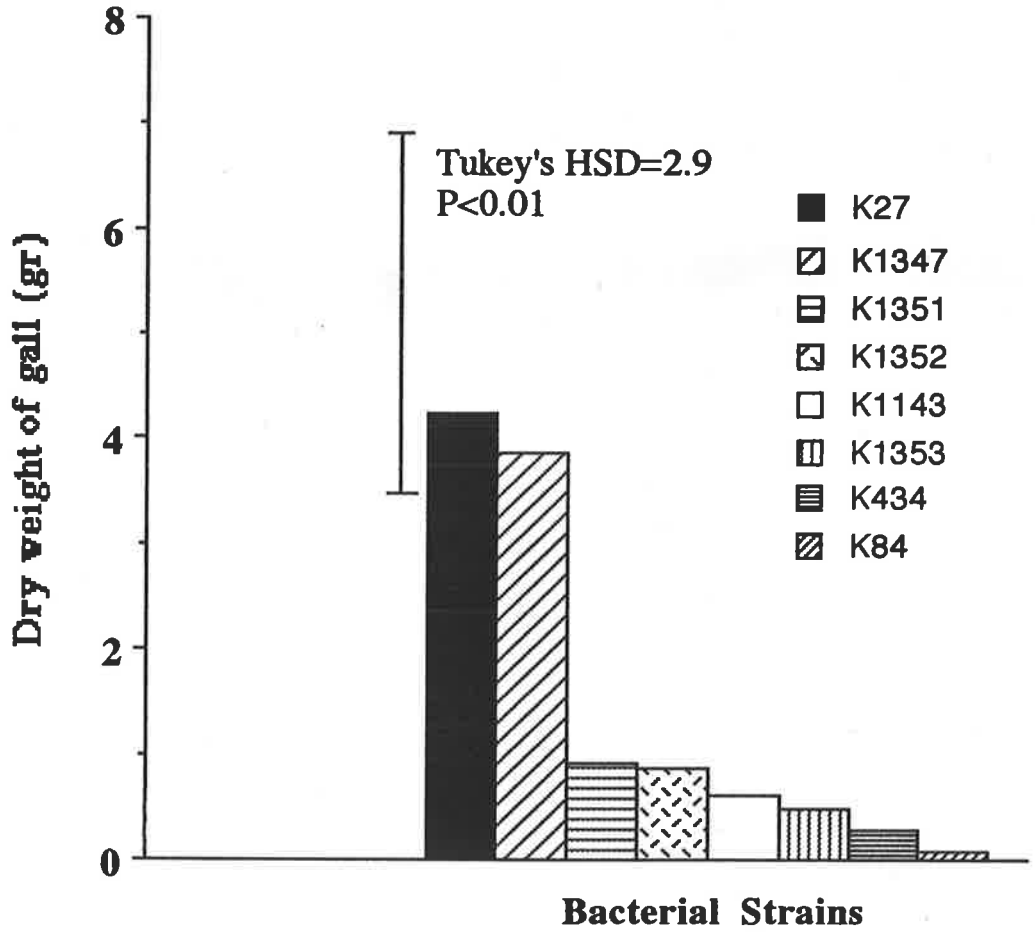


Fig No.4.6

Gall formation on root of the almond seedlings cv. Fritz with pathogenic strain K27±non-pathogenic strains

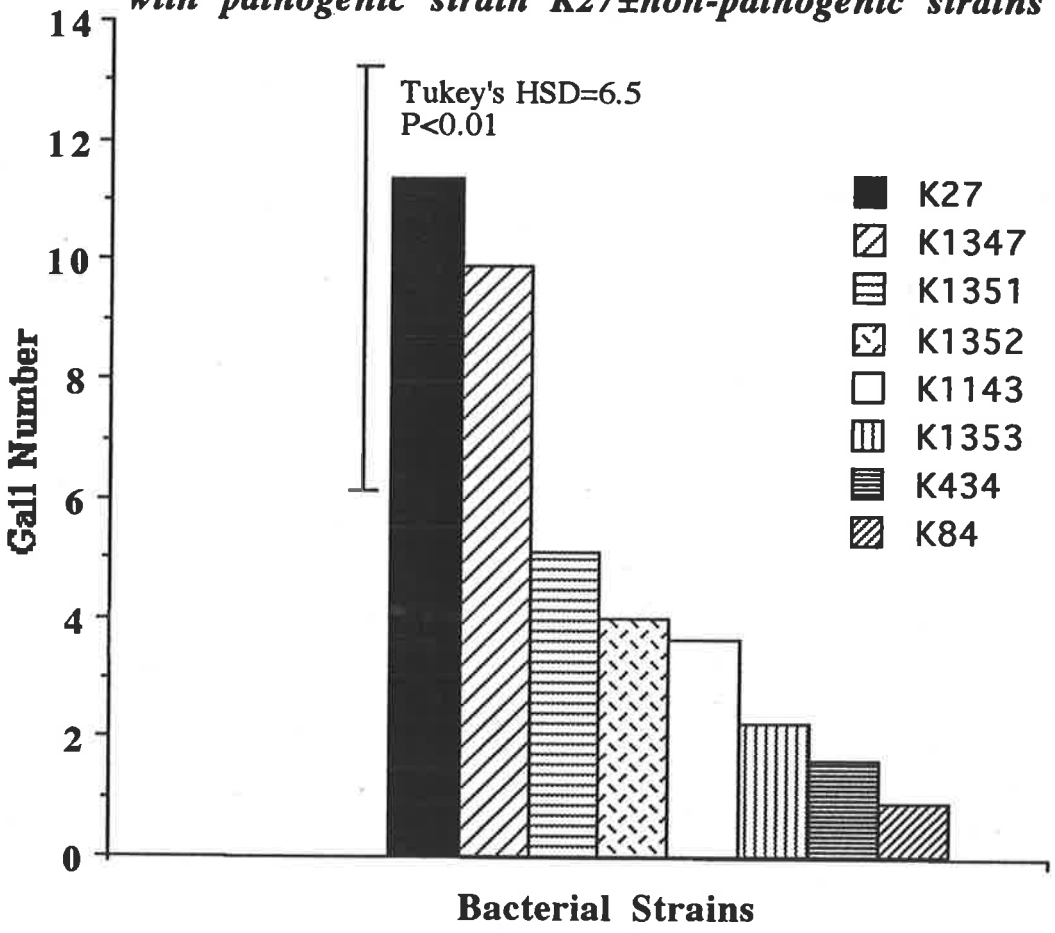
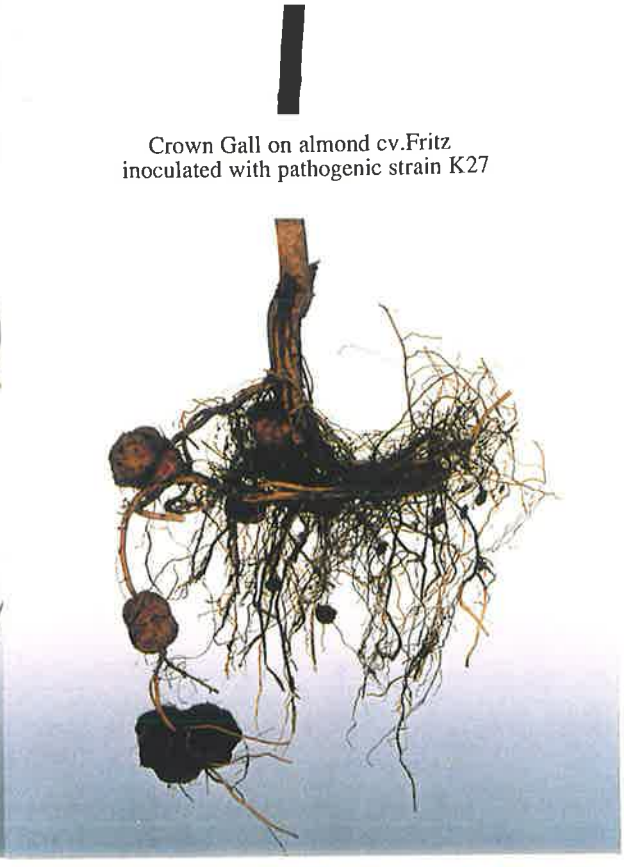


Plate 4.2 Biological control of crown gall on roots of almond *cv*.Fritz inoculated with pathogenic strain K27±non-pathogenic strains (K1143 with pAgK434 and K1347 plasmid free strain).

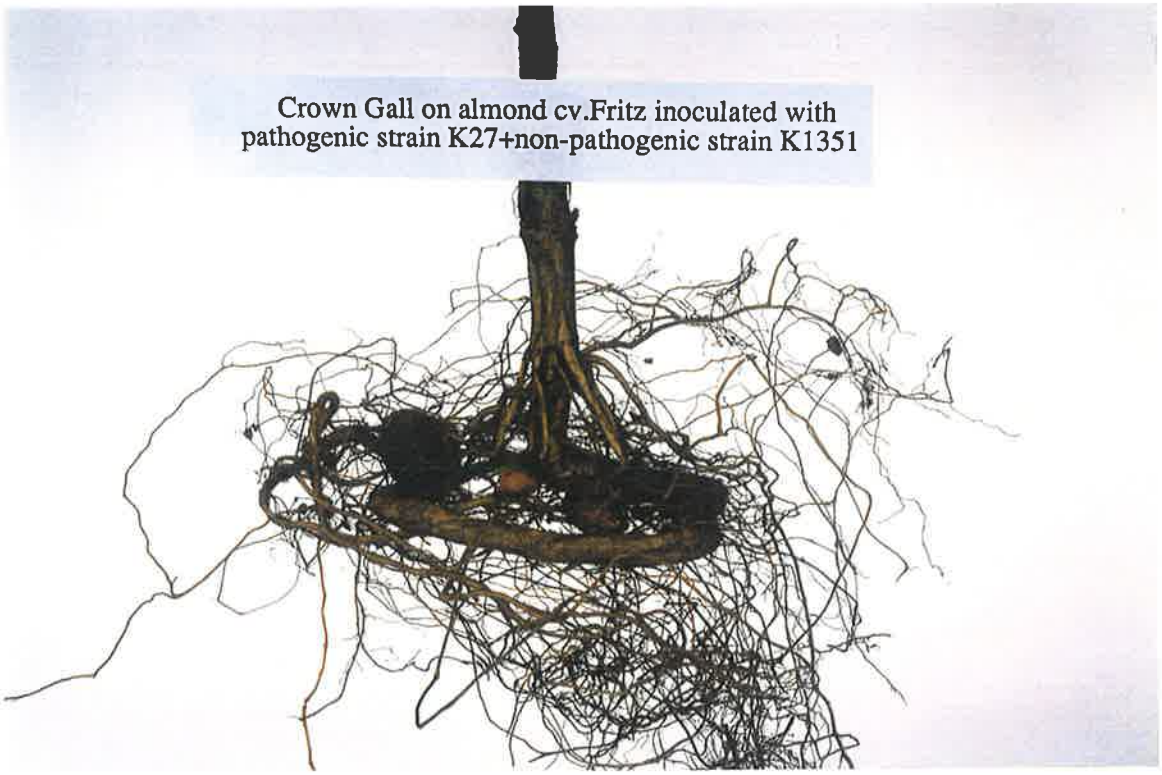

Almond cv.Fritz inoculated with water
(Control)


Crown Gall on almond cv.Fritz
inoculated with pathogenic strain K27


Crown Gall on almondcv.Fritz inoculated with
pathogenic strain K27+non-pathogenic strain K1143


Crown Gall on almondcv.Fritz inoculated with
pathogenic strain K27+non-pathogenic strain K1347

Plate 4.3 Biological control of crown gall on roots of almond *cv*.Fritz inoculated with pathogenic strain K27±non-pathogenic strains (K1351 with pAtK84b and K84 with pAgK84, pAtK84 and pAgK434).



Crown Gall on almond cv.Fritz inoculated with pathogenic strain K27+non-pathogenic strain K1351

Crown Gall on almond cv.Fritz inoculated with pathogenic strain K27+non-pathogenic strain K84

confirmed by root bioassay results. This may be partially due to the different environmental conditions. The result of this study also confirms the biological activity of agrocin 434, which is produced by K1143, K434 and K84 (Plate 4.2, 4.3). The strains K1143 (agrocin 434 producer) and strain K1351 (with plasmid pAtK84b), have the ability to inhibit gall formation as well as strain K1352, (agrocin 84 producer).

4.3.3.1 *Root inoculation and culture under greenhouse condition swith genetically modified biocontrol strains*

The suppressive effect of non-pathogenic strains of *A. rhizogenes* on tumor formation by an *A. rhizogenes* pathogenic strain was examined by mixing control strains K1143, K1347, K1356 and K1357 with pathogenic strain K27 and inoculating almond roots. This experiment was done in a containment glasshouse. After six months, the galls were removed from the roots scored, dried (three days at 65°C) and weighed individually. Results are presented in Fig. 4.7 and 4.8. The Genstat 5 program and HSD was used for statistical analysis. As indicated in Fig 4.8, fewer galls formed on almond roots after treatment with the agrocin 434 producer strain, K1143, compared with other treatments. The result of this experiment determined that strain K1347 was unable to control gall formation by the pathogenic strain K27, and that there was no significant difference in biocontrol ability between strain K1347 and strains K1356 and K1357 which carry pAgK434 with Tn*5* insertions (Agrocin 434^{-}).

Fig No.4.7

Gall formation on root of the almond cv.Fritz inoculated with pathogenic strain K27±non-pathogenic strains

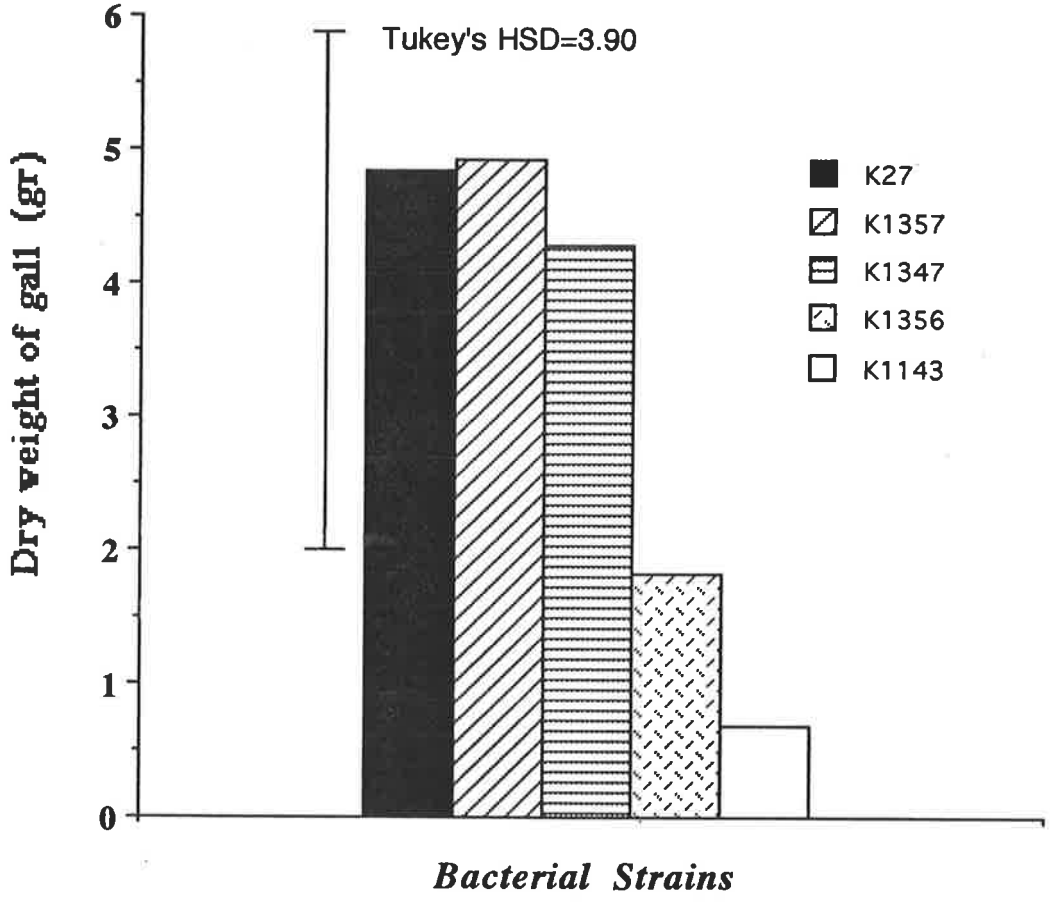
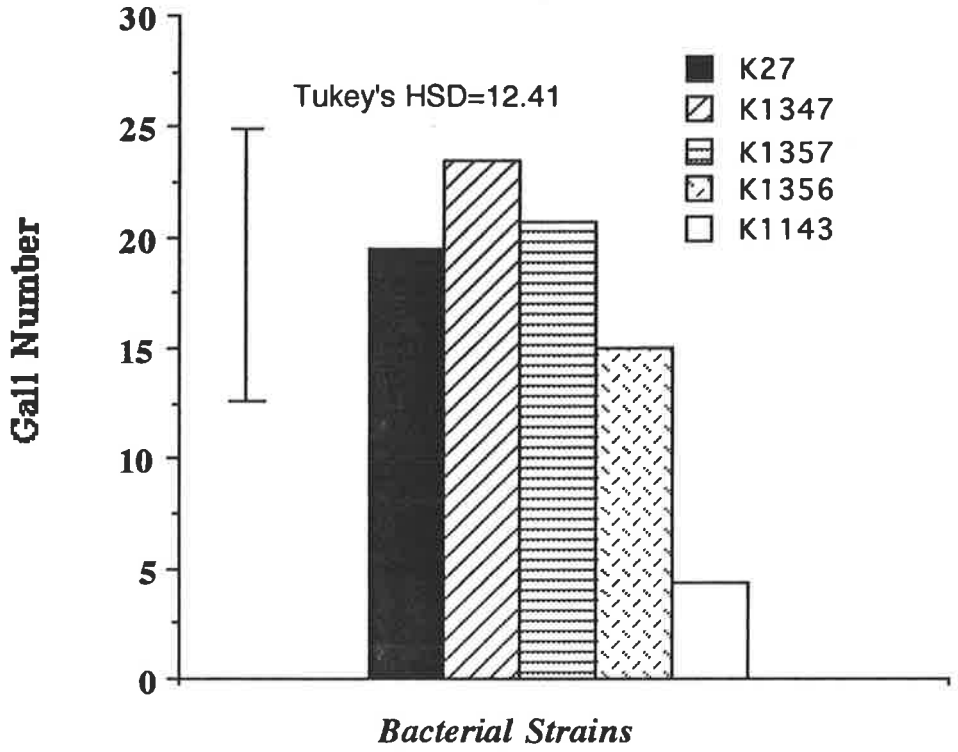


Fig No.4.8

Gall formation on root of the almond cv.Fritz inoculated with pathogenic strain K27±non-pathogenic strains.



4.4 *Conclusions*

The main aim of this study was to compare the efficacy of newly constructed K84 derivatives to control pathogenic strains by different bioassay methods. The results of previous studies by Ellis *et al.*, 1979 and Shim *et al.*, 1987 indicated that production of agrocin 84 by a strain does not automatically make it an efficient biocontrol agent. Strain K1352 (agrocin 84 producer) was not significantly more efficient than strain K1143 (agrocin 434 producer) in all the bioassay methods used. Results from stem inoculation and leaf disc culture assays showed that all the derivatives of K84, including the plasmid free strain, K1347, significantly reduced galling by the pathogen *A. rhizogenes* K27. Strains carrying one or more of the three plasmids of K84 showed a significantly greater biocontrol ability than strain K1347. Results from root inoculation assays indicated that strains harbouring pAgK84, pAtK84b or pAgK434 significantly reduced gall formation by the pathogen K27. Gall formation on almond roots following treatment with strain K1347 was not significantly different than with the pathogen alone. This is in contrast to the result with the stem inoculation and may reflect differences in the assay methods used. The plasmidless strain K1347 reduced gall formation significantly when it was mixed with the agrocin 84 and 434 sensitive strain, K27, and inoculated on tomato stems (Plate 4.1).

The mechanisms of action of K1351 may be due to production of unknown inhibitory compounds and/or competition for nutrients and sites, or other factors. This study provides further evidence for the role of multiple factors in the biological control of *Agrobacterium* pathogens . The finding that the deleted Ti-plasmid, pAtK84b, also enhances biocontrol ability is novel. The insertion of Tn*5* into pAgK434 stopped agrocin 434 production by strains K1356 and K1357. The biological activity of these

new strains was tested using the different bioassays and indicated that these strains can significantly reduce gall formation or tumor induction in leaf disc culture and/or stem inoculation bioassays, but they are unable to control the pathogen in almond root bioassays. This may be due to lack of agrocin 434 production.

The results of assessment of efficacy of potential biocontrol strains to control same the pathogen in different bioassay methods showed some variation i.e. the results of root inoculation were different from other methods tested with strains K1347, K1356 and K1357. This variation may due to the influence of the following factors under root conditions: the ability to produce sufficient antibiotic, chemotaxis and osmotolerance, motility, surface properties of bacterial cells, the species, cultivar, the growth stage of the host plant, soil microbial interactions including competition, mutualism and antagonism, abiotic conditions in the soil and rhizosphere such as availability of O_2, soil matrix, diffusion of nutrients and soil temperature, and population dynamics of the biocontrol agent (Moore & Warren, 1979; Parke 1991).

Chapter Five

Assessment of biological control capabilities of agrocin 434 producer strains with different pathogens.

5.1 Introduction

The initial stages of the pathogenic process for soil-borne bacteria such as *Agrobacterium spp.* are the attraction to the plant root (chemotaxis) and colonizing of the root system. The latter involves attachment, multiplication of bacteria on the root surface and spreading throughout the root system or other parts of the plant. It is interesting that *A. vitis* isolates systemically colonize their hosts as do the *A. rubi* isolates. In this regard *A. vitis* and A. *rubi* have a similar strategy. They cannot survive well in the soil which may be due to lack of saprophytic ability and is the possible reason for increased host specificity in both cases. They have less motility than strains of *A. tumefaciens* and *A.rhizogenes*. *A. vitis* and *A. rubi* pathogenic strains are not controlled by biocontrol strain K84 and when control measures are considered, the systemic natures of biovar 3 strains and *A. rubi* will be an important factor to be consider (Ophel, K.M, Thesis, 1987).

Cooksey and Moore (1982) showed that a mutant of strain K84 that was unable to produce agrocin 84 reduced tumour formation by an agrocin 84 resistant strain. They concluded that other mechanisms, such as physical blockage of infections sites, competition for attachment sites could be responsible for this result. Similarly Lopez *et al.*, (1989) reported that both strain K84 and non-agrocin 84 producer mutant of strain K84, controlled strains resistant to agrocin 84 in field conditions. The previous

studies indicated that strain K84 produces some other inhibitory substances besides agrocin 84. Dhanvantari (1983) reported that some strains *A. vitis* (bv3) resistant to agrocin 84 were inhibited by strain K84 when the assay medium was modified with a different nitrogen source.

The production of second antibiotic compound, called agrocin 434, by strains K84, K1026, K434 and K1143 has been reported. Strains 434 and K1143, a derivative of strain K84, which are lacking agrocin 84 producer plasmid, pAgK84 (Fajardo *et al* 1994, Donner 1993, Clare 1995). These data suggest that agrocin 434 is effective against *A. rhizogenes* pathogens. In addition, Penalver *et al.* (1994) reported the production of an antibiotic like compounds other than agrocins 84 and 434, which is produced *in vitro* by strains K84 and K1026 and a spontaneous mutant of K84 lacking pAgK84. The assay medium used to study inhibition of pathogenic strains can affect the sensitivity patterns of strains tested. Strain K84 and its derivatives, including agrocin 434 producer strains on Danvantari's medium inhibited the biovar 3 pathogenic strain K252. This may be due to inability of strain K252 to grow in the acidic environment surrounding the producer colony or possibly production of higher concentrations of an inhibitory compound such as agrocin 434 in Danvantari's medium compared to Stonier medium (Fajardo, thesis 1995). The main aim of this study is to assess the efficacy of agrocin 434 producer strains to control pathogenic *Agrobacterium* strains from different species with rapid bioassay methods, leaf disc tumorigenesis bioassays and stem inoculation. Almond root bioassays cannot be used as the pathogens are specific for host plants.

5.2 Materials and Methods

5.2.1 *Bacterial Strains.*

The *Agrobacterium* strains used in this study, and their characteristics are shown in table 5.1.

5.2.2 *Agrocin 84 Bioassay*

To test agrocin 84 sensivity, the method of Kerr and Htay (1974) was used as described in chapter 2. The indicator strains K230, K1046, K27 and K252 were tested for agrocin 84 sensivity from strain K84. After 1 or 2 days of incubation at 28°C the plates were examined and sensitive strains showed a clear zone of inhibition around the K84 colony.

5.2.3 *Tomato seedling assay for biological control of crown gall.*

The method of Kerr & Htay (1974) as described previously in chapter 2 was used. Stems of six weeks old tomato cv Quick Pick plants were inoculated with *Agrobacterium* strains. Strains K1143 and K434, agrocin 434 producer strains, were tested for their ability to control gall formation by the pathogenic strains K230 (*A.*

Table No.5.1.

Agrobacterium strains tested for tumor induction and/or gall formation with pathogenic strains ± non-pathogenic strains (agrocin 434 producer strains).

Strain	Biovar	Pathogen	Plasmid	Agrocin sensitivity		Description/ Source
				84	434	
K230	1	yes	C58			R.Hamilton, USA
K27	2	yes	pTiK27, pAtK27	+	+	Kerr, Australia
K252	3	yes	pTiAg57	-	+	Panagopoulos, Greecs
K1046	*A.rubi*	yes	pTi	–	–	ATCC-13335, TR3, Ophel & Kerr, 1990)
K1143	2	no	pAgK434	_	_	Donner *et al.*, 1993
K434	2	no	pAgK434, pAtK84b	_	_	Donner *et al.*, 1993

tumefaciens), K27 (*A. rhizogenes*), K252 (*A. vitis*) and K1046 (*A. rubi*). Bacteria from 48h TY broth were diluted in sterile distilled water, then the test strains were mixed with pathogenic strains at ratio of 1:5. Six week after inoculation , the galls were removed from the stems, dried and weighed individually.

5.2.4 *Assessment of biological control capabilities of agrocin 434 producer strains by leaf disc tumorigenesis bioassays.*

Leaf discs were prepared and inoculated according to the protocol described previously in chapter 3. The strains K1143 and K434 (agrocin 434 producer strains) were tested for ability to control tumor induction by pathogenic strains K230 (*A. tumefaciens*), K27 (*A. rhizogenes*), K252 (*A. vitis*) and K1046 (*A. rubi*) on hormone free medium MSO. The test strains were mixed with pathogenic strains at ratios of 1:1 and 1:10. There were five replicates of each treatment. In this experiment the tobacco plant cultivar White Burley was used for the leaf disc culture. Three weeks after inoculation the discs were removed from the plates, dried for three days at 65°C, and weighed individually.

5.3 Results and Discussion

5.3.1 *Agrocin 84 sensitivity*

The representative strains from *A. tumefaciens*, *A. rhizogenes* and A. rubi were tested for agrocin 84 sensitivity in Stonier's medium. The results are presented in Table 5.2 and plate 5.1.

TableNo 5.2. *In vitro* sensitivity of selected *Agrobacterium* strains to agrocin 84 and 434 on Stonier's medium.

Strain/species	Agrocin 84	Agrocin 434*	Agrocin production
K230(*A. tumefaciens*)	+	-	-
K27 (*A. rhizogenes*)	+	+	-
K252 (*A. vitis*)	+/-**	-	-
K1046 (*A. rubi*)	+	-	-

* results for agrocin 434 senisivity extracted from Fajardo, thesis (1995)

** complex response- concentic zones of growth and inhibition.

5.3.2 *Tomato stem inoculation*

The strains listed in table 5.1 were tested for their ability to control gall induction by pathogenic *Agrobacterium* strains. Results are presented in table 3 and Fig. 5.1. Analysis of the tomato seedling bioassays data showed that the ability of strain K1143 to control the pathogenic strains was slightly lower than strain K434 (plate 5.2).

Treatments with mixture of agrocin 434 producer strains, K1143, K434 with pathogenic strains K230, K27, K252 and K1046 significantly reduced gall formation by the pathogens, compared with the pathogens alone with $P<0.01$.

The ability of strain K1143 to control the bv1 (K230), bv3 (K252) and *A. rubi* (K1046) was slightly lower than control of the bv2 pathogenic strain.

Plate 5.1 Inhibition of *A. rubi* strain K1046 and biovar 1 strain K230 by K84 (produces agrocin 434 and 84) on Stonier's medium.

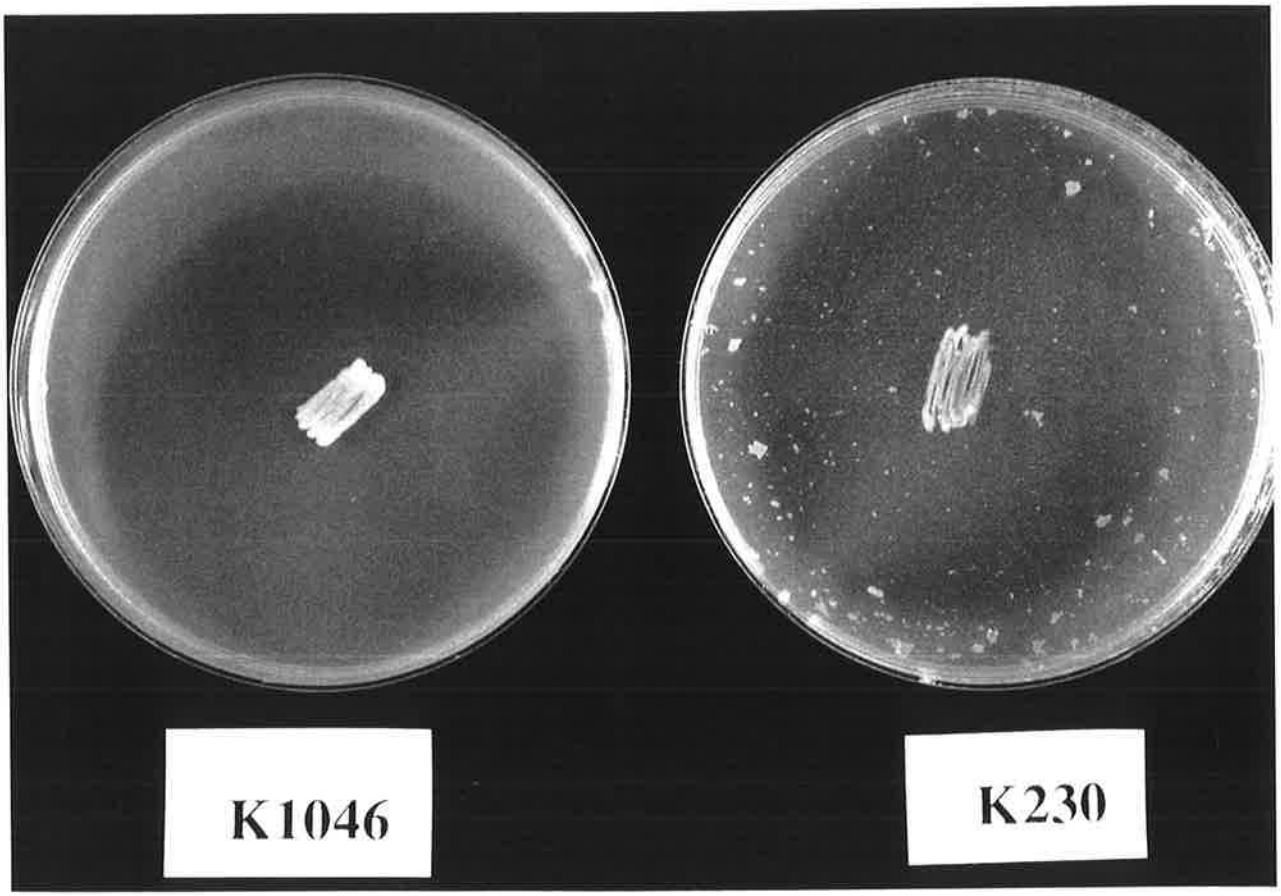

K1046
K230

Table No.5.3 The effect of treating tomato cv Quick Pick with *Agrobacterium* pathogenic strains ± agrocin 434 producer strains (Tukey's HSD= 29.6, P<0.01).

	Dry Weight of galls (mg)	
Treatment	**Means**	**Range**
A. tumefaciens		
K230	149.8	123-201
K230+K1143	19.5	15-25
K230 + K434	0.3	0-2
A. rhizogenes		
K27	218.8	185-254
K27+K1143	2.5	0-5
K27+K434	0	0-0
A. vitis		
K252	157.7	136-189
K252+K1143	23.2	16-32
K252+K434	1.2	0-2
A. rubi		
K1046	168.7	113-198
K1046+K1143	22.8	18-32
K1046+K434	0.8	0-5

Fig No.5.1

Gall formation on tomato cv.Quick Pick with pathogenic strains±non-pathogenic strains.

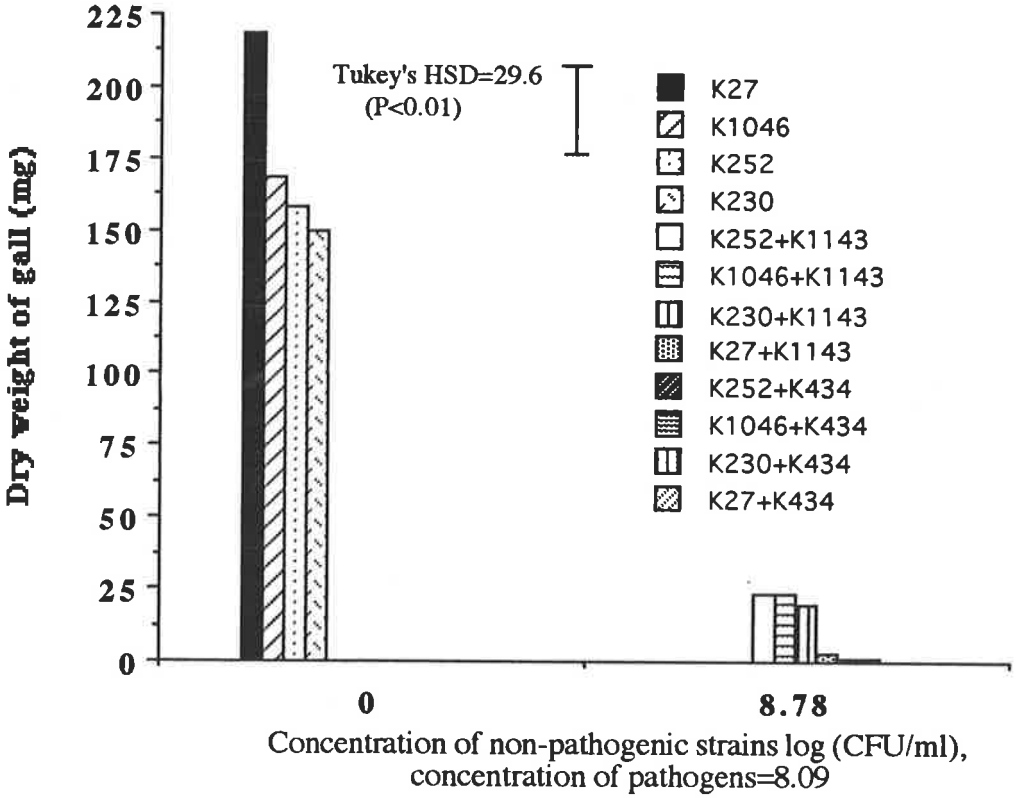


Plate 5.2 Biological control of crown gall on stems of tomato *cv.*Quick Pick inoculated with different pathogen biovars (K230 biovar 1; K27 biovar 2; K252 biovar 3; and K1046 *A. rubi*) ±agrocin 434 producer strains (K434 with pAtK84b and pAgK434; and K1143 with pAgK434).


K252 (bv3)
K434 X K252 (5:1)
K1143 X K252 (5:1)
K1046 (A.rubi)
K434 X K1046 (5:1)
K1143 X K1046 (5:1)
K230 (bv1)
K434 X K230 (5:1)
K1143 X K230 (5:1)
K27 (bv2)
K434 X K27 (5:1)
K1143 X K27 (5:1)

5.3.3 *Leaf disc culture bioassays*

The test strains K1143 and K434 were used as biocontrol agents to control the pathogenic strains K230 (*A. tumefaciens*), K27 (*A. rhizogenes*), K252 (*A. vitis*) and K1046 (*A. rubi*). The test strains were mixed with pathogenic strains at ratios of 1:1 and 1:10. Three weeks after incubation the discs were removed from the plates, dried and weighed. Randomised Complete Block Design in the Genstat 5 program was used for statistical analysis. The results indicated that there is a slight difference between K434 and K1143 in biocontrol of pathogenic strains K230, K27, K252 and K1046 but it was not significant. The result of the treatment with mixture of agrocin 434 producer strains, K434 and K1143 showed a significant reduction of tumor induction by the pathogens, compared with the pathogen alone, with $P<0.01$.

From these results it is concluded that the ability of strain K434 with pAgK434 and pAtK84b to control the pathogenic strains K230 (*A. tumefaciens*), K252 (*A. vitis*) and K1046 (*A. rubi*) was higher than strain K1143 at ratio 1:1. The result indicated that dry weight of tumors in treatment with bv1 (K230), bv3 (K252) and *A.rubi* (K1046) was slightly higher than treatment with bv2 (K27) pathogenic strain. This may be due to greater susceptibility of tobacco *cv* White Burley to these pathogens, which was not expected, especially with *A.rubi* and *A. vitis* (plate 5.3).

Table No.5.4. Tumor induction on leaf disc of tobacco *cv* White Burley with *Agrobacterium* pathogenic strains±agrocin 434 producer strains, in MSO medium without hormone (Tukey's HSD=10.47 with P<0.01).

	Dry weight of tumors (mg)	
	Bacterial Concentration log (CFU/ml), ratios of pathogen±biocontrol agent	
Treatment	**8.09 : 8.09 (1:1)**	**8.09 : 9.09 (1:10)**
A. tumefaciens		
K230	52	
K230+K1143	18.6	11.6
K230 + K434	9.6	5.8
A. rhizogenes		
K27	44.8	
K27+K1143	10.4	5.4
K27+K434	9.4	4.4
A. vitis		
K252	50.2	
K252+K1143	17.4	10.2
K252+K434	10.6	5
A. rubi		
K1046	64.6	
K1046+K1143	18.6	11.4
K1046+K434	11.8	6.6

Fig No.5.2

Callus induction on leaf discs of the tobacco cv.White Burley inoculated with the pathogenic strains±non-pathogenic strains.

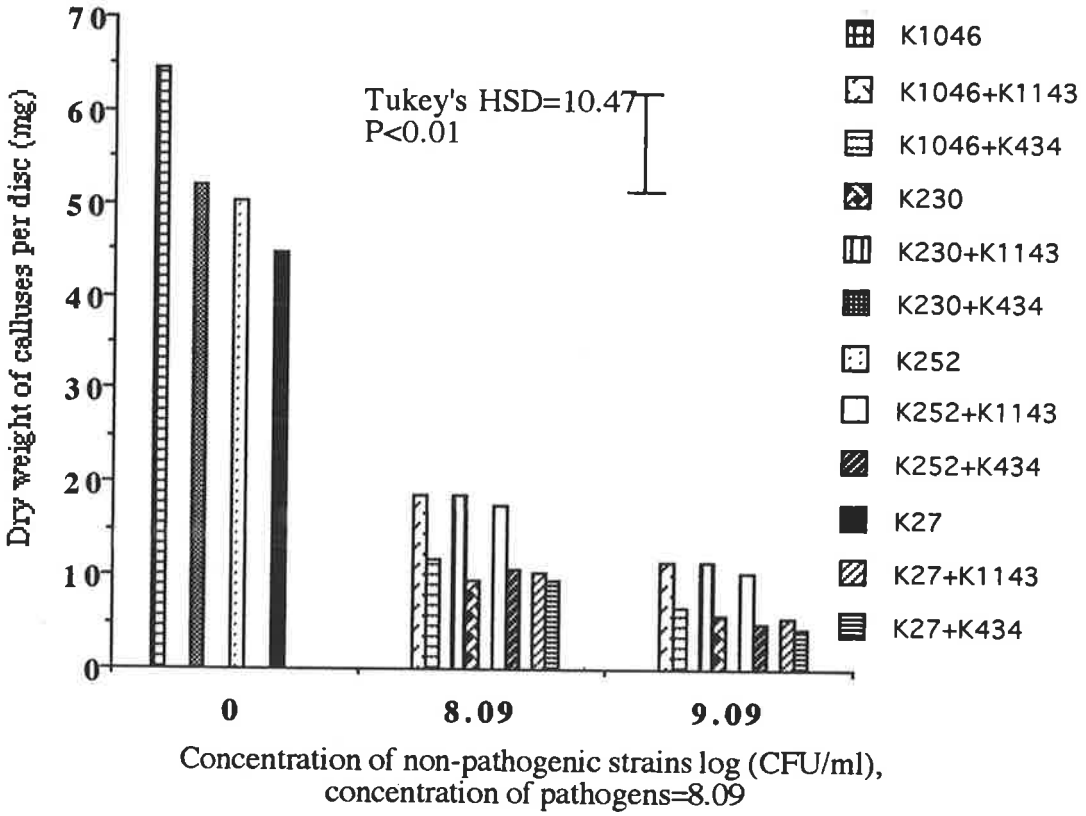
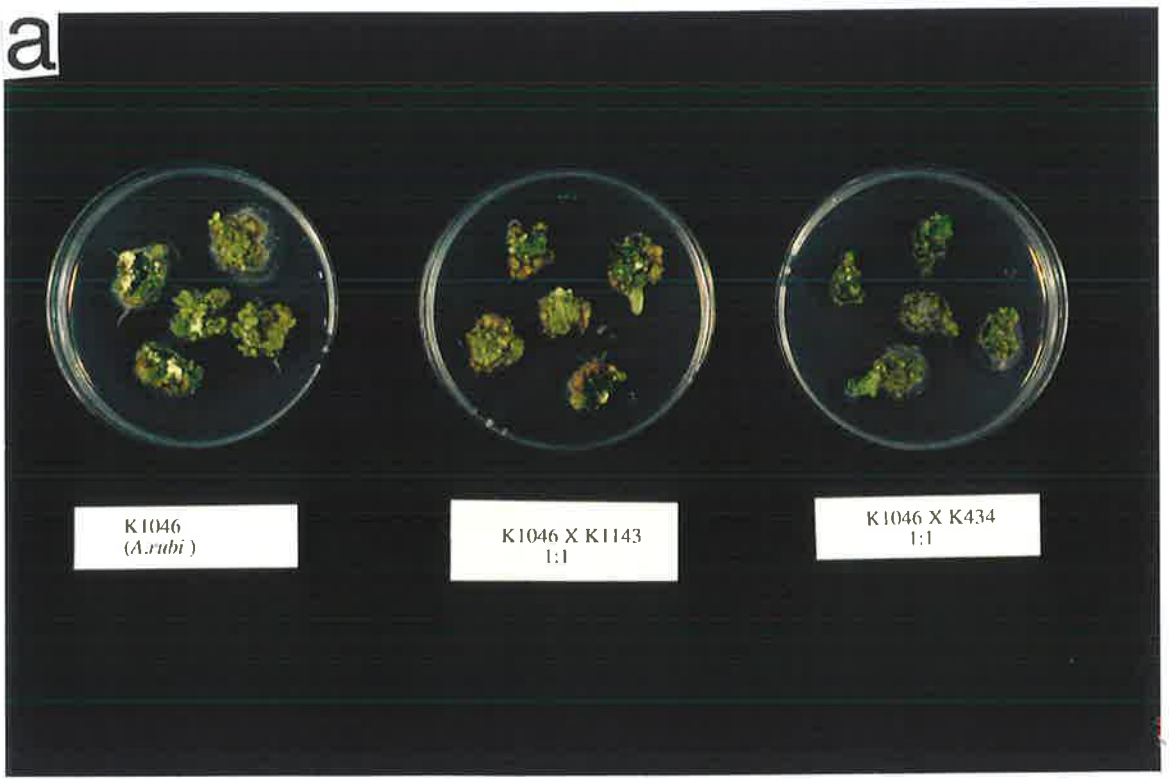


Plate 5.3 Callus induction on leaf disc of tobacco *cv* White Burley inoculated with the pathogen K1046 (*A. rubi*) ± biocontrol strains (K434 with pAgK434 and pAtK84b; and K1143 with pAgK434) at raios 1:1 and 1:10 (pathogen to biocontrol) in MSO media without hormone.


a
K1046
(A.rubi)
K1046 X K1143
1:1
K1046 X K434
1:1


b
K1046
(A.rubi)
K1046 X K1143
1:10
K1046 X K434
1:10

5.4 Conclusions

The most important result from this study is that strain K434 is almost as efficient in controlling crown gall by *A. tumefaciens* and *A. vitis* and *A.rubi* in the stem inoculation and/or leaf disc tumorigenesis bioassays as in controlling crown gall induction by *A. rhizogenes* pathogen (plate 5.2 and 5.3). Strain K1143 significantly reduced gall formation and/or tumor induction by the representative pathogenic strains from bv1, bv3 and *A. rubi* . It was more effective in higher concenteration at ratio 5:1 or at 10:1 (Fig 5.2 and table 5.4).

Biovar 3 and *A. rubi* do not naturally infect the plant species used in this experiments, tomato and tobacco, therefore the results must be viewed with cation, however results are still interesting. An effective biocontrol agent for biovar 3 pathogens (*A. vitis*) needs to colonise grapevine as well as the pathogens. The results of Pu and Goodman (1993) shown that strain K84 is unable to attach to grapevine cells and it was failed to suppress grape crown gall infection. Ophel (1987) reported that K84 produced no inhibition zones at all against any of the *A. rubi* strains tested and also all biovar 3 strains tested were less sensitive to agrocin 84 compared to *A. rhizogenes* strains when tested by the method of Stonier. However, results of our plate assay in Stonier's medium show that strains K230 (*A, tumefaciens*), K1046 (*A. rubi)* were inhibited by K84 but *A. vitis* strain K252 has previously showed a complex response, concentric zones of growth and inhibition (Fajardo, 1995). The result of our study determined that strain K434 can reduce gall formation on tomato and tobacco plants in the two different methods used. However results of these methods do not show the real situation in soil conditions where other factors such as competition for nutrients, motility of bacterial strains and attachment to wound sites may play major roles.

Chapter Six

6.1 *General Discussion*

This study has concentrated on: investigation of the biological properties and role of agrocin 434 and other factors in biocontrol process of crown gall disease; isolation of the complete the range of the K84 derivatives carrying different combinations of plasmids pAgK84, pAgK434 and pAtK84b in the same host background; development of a rapid plant bioassay to assess the efficacy of biocontrol strains by leaf disc tumorigenesis assay; and characterization of agrocin 434 synthesis and immunity genes.

Prior to this study one of the major factors shown to play a role in the biological control of *Agrobacterium* pathogens by strain K84 was production of an antibiotic-like substance known as agrocin 84. Results of Wang *et al.* (1993) have shown that agrocin 84 genes (*agn)* are expressed in *planta.* Agrocin 84 production by strain K84 is encoded by a 47.7 kb conjugal plasmid, designated pAgK84 (Ellis *et al.*, 1979; Slota and Farrand 1982, Farrand *et al.*, 1985; Clare *et al.*, 1990). Agrocin 84 is a disubstituted adenine nucleotide analogue (Ellis and Murphy, 1981). It contains 3'-deoxy-D-arabinose in place of ribose and thus lacks a 3' OH group. The *acc* genes on nopaline Ti-plasmid pTiC58 are responsible for transport and catabolism of the opines agrocinopine A and B (Ellis and Murphy, 1981; Hayman and Farrand, 1988; Ryder *et al.*, 1984; Hayman *et al.*, 1993). Transport of these opines involves a periplasmic permease which has also been shown to take up agrocin 84 due to its N^6-D-glucofuranosyloxysphoramidate substituent. *Agrobacterium* pathogenic strains with *acc*

genes are therefore sensitive to agrocin 84 (Murphy & Roberts, 1979; Ellis and Murphy, 1981; Murphy *et al.*, 1981; Hayman and Farrand, 1988; Hayman *et al.*, 1993). pAtK84b encodes nopaline and agrocinopine catabolism (Ryder *et al.*, 1987; Clare *et al.*, 1990). This plasmid also causes incompatibility to tumor inducing Ti plasmids of the Inc Rh1 group in *Agrobacterium* pathogens (Clare *et al.*, 1990). However, other factors of control other than agrocin 84 production are thought to be involved in the biocontrol process, particularly against pathogens insensitive to agrocin 84 (Cooksey & Moore, 1982; Lopez *et al.*, 1989; Vicedo *et al.*, 1993; Penalver *et al.*, 1994). Recently a new agrocin, designated agrocin 434 has been identified, produced by strains K84, K1026 and derivative strains such as K1143 and K434 both of which have lost pAgK84, the agrocin 84 producer plasmid (Donner *et al.*, 1991, 1993). Initial studies have shown that genes involved in the biosynthesis of agrocin 434 are located on a large cryptic plasmid (300-400 kb) of strain K84 and derivatives (Donner *et al.*, 1993). The chemical structure of agrocin 434 is a disubstituted cytidine (Fajardo *et al.*, 1994; Clare, 1995; Tate *et al.*, 1995). Results of *in vitro* plate assays in plate show that agrocin 434 is less inhibitory than agrocin 84 but inhibits a wider range of *A. rhizogenes* strains (Donner *et al.*, 1993; Fajardo *et al.*, 1994; McClure *et al.*, 1994). Only *A. rhizogenes* strains that produced agrocin 434 were resistant to agrocin 434. However, no *A. tumefaciens* , *A. vitis* or *A. rubi* strains were sensitive to agrocin 434 on Stonier's medium.

The results of this study indicate that genes involved in the immunity/resistance to agrocin 434 are also carried by pAgK434. This has been demonstrated by transferring the plasmid pAgK434 to the agrocin 434 sensitive strain, K27. The resulting strain became resistant to agrocin 434 and had acquired the ability to produce agrocin 434 as well. In this regard, pAgK434 resembles pAgK84, which encodes both agrocin 84 immunity and synthesis genes (Farrand *et al.*, 1985; Ellis *et al.*, 1979; Ryder *et al.*, 1987; Wang *et al.*, 1994). Ellis *et al.* (1979) have demonstrated that agrocin producer

plasmid pAgK84 can be transferred from one strain of *Agrobacterium* to another *Agrobacterium*. Similarly, Vicedo *et al.* (1995) have shown that pAgK84, pAtK84b and pTi can transfer between *Agrobacterium* strains, and in the case of transfer of pAgK84 to pathogens, resultant transconjugants became resistant to agrocin 84. When pTi was transferred into strain K84 it became sensitive to agrocin 84. The result of experiments with cured derivative of K84, K1347 (McClure *et al.*, 1994) show that this strain is resistant to agrocin 434. All *A. rhizogenes* strains ever tested are sensitive, except agrocin producer strains (Donner *et al.*, 1993; Fajardo, 1995). This result suggests that additional resistance/immunity functions to agrocin 434 are chromosomally borne in this strain. The result of this study provides the first evidence that immunity/resistance functions to agrocin 434 are carried by pAgK434.

Another derivative of K84, strain K1318 with pAgK1318, carries a deleted version of pAgK434 (Rosewarne & Jones, unpublished). This strain is unable to produce agrocin 434 but produces a modified agrocin 434 (nucleoside 4176) which has no inhibitory activity. The kanamycin resistance transposon Tn*5* was introduced into derivatives of pAgK1318 marked with antibiotic resistance markers and the resulting plasmid transferred to an agrocin 434 sensitive strain. The resulting transconjugants were able to produce modified agrocin 434 as did K1143 pAgK1318. The result of this study shows that resistance/immunity functions are also carried on pAgK1318, as transconjugant strains were resistant to agrocin 434. Strain K1318 is resistant to agrocin 434, this strain can be used for further characterization and localization of agrocin 434 genes that encode immunity to agrocin 434, the smaller size of pAgK1318 make this plasmid an easier target for mutagenesis and genetic characterization compared to pAgK434. A restriction map for pAgK1318 has recently been completed (Fajardo, 1995).

To study the role of agrocin 434 in the biocontrol process a strain cured of pAgK434 was used, K1347, but it is uncertain whether there are other factors which may play a role located on this plasmid. Therefore, we tested the Tn*5* insert mutants of pAgK434. As a result of Tn*5* mutagenesis of pAgK434 in strain K1143, two isolates strain K1356 and K1357 had been obtained, however it is important to show that loss of ability to produce agrocin 434 was caused by a Tn*5* insertion in genes essential for production. Verification of Tn*5* insertions causing the loss of agrocin 434 production is still needed. To prove that Tn*5* insertion into K1356/K1357 was responsible for stopping agrocin 434 synthesis Southern hybridization analysis was considered. The steps needed to verify Tn*5* inserts are as follows: clone fragment with Tn*5* into vector, reintroduce into wild type strain via recombination to regenerate mutant. In this study a potential clone carrying a fragment from strain K1356 was analysed. Results of this analysis suggests that the cloned band does have a Tn*5* insertion but that some sort of rearrangement and/or instability had occurred during the original cloning procedure, therefore this was not continued. Verification of strains K1356 and K1357 will require recloning of mutant fragments from these strains.

To understand the role of each of the K84 plasmids, pAgK84, pAtK84b and pAgK434 in the biocontrol process, a range of derivatives of strain K84 harbouring all combinations of these three resident plasmids in the same host background were constructed. Previously, strains K84 (New and Kerr, 1972), K434 (Shim *et al.*, 1987), K1143 (Donner *et al.*, 1993), K1347 (McClure *et al.*, 1994), K1356 and K1357 (NC McClure, unpublished) had been isolated. In this study strain K1351 with pAtK84b, strain K1352 with pAgK84, strain K1353 with pAtK84b and pAgK84 and strain K1355 with pAgk84::Tn*5* and pAgK434 were constructed, to complete the possible plasmid combinations in the K84 host background. Transfer frequencies of pAgK84 from donor strain K325 to recipient strain K1347 was very low an average 2.5×10^{-3} per recipient and the transfer frequency of pAgK84 and pAtK84b from *A. tumefaciens*

donor to *A. rhizogenes* recipient was 6.6 x 10^{-3}. It was difficult to select the transconjugants because there is no positive selective media for pAgK84 which carries no selective marker. For this reason transconjugants were identified on *A. rhizogenes* selective media, verified as *A. rhizogenes* using chemical tests for 3-ketolactose production and potential transconjugants screened for agrocin 84 production. Presence of the plasmid was verified by gel electrophoresis.

To compare methods that can be used to evaluate the efficacy of biocontrol strains, the following techniques were used: (1) leaf disc tumorigenesis assay; (2) stem inoculation bioassay and (3) tobacco root inoculation.

A rapid efficient method for testing pathogenicity and/or the efficacy of biocontrol strains was developed using a leaf disc tumorigenesis assay. In this method MSO hormone free medium (Horsch *et al.*, 1988) was used because when T-DNA is integrated into the plant genome the transferred DNA encodes genes for synthesis of the phytohormones such as indole-3-acetic acid, an auxin, and cytokinin (Schröder *et al.*, 1984; Barry *et al.*, 1984; Costacurta and Vanderleyden 1995). Auxins and cytokinin are involved in: cell division, cell elongation, tissue differentiation and apical dominance of plant growth and development (Costacurta and Vanderleyden 1995). However, crown gall tumors do grow in culture in the absence of added auxin or cytokinin, following tobacco pith cell transformation into crown gall tumor cells, both of these plant hormones were persistently synthesized and typical tumors developed. That these plant growth regulators were, in fact, synthesized persistently by the tumor cells was later indicated by isolating and characterizing them chemically (Braun, 1982). The leaf disc tumorigenesis system permits simple, efficient, and reproducible examination of *Agrobacterium* gene transfer into plant cells in the disc. Whole discs provide a convenient unit for replicating treatment samples and this method permits a more sensitive measurement of pathogenicity, gene transfer,

expression, and integration than widely used transformation assays on plant stems or leaves. A range of tobacco and tomato cultivars were tested to determine which plant cultivars gave the most rapid and reproducible tumor formation with different concentrations of pathogen. Tobacco cultivars affected transformation although the differences between the cultivars examined in this study were generally small. Tobacco cultivar White Burley transformed more easily and produced tumors more rapidly than other cultivars as seen by a higher gall induction for all bacterial concentrations. The accelerated development of necrosis following infection was observed in tomato cultivars for high bacterial concentrations. This necrotic response was possibly due to a hypersensivity of the tomato tissue or the nature of the tissues which presumably lacked the structural and biochemical barries to infection operating in leaf disc material (Charest *et al.*, 1988; Sharma and Skidmore, 1988; Scott *et al.*, 1992). It was suggested that slight differences in environmental conditions may have been responsible for the development of necrosis. To use leaf disc culture tumorigenesis many factors should be considered such as temperature, inoculum concentration and light conditioning. In addition any *in vitro* model systems, whether for biocontrol agent screening or for pathogenicity, will be of value only when the reactions observed are analogous to whole plants bioassays at the *in vivo* level. Bacterial strains showing biological control activity or pathogenicity in leaf disc culture tumorigenisis bioassay must, therefore be further evaluated under field and/or glasshouse conditions. For example the effect of host factors including host specificity, root exudation, root colonization ability of bacterial cell and the effects of the soil environment, have been shown to modify the result in the field (Moore & Warren, 1979). Comparison between pathogen concentration indicated that 10E 8.09 (CFU/ml) of inoculum is the optimum concentration for tumor induction with tobacco and 10E 7.70 (CFU/ml) of inoculum is the optimum concentrations for tumor induction with tomato.

Initially an assessment of pathogenicity and biocontrol efficacy was tested by enumerating the tumors but it was very hard to score the tumors due to size variation.

For this reason dry weights of tumors were measured to analyse the results of tumor induction and this gave less variation and was easier to score than using tumor numbers.

Screening using a tissue culture technique, leaf disc tumorigenesis assay has several advantages over conventional methods as follows: many plants may be assessed in a small space where environmental and nutritional factors can be strictly controlled; plant material may be inoculated with isolates of pathogen/biocontrol agent in the absence of biotic contaminants; plant material *in vitro* often gives a rapid response compared with other methods and hence preliminary assessment may be completed more rapidly than if undertaken in the glasshouse or field. Disadvantages of leaf disc culture; possibility of hypersensitivity response and necrosis of and contamination of discs by fungi and bacteria, this method needs special equipment such as a tissue culture room and materials are very expensive. In addition pathogens/biocontrol agents may overgrow the culture medium used for the host plant and obscure the assessment of efficiency of biocontrol agent or pathogenicity of pathogen.

The results of stem inoculation bioassays determined that tomato *cv.* Quick Pick is the best plant for stem inoculation, because gall formation by stem inoculation of tobacco requires a longer incubation period than tomato and this cultivar produced more galls than other tomato cultivars. Comparison between leaf disc culture and stem inoculation indicated that leaf disc culture is a rapid method for assessment of the efficacy of biocontrol strains. This method produced results after 15-21 days compared with 4-5 weeks for tomato and 10 weeks for the tobacco stem inoculation assay method. The results indicated significant differences between different population ratios of biocontrol strains to pathogen in controlling pathogens. For example, strain K1347, the plasmid free derivative of K84, at higher concentration (10:1) was more effective compared to lower concentration (1:1). This may be due to differences in production of another inhibitory compound and/or competition for sites. In 1977, Moore reported that strain K84 reduced incidence of galling to 5% at ratio 1:10 compared 31% at ratio

1:100, biocontrol to pathogen. Results of stem inoculation and leaf disc tumorigenesis assays with strains K1357 and K1356 were similar to strain K1347. At higher concentration (10:1) they controlled tumor formation by pathogen compared to lower concentration (1:1).

Tobacco roots exhibited a necrotic response to bacterial infection by *A. rhizogenes* strain K27 and gall formation was not observed. Similarly, in some case *A. tumefaciens* induced a necrotic response on some *Vitis* cultivars, while they induced tumor formation on other cultivars of grapevine (Yanofsky *et al.*, 1985; Pu & Goodman, 1992). Therefore, the response of a plant to infection by a specific strain of *Agrobacterium* can be tumor formation, resistance, or necrosis, depending on the properties of the *Agrobacterium* strain and the particular plant species infected. Mutations within the *virC* locus of wide host range strains prevent the necrotic response and allow the formation of tumors by the strain, implying that high T-DNA transfer efficiency is important for inducing the necrotic response (Yanofsky *et al.*, 1985). The gall formation results from the active production of plant growth regulators, auxin and cytokinin, encoded by T-DNA genes (Thomashow *et al.*, 1986). Auxin and cytokinin must be at appropriate levels and ratios for *Agrobacterium* to induce tumors on its host plant, since accumulation of the phytohormones at high levels could cause cell death. Some plants may have evolved mechanisms that overcome drastic fluctuations in growth regulators levels, and thus have become insensitive to tumor formation. On the other hand, some plants may be extremely sensitive to such changes and respond to such alterations in a way other than tumorigenesis. However, it is important to note that the necrotic response in not the typical hypersensitive response, the well known plant defence system which results from an incompatible interaction with a plant pathogen. This reaction is characterized by the rapid, localized death of plant cells at the site of pathogen invasion (Klement 1982; Goodman & Novacky, 1994). The hypothesis that the necrotic response is different from the hypersensitive response is supported by the report that some *A. tumefaciens* can in fact inhibit the development of the

hypersensitivity caused by *Pseudomonas syringae pv. phaseolicola* on tobacco (Robinette & Matthysse 1990). It should be mentioned that to find a fast growing test plant for root inoculation assays to enable quicker root-based assays, tobacco plant were selected as a test plant for root inoculation assay. Previously, most investigators used tomato (Kerr & Htay, 1974) or almond seedlings (Jones & Kerr, 1989) as the test plant although this latter plant needs at least 9 month to demonstrate the results. After the necrosis reaction in tobacco plants was observed almond seedlings were used for further experiments.

Screening bioassay techniques can be employed to save time and reduce costs when screening for biological control agents. Several *in vitro* bioassays were tested plate assays for agrocin production on Stonier's medium and leaf disc tumorigenesis. The results of these methods were not sufficient to predict the prevention of gall formation in pot trails. The efficacy of all derivatives of strain K84 in controlling the pathogenic biovar strain K27 was assessed using several *in vitro* and *in vivo* bioassays including leaf disc tumorigenesis, stem inoculation and root inoculation. There is evidence that strain K84 produces at least one other inhibitory compound other than agrocin 84 and 434 (Vicedo *et al,* 1993; Penalver *et al.*, 1994). Results from stem inoculation and leaf disc culture assays indicated that all the derivatives of K84, including the plasmid free strain, K1347, significantly reduced galling by the pathogen *A. rhizogenes* K27. The results of these experiments indicated that plasmid free derivative of K84, strain K1347, was able to control gall formation and/or tumor induction by the pathogenic strain. This capacity may be due to competition for site and/or nutrient between the pathogenic strain and the cured strain, K1347. One important point is that there is the possibility that other chromosomally encoded inhibitory compounds are produced by the cured strain K1347. In this regard, Vicedo *et al.* (1993) observed that Agr^- derivatives of K84 and K1026 were able to inhibit an agrocin 84 resistant pathogen *in vitro*. They concluded that strain K84 and K1026 produced other inhibitory

compounds other than agrocin 84 and 434. Similarly Lopez *et al.* (1989) reported that strain K84 and an agrocin 84$^-$ mutant of K84 controlled tumor formation on plum and peach trees in a root inoculation bioassay with pathogenic strains of *A. tumefaciens* sensitive or resistant to agrocin 84. The effect of different media on sensitivity to agrocin 84 and 434 showed that strain K1347 as well as strains K1143 and K1352 produced inhibition zone in Danvantri's medium against a pathogenic strain (*A. tumefaciens*) but it was unable to inhibit the same pathogen in Stonier's medium (Fajardo, 1995). It is suggested that this may be due to effect of pH or production of another inhibitory compound and that the effect and/or production is dependent on the assay medium used. Similarly, Penalver *et al.* (1994) reported that *A. rhizogenes* strain K84, K1026 and a K84 non-agrocin producing mutant (K84 Agr$^-$) produced an antibiotic-like substance in MG medium, called ALS 84. They did not observe production of an inhibitory substance following growth in Stonier's medium. They found that strain K84 and its derivative, K84 Agr$^-$ had an antagonistic effect against some agrocin 84-resistant strains which they did not observe on Stonier's medium. They concluded that the classification of *Agrobacterium* pathogens into sensitive or resistant to agrocin 84 producer strain was correlated to the types of medium and utilization method, because strains resistant to agrocin 84 *in vitro* were inhibited by adding glucose to MG medium (Penalver *et al.*, 1994). It is concluded that activity of biocontrol agents under *in vitro* conditions may be affected by media conditioning (Moore & Warren, 1979).

Strains carrying one or more of the three plasmids of K84 showed a significantly greater biocontrol ability than strain K1347. Results from root inoculation assays indicated that strains harbouring pAgK84, pAtK84b or pAgK434 significantly reduced gall formation by the pathogen K27. In contrast, the inhibitory effect of plasmidless strain K1347 on biocontrol of K27 in stem inoculation and leaf disc culture bioassays could not be confirmed by root bioassay results. This may be partially due to the

different environmental conditions and may reflect a differences in the assay methods used. The poor biocontrol activity of strain K1347 in the root zone may be due to lower ability to colonize the roots of host plants, soil pH, effect of other soil microorganisms, soil structure, root exudates, ability of pathogen to produce inhibitory compounds against this strain in soil conditions, inability to compete with pathogens for nutrients or sites, or combinations of these factors.

In some cases the results of root inoculation were different to other methods tested e.g. with strains K1347, K1356 and K1357. Under root conditions the following factors may influence the capability of biological control agents: the ability to produce sufficient antibiotic, chemotaxis and osmotolerance, motility, surface properties of bacterial cells, the species, cultivar, the growth stage of the host plant, soil microbial interactions including competition, mutualism and antagonism, abiotic conditions in the soil and rhizosphere such as availability of O_2, soil matrix, diffusion of nutrients and soil temperature, and population dynamics of the biocontrol agent (Moore & Warren, 1979; Parke 1991).

The results of this study provide the first evidence for the biological activity of agrocin 434, which is produced by K1143, K434 and K84. The strains K1143, (agrocin 434 producer) and strain K1351 (with plasmid pAtK84b), inhibited gall formation at a level equivalent to strain K1352, (agrocin 84 producer), with the test pathogen used.

The mechanisms of action of K1351 with pAtK84b may be due to production of unknown inhibitory compounds and/or competition for nutrients and sites, or other factors. In addition, pAtK84b causes incompatibility to Inc Rh1 Ti plasmid of pathogenic *Agrobacterium* strains, by preventing transfer of the Ti plasmid to the

biological control agent and it may also reduce occurrence of further infections (Clare *et al.*, 1990). The result of Clare *et al.* (1990) shown that there is strong and extensive DNA homology between pAtK84b of strain K84 and tumor inducing plasmid pTiC58 of *A. tumefaciens* C58. This homologous region codes for Noc, nopaline catabolism, Incompatibility /Origin of replication, and Tra, conjugal transfer, in both plasmids, pAtK84b and pTiC58. In contrast there was no detectable homology between pAtK84b and oncogenic T-DNA and virulence (*vir*) regions of Ti plasmid. They suggested that pAtK84b is a deleted version of pTi58 plasmid which has been disarmed in both T-DNA and *vir* regions. Results of hybridization analysis between pTi and pAtK84b has shown that there may be recombination between pTi and pAtK84b, and also pAtK84b can transfer from K84 to a virulent strain and cause the replacement of pTi by pAtK84b in *A. tumefaciens*. The resultant transconjugant became avirulent (Vicedo *et al.*, 1995).

Wang *et al.* (1994) demonstrated that presence of the pAtK84b does not affect *agn* expression or root inoculation, *in planta*, indicating that the plasmid may not contribute to either to these phenotypes. This study provide the first evidence that pAtK84b does play a role in the biological process. The results of Wang *et al.* (1994) cannot be directly compared with results presented here because they used a different tests system to show the expression of agrocin production genes. In the study described by Wang *et al.* (1994) they did not use wounded roots. Root exudates are involved in chemotaxis and wound sites are important for attachment initial steps in the interaction between *Agrobacterium* and potential host plants and bacterial cells may respond chemotactically to chemicals released into the rhizosphere by wounded plant cells, used in this study. They also used a different bacterial host background for pAtK84b and host background plays an important role in the efficacy of the biocontrol agent. Also they did not assess the role of pAtK84b in binding in the presence of pathogens. In this study experiments were conducted with mixtures of biocontrol and pathogen.

There are several potential explanations for the role of pAtK84b in control of pathogen K27: pAtK84b carries genes involved in catabolism of nopaline and agrocinopine, for this reason, strains carrying this plasmid can compete with pathogen for nopaline as a carbon source. However this advantage only takes effect after transformation and gall formation. A more likely reason is that pAtK84b caries genes important in early stages of the pathogenic process. This may be part of the attachment process and this conclusion is supported by the papers which have shown that nopaline type Ti plasmids (of which pAtK84b is a deleted version) enhance attachment (Smith & Hindley, 1978; Whatley *et al.*, 1978; Matthysse *et al.*, 1978; Tanimoto *et al.*, 1979; Pu & Goodman, 1993). Alternatively chemotaxis of strains harbouring pAtK84b towards wound sites may be enhanced compared to plasmid-free strains. Ti plasmids genes have been shown to play a role in chemotaxis to wound exudates (Ashby et al., 1987, 1988). In addition, pAtK84b may encode another inhibitory compound which affects the pathogenicity of pathogenic agrobacteria. This study provides further evidence for the role of multiple factors in the biological control of *Agrobacterium* pathogens. The finding that the deleted Ti-plasmid, pAtK84b, also enhances biocontrol ability is novel and warrants further investigation. The insertion of Tn*5* into pAgK434 resulted in loss of agrocin 434 production by strains K1356 and K1357 (McClure, unpublished). The biological activity of these new strains was tested using the different bioassays and indicated that these strains can significantly reduce gall formation or tumor induction in leaf disc culture and/or stem inoculation bioassays, but they are unable to control the pathogen in almond root bioassays. In this respect they acted like the cured strain K1347 which suggest that agrocin 434 production is the major factor encoded by pAgK434 involved in biological control.

The efficacy of agrocin 434 producer strains to control pathogenic *Agrobacterium* strains from different species was assessed with a rapid bioassay method, leaf disc tumorigenesis bioassays and stem inoculation. Almond root bioassays cannot be used

as biovar 3 and *A. rubi* pathogens are specific for host plants. The host range of *Agrobacterium* strains is commonly characterized as wide or narrow (Thomashow *et al.*, 1980). Most *Agrobacterium* strains have a wide host range such as *A. tumefaciens* and 2, but others, *A. vitis* and *A. rubi,* are very restricted, frequently to their natural host. The host range of *Agrobacterium* isolates is determined by characteristics of Ti plasmid (Knauf *et al.*, 1982; Burr and Katz, 1983). The most important result from this section is that strain K434 is effective in controlling tumor formation by *A. tumefaciens* , *A. vitis* and *A. rubi* in the stem inoculation and/or leaf disc tumorigenesis bioassays as well as by *A. rhizogenes* pathogens. Strain K1143 with pAgK434 was able to control the pathogens from *A. tumefaciens, A.rhizogenes, A.vitis* and *A. rubi* in higher concentration at ratio 5:1 or at 10:1. However results of these methods do not show the real situation because the test plants were artificial hosts and these experiments should be conducted with real host plants in root inoculation bioassay under soil conditions where other factors such as competition for nutrients, motility of bacterial strains, attachment to wound sites may play major roles. Biovar 3 and *A. rubi* pathogenic strains are not controlled by biocontrol strain K84 and when control measures are considered, the systemic natures of biovar 3 strains and *A. rubi* will be an important factor to be considered (Ophel, K.M, Thesis, 1987). However, results of our plate assay in Stonier's medium show that strains K230 (bv1), K1046 (*A. rubi)* were inhibited by strain K84 but strain K252 (bv3) has previously showed a complex response, concentric zones of growth and inhibition (Fajardo, thesis 1995). Similarly, Dhanvantari (1983) reported that some strains of *A. vitis* (bv3) resistant to agrocin 84 were inhibited by strain K84 when the assay medium was modified with a different nitrogen source. Vicedo *et al.* (1993) have reported that strain K84 does produce another antibiotic substance other than agrocin 84 or 434. The results of their experiments showed that this inhibitory compound inhibited several *A. tumefaciens* strains, which were resistant to agrocin 434. They used a derivative of strain K84 which did not produce agrocin 84 due to lack of pAgK84.

In summary, it has been shown that genes involved in the immunity/resistance to agrocin 434 are carried by pAgK434 and pAgK1318. A range of strain K84 derivatives has been isolated, strains K1352 with pAgK84, K1353 with pAgK84 and pAtK84b, K1355 with pAgK84:Tn*5* and pAgK434; and K1351 with pAtK84b. This completes the range of derivatives of strain K84 with every combination of the three residents plasmids. A rapid plant bioassay method for assessing the efficacy of biocontrol strains was developed by using leaf disc tumorigenesis assay. The results of this study provide the first evidence for biological activity of pAgK434, the agrocin 434 producer plasmid. The results of this study also demonstrated that pAtK84b may play a role in the biocontrol process. Strain K434 with pAgK434 and pAtK84b controlled pathogens from different biovars of *Agrobacterium* strains, in leaf disc and stem bioassays. It has also been demonstrated that care should be taken when extrapolating biocontrol ability from *in vitro* or "artificial" bioassays to the "real" environment.

Future work should be conducted to: find out the exact role of pAtK84b in the biocontrol process; verify the location of Tn*5* inserts in pAgK434; further molecular analysis of agrocin 434 production/immunity; colonization and competition need to be investigated in order to find the exact role of these factors in biological control by K84. This thesis has contributed to our understanding of the biological control of *Agrobacterium* pathogens by the successful biocontrol agent, K84. Such studies are important in understanding why the control strain is not effective in some cases and may led to the development of new or improved biological control agents.

References

Alconero R. 1980. Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. *Plant Disease* **64:** 835-838.

Ashby AM, Watson MD and Shaw CH. 1987. A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. *FEMS Microbiology Letters* **41:** 189-192.

Ashby AM, Watson MD, Loake GJ and Shaw CH. 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C^1 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. *Journal of Bacteriology* **170:** 4181-4187.

Bakanchikova TI, Lobanok EV, Pavlova-Ivanova LK, Red'kina TV, Nagapetyan JA and Maisuryan. 1993. Inhibition of tumor formation in dicotyledonous plants by *Azospirillum brasilense* strans. *Microbiology* **62(3):** 319-323.

Barry GF, Rogers SG, Fraley RT and Brand L. 1984. Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Academy of Sciences USA* **81:** 4776-4780.

Bergersen FJ. 1961. The growth of *Rhizobium* in synthetic media. *Australian Journal of Biology Sciences* **14:** 349-360.

Beringer JE. 1974. R factor transfer in *Rhizobium leguminosarum*.. *Journal of General Microbiology* **84:** 189-198.

Bernaerts MJ and De Ley J. 1963. A biochemical test for crown gall bacteria. *Nature*. London, **197**: 406-407

Binns AN and Thomashow MF. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. *Annual Review of Microbiology* **42:** 575-606.

Bolten GW, Nester EW and Gordon MP. 1986. Plant phenolic compounds induce expression of *Agrobacterium tumefaciens* loci needed for virulence. *Science* **232:** 983-985.

Bouzar H and Moore LW. 1987. Isolation of different *Agrobacterium* biovars from a natural oak savanna and tallgrass prairie. *Applied Environmental Microbiology* **53:** 717-721.

Braun AC. 1982. A history of the crown gall problems, pp. 155-210 in: *Molecular Biology of Plant Tumors*. G Kahl and JS Schell, eds. Academic Press, New York.

Broer I, Droege-Laser W, Barker RF, Neumann K, Kipp W and Puehler A. 1995. Identification of the *Agrobacterium tumefaciens* C58 T-DNA genes *e* and *f* and their impact on crown gall formation. *Plant Molecular Biology* **27(1):** 41-57.

Burr TJ and Katz BH. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap and vineyard soil. *Phytopathology* **73:** 163-165.

Burr TJ and Reid CL. 1994. Biological control of grape crown gall with non-tumorigenic *Agrobacterium vitis* strain F2/5. *American Journal of Enology and Viticulture* **45(2):** 213-219.

Burr TJ, Reid CL and Katz BH. 1993. Failure of *Agrobacterium radiobacter* strain K-84 to control crown gall on raspberry. *HortScience* **28(10):** 1017-1019.

Bush AL and Pueppke SG. 1991. Cultivar-strain specificity between *Chrysanthemum morifolium* and *Agrobacterium tumefaciens*. *Physiological and Molecular Plant Pathology* **39:**309-323.

Bytebier B, Deboeck F, De Greve H, Van Montagu M and Hernalsteens JP. 1987. T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Aspargus afficinalis*. *Proceedings of the National Academy of Sciences USA* **84:** 5345-5349.

Charest PJ, Holbrook LA, Gabard J Iyer VN and Miki BL. 1988. *Agrobacterium*-mediated transformation of thin cell layer explants from Brassica napus L. *Theoretical Applied Genetics* **75:** 438-445.

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

Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, Gordon MP and Nester EW. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11:** 263-271.

Chilton MD, Saiki RK, Yadav N, Gordon MP and Quetier F. 1980. T-DNA from *Agrobacterium* Ti plasmid is in the nuclear DNA fraction of crown gall tumor cells. *Proceedings of the National Academy of Sciences USA* **77:** 4060-4064.

Chyi YS and Philips GC. 1987. High efficiency of *Agrobacterium*-mediated transformation of Lycopersicun based on conditions favorable for regeneration. *Plant Cell Reports* **6:** 105-108.

Chyi YS, Jorgensen RA, Goldstein D, Tanksley SD. and Loaiza-Figueroa, F. 1986. Locations and stability of *Agrobacterium*-mediated T-DNA insertions in the *Lycopersicon* genome. *Journal of Bacteriology* **170**: 2659-2667.

Clare BG and McClure NC. 1995. *Agrobacterium*. pp. 221-236 in: *Pathogenesis and Host Specificity in Plant Diseases, Vol. I: Prokaryotes*. US Singh, RP Singh and K Kohmoto, eds. Elsevier Science Ltd, Oxford.

Clare BG, Kerr A and Jones DA. 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.

Clare BG. 1990. *Agrobacterium* in plant disease, biological disease control and plant genetic engineering. *Science Progress*, Oxford **74:** 1-13.

Clare BG. 1993. *Agrobacterium*: biological plant disease control, pp. 129-146 in: *Advanced Engineered Pesticides*. L Kim, ed. Marcel Dekker, Inc., New York.

Clare BG. 1995. Agrocins, pp. 619-632 in: *Genetics and Biochemistry of Antibiotic Production*. LC Vining and C Stuttard, eds. Butterworth-Heinemann, Boston.

Cook RJ and Barker KF. 1983. The Nature and Practical of Biological Control of Plant Pathogens. *American Phytopathological Society*. St. Paul. 539 pp.

Cooksey DA and Moore LW. 1980 Biological control of crown gall with fungal and bacterial antagonists. *Phytopathology* **70:** 506-509.

Cooksey DA and Moore LW. 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. *Phytopathology* **72:** 919-921.

Costacurta A and Vanderleyden J. 1995. Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* **21(1):** 1-18.

Day AG and Lichtenstein CP. 1991. Plant genetic transformation. pp.151-181 in: *Plant Biotechnology*. Fowler MW, Warren GS and Moo-Young, eds. Pergamon Press.

De Cleene M and De Ley J. 1976. The host range of crown gall. *The Botanical Review* **42:** 389-466.

Dessaux Y, Petit A and Tempé J. 1992. Opines in *Agrobacterium* biology, pp. 109-136 in: *Molecular Signals in Plant-Microbe Communications*. DPS Verma, ed. CRC Press, Inc.

Dhanvantari BN. 1983. Etiology of grape crown gall in Ontario. *Canadian Journal of Botany* **61:** 2641-2646.

Dickie GA and Bell CR. 1995. A full factorial analysis of nine factors influencing *in vitro* antagonistic screens for potential biocontrol agents. *Canadian Journal of Microbiology* **41:** 284-293.

Donner SC, Jones DA, McClure NC, Rosewarne GM, Tate ME, Kerr A, Fajardo NN and Clare BG. 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.

Donner SC, Tate ME, Jones DA, Rowarne GM Fajardo NN, Kerr A and BG Clare. 1991. Agrocin 434, inhibitory to *Agrobacterium* biovar 2 pathogens, is encoded by the cryptic plasamid pAtK84. Presented at the International Conference on *Pathology and Molecular Biology of Crown Gall*, Paris , France. CNRS,Gif Sur Yvette, France.

Douglas CJ, Halperin W and Nester EW. 1982. *Agrobacterium tumefaciens* mutants affected in attachment to plant cells, *Journal of Bacteriology* **152**:1265-1275.

Douglas CJ, Staneloni RJ, Rubin RA and Nester EW. 1985. Identification and genetic analysis of an *Agrobacterium tumefaciens* chromosomal virulence region. *Journal of Bacteriology* **161:** 850-860.

Dowling DN and O'Gara F. 1994. Metabolites of *Pseudomonas* involved in the biocontrol of plant disease. *Trends in Biotechnology* **12:** 133-141.

Eapen-Kohler SF, Gerdemann M and Scieder O. 1987. Cultivar dependence of transformation rates on moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*. *Theoretical Applied Genetics* **75:** 207-210.

Eckhardt T. 1987. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* **1**:584- 588.

El-Fiki F and Giles KL. 1981. *Agrobacterium tumefaciens* in agriculture and research. In *Bilogy of the Rhizobiaceae*: eds. K.L. Giles and A.G. Atherly, *International review of cytology supplement.* **13**: 47-58.

El-Masry MH, Mostafa MH, Mohamed Ibrahim A and El-Naggar MMA. 1995. Marine algae that display anti-tumorigenic activity against *Agrobacterium tumefaciens*. *FEMS Microbiology Letters* **128:** 151-156.

Elad Y and Chet I. 1987. Possible role of competition for nutrients in biocontrol of *Pythium* damping-off by bacteria. *Phytopathology* **77:** 190-195.

Ellis JG and Kerr A. 1979. Transfer of agrocin 84 production from strain 84 to pathogenic recipients: a comment on the previous paper. pp. 579-583 in: *Soil-Borne Plant Pathogens*. B Schippers and W Gams, eds. Academic Press, London.

Ellis JG and Murphy PJ. 1981. Four new opines from crown gall tumours – their detection and properties. *Molecular and General Genetics* **181:** 36-43.

Ellis JG, Kerr A, van Montagu M and Schell J. 1979. *Agrobacterium:* genetic studies on agrocin 84 production and the biological control of crown gall. *Physiological Plant Pathology* **15:** 311-319.

Endoh H, Aoyama T and Oka A . 1993. Transcription *in vitro* promoted by the *Agrobacterium Vir*G protein. *FEBS Letters* **334(3):** 277-280.

Escudero J, Neuhaus G and Hohn B. 1995. Intracellular *Agrobacterium* can transfer DNA to the cell nucleus of the host plant. *Proceedings of the National Academy of Sciences USA* **92(1):** 230-234.

Fajardo NN, Tate ME and Clare BG. 1994. Agrocin 434: an additional biological control component for crown gall. pp. 128-130 in: *Improving Plant Productivity with Rhizosphere Bacteria*. MH Ryder, PM Stephens and GD Bowen, eds. CSIRO Division of Soils, Adelaide.

Fajardo NN. 1995. Biological and chemical aspects of agrocin 434 as a supplementary biocontrol agent for crown gall. *PhD thesis*, University of Adelaide, Adelaide, South Australia.

Farrand SK and Wang C. 1992. Do we really understand crown gall control by *Agrobacterium radiobacter* strain K84?, pp. 287-293 in: *Biological Control of Plant Diseases.* EC Tjamos, GC Papavizas and RJ Cook, eds. Plenum Press, New York.

Farrand SK, Slota JE, Shim J-S and Kerr A. 1985. Tn*5* insertions in the agrocin 84 plasmid: the conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. *Plasmid* **13:** 106-117.

Farrand SK. 1990. *Agrobacterium radiobacter* strain K84: a model biocontrol system, pp. 679-691 in: *New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases.* RR Baker and PE Dunn, eds. Wiley-Liss, New York.

Formica JV. 1990. Conditions for the production of agrocin 84 by *Agrobacterium radiobacter* K84. *Journal of Industrial Microbiology* **5:** 33-44.

Glogowski W and Galsky AG. 1978. *Agrobacterium tumefaciens* site attachment as a necessary prerequisite for crown gall tumour formation on potato discs. *Plant Physiology* **61**: 1031-33.

Goodman RN and Novacky AJ. 1994. The hypersensitive reaction in plants to pathogens: A resistance phenomenon. APS Press, St. Paul, Minnesota.

Gould WD. 1990. Biological control of plant root diseases by bacteria. pp. 287-317. in: Biotchnology of Plant-Microbe Interactions. Nakas JP and Hagedorn C,eds. McGraw-Hill Publishing Company.

Grant JE, Dommisse EM, Christey MC and Conner AJ. 1991. Gene transfer to plants using *Agrobacterium* . In Murray DR, ed. Advanced methods in plant breeding and biotechnology. *C.A.B.International*, 50-73.

Graves ACF and Goldman SL. 1986. The transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*. *Plant Molecular Biology* **7:** 43-50.

Graves AE, Goldman SL, Banks SW and Graves ACF. 1988. Scanning microscope studies of *Agrobacterium tumefaciens* attachment to *Zea mays*, *Gladious sp.*, and *Triticum aestivum*. *Journal of Bacteriology* **170:** 2395-2400.

Groebner DF and Shannon PW. 1990. Business statistics: A decision-making approch. Boise State University, Merrill Publishing Company, an imprint of Macmillan Publishing Company. Third edition, pp.520-527.

Guyon P, Petit A, Tempé J and Dessaux Y. 1993. Transformed plants producing opines specifically promote growth of opine-degrading agrobacteria. *Molcular Plant-Microbe Interaction* **6:** 92-98.

Hawes, MC, Smith LY and Howarth AJ. 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Molcular Plant-Microbe Interaction* **1**: 182-186.

Hayman GT and Farrand SK. 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. *Journal of Bacteriology* **170:** 1759-1767.

Hayman GT, von Bodman SB, Kim H, Jiang P and Farrand SK. 1993. Genetic analysis of the agrocinopine catabolic region of *Agrobacterium tumefaciens*Ti plasmid pTiC58, which encodes genes required for opine and agrocin 84 transport. *Journal of Bacteriology* **175:** 5575-5584.

Hemstad PR and Reisch BI. 1985. *In vitro* production of galls induced by *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* on *Vitis* and *Rubus*. *Journal of Plant Physiology 120:* 9-17.

Hendson M, Askjaer L, Thomson JA and van Montagu M. 1983. Broad-host-range agrocin of *Agrobacterium tumefaciens*. *Applied and Environmental Microbiology* **45:** 1526-1533.

Hoekema A, DePater BS, Fellinger AJ, Hooykaas PJJ and Schilperoort R A. 1984. The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. *EMBO Journal* **3:** 3043-3047.

Hooykaas PJJ and Beijersbergen AGM. 1994. The virulence system of *Agrobacterium tumefaciens*. *Annual Review of Phytopatholology* **32:** 157-179.

Hooykaas PJJ and Mozo T. *Agrobacterium* molecular genetics. *Plant Molecular Biology Manual* **B3:** 1-9. Kluwer Academic Publishers. Printed in Belgium.

Hooykaas-Van Slogteren GMS, Hooykaas PJJ and Schilperoort RA. 1984. Expression of Ti plasmid in monocotyledonous plants infected with *Agrobacterium tumefaciens*. *Nature* **311:** 763-764.

Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG and Fraley RT. 1988. Leaf disc transformation. in: *Plant molecular biology manual.* SB Gelvin, RA Schilperoot, APS Verma,(eds). Kluwer Academic **A5**:1-9

Htay K and Kerr A. 1974. Biological control of crown gall: seed and root inoculation. *The Journal of Applied Bacteriology* **37:** 525-530.

Inze D, Follin A, Van Lijsebettens M, Siomons C, Gentello C, Van Montagu MH and Schell J. 1984. Genetic analysis of the individual T-DNA genes of *Agrobacterium tumefaciens*: further evidence that two genes are involved in indole-3-acetic acid biosynthesis. *Molecular General Genetics* **194:** 265-274.

Jones DA and Kerr A. 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. *Plant Disease* **73:** 15-18.

Jones DA, Ryder MH, Clare BG, Farrand SK and Kerr A. 1988. Construction of a Tra$^-$ deletion mutant of pAgK84 to safeguard the biological control of crown gall. *Molecular and General Genetics* **212:** 207-214.

Jordan DC. 1984. Family III. Rhizobiaceae Conn 1938, pp. 234-256 in: *Bergey's Manual of Systematic Bacteriology, vol. 1.* NR Krieg and JG Holt, eds. Williams and Wilkins, Baltimore.

Kalogeraki VS and Winans SC. 1995. The octopine-type Ti plasmid pTiA6 of *Agrobacterium tumefaciens* contains a gene homologous to the chromosomal virulence gene *acv*B. *Journal of Bacteriology* **177(4):** 892-897.

Kanemoto RH, Powell AT, Akiyoshi DE, Regier DA and Kersteter RA. 1989. Nucleotide sequence and analysis of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens* . *Journal of Bacteriology* **171**: 2506-2512.

Kerr A and Brisbane PG. 1983. *Agrobacterium*, pp. 27-43 in: *Plant Bacterial Diseases*. A Diagnostic Guide. PC Fahy and GJ Persley, eds. Academic Press, Australia.

Kerr A and Htay K. 1974. Biological control of crown gall through bacteriocin production. *Physiological Plant Pathology* **4:** 37-44.

Kerr A and Panagopoulos CG. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathology* **90:** 172-179.

Kerr A and Tate ME. 1984. Agrocins and the biological control of crown gall. *Microbiological Sciences* **1:** 1-4.

Kerr A. 1969. Transfer of virulence between isolates of *Agrobacterium*. *Nature* **223:** 1175-1176.

Kerr A. 1971. Acquisition of virulence by non-pathogenic isolates of *Agrobacterium radiobacter*. *Physiologycal plant pathology* **1**:241-246.

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

Kerr A. 1982. Biological control of soil-borne microbial pathogens and nematodes, pp. 429-463 in: *Advances in Agricultural Microbiology.* NS Subba Rao, ed. Butterworth Scientific, London.

Kerr A. 1989. Commercial release of a genetically engineered bacterium for the control of crown gall. *Agricultural Science* **2:** 41-44.

Kerr A. 1992. The genus *Agrobacterium*, pp. 2214-2235 in: *The Prokaryotes,* 2nd edition. A Balows, HG Touper, M Dworkin, W Harder and KH Schleifer, eds. Springer-Verlag, New York.

Kersters K and De Ley J. 1984. Genus III. *Agrobacterium*, pp. 244-254 in: *Bergey's Manual of Systematic Bacteriology,* vol. 1. NR Krieg and JG Holt, eds. Williams and Wilkins, Baltimore.

Khmel IA, Sorokina TA, Metliski OZ and Burdeinaya TV. 1995. Biological control of crown gall and other plant diseases by *Pseudomonas* strains with wide host range spectrum of antagonistic activity. Presented at the International Conference on *Agrobacterium and beyond.* Paris , France. CNRS,Gif Sur Yvette, France.

Klement Z. 1982. Hypersensitivity. in: *Phytopathogenic procaryotes*. pp.150-178. Mount MS and Lacy, eds. Academic Press, New York.

Knauf VC, Pangopoulos CG and Nester EW. 1982. Genetic factors controlling host range of *Agrobacterium tumefaciens*. *Phytopathology* **72:** 1545-1549.

Körber H, Strizhof N, Staiger D, Feldwisch J, Olsson O, Sandberg G, Palme K, Schell J and Koncz C. 1991. T-DNA gene 5 of *Agrobacterium tumefaciens* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants, *EMBO Journal* **10:**3983-3991.

Lippincott JA and Lippincott BB. 1969. Bacterial attachment to a specific wound site as an essential stage in tumor initiation by *Agrobacterium tumefaciens*. *Journal of Bacteriology* **97**: 620-628

Lippincott JA and Lippincott BB. 1975. The genus *Agrobacterium* and plant tumorigenesis. *Annual Review of Microbiology* **29:** 377-405.

López MM, Gorris MT, Salcedo CI, Montojo AM and Miró M. 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. *Applied and Environmental Microbiology* **55:** 741-746.

Matthysse AG, Holmes KV and Gurlitz RHG. 1981. Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* . *Journal of Bacteriology* **145:** 583-595.

Matthysse AG, Holmes KV and Gurlitz RHG. 1982. Binding of *Agrobacterium tumefaciens* to carrot protoplasts. *Physiological Plant Pathology* **20:** 27-33.

Matthysse AG, Thomas DL and White AR. 1995. Mechanism of cellulose synthesis in *Agrobacterium tumefaciens* . *Journal of Bacteriology* **177(4):** 1076-1081.

Matthysse AG, White AR and Lightfoot R. 1995. Genes required for cellulose synthesis in *Agrobacterium tumefaciens* *Journal of Bacteriology* **177(4):** 1069-1075.

Matthysse AG, Wyman PM and Holmes FV. 1978. Plasmid-dependent attachment of *Agrobacterium tumefaciens* to plant tissue culture cells. *Infection and Immunity* **22:** 516-522.

Matthysse AG. 1983. Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection, *Journal of Bacteriology* **154:**906-915.

Matthysse AG. 1984. Interaction of *Agrobacterium tumefaciens* with the plant cell surface. p. 33-54. In: *Plant gene research-basic knowledge and application.* DPS Verma and T Hohn, eds. Springer Verlag.

Matthysse AG. 1986. Initial interactions of *Agrobacterium tumefaciens* with plant host cells. *CRC Critical Review in Microbiology* **13:** 313-323.

Matthysse AG. 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens. Journal of Bacteriology* **169:** 313-323.

Matthysse AG. 1988. Attachment of *Agrobacterium tumefaciens* to the host cells. In: *Molecular genetics of plant microbe interactions.* pp. 63-68. Palacios R and Verma PDS, eds. American Phytopathological Society Press.

Matthysse AG. 1994. Conditioned medium promotes the attachment of *Agrobacterium tumefaciens* strain NT 1 to carrot cells. *Protoplasma* **183:** 131-136.

McClure NC, Ahmadi AR and Clare BG. 1994. The role of agrocin 434 produced by *Agrobacterium* strain K84 and derivatives in the biological control of *Agrobacterium* biovar 2 pathogens. pp. 125-127 in: *Improving Plant Productivity with Rhizosphere Bacteria.* MH Ryder, PM Stephens and GD Bowen, eds. CSIRO Division of Soils, Adelaide.

McClure NC, Saint CP, Fry JC and Weightman AJ. 1990. The construction of broad host range of genetic markers and their use in monitoring the release of catabolic

GEMs to aquatic environments. pp 169-172. Christiansen C, Murk L and Villadsen J. eds. *Proceedings Vol 1.*

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R and Fraley R. 1986. Leaf disc transformation of cultivated tomato. (L. esculentum) using *Agrobacterium tumfaciens*. *Plant Cell Reports* **5:** 81-84.

Messens E, Dekeyser R and Stachel SE. 1985. Genetic basis for opine secretion from crown gall tumor cells. *Molcular and General Genetic* **199**: 344-348.

Moore LW and Warren G. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. *Annual Review of Phytopathology* **17:** 163-179.

Moore LW. 1977. Prevention of crown gall on Prunus roots by bacterial antagonists. *Phytopathology* **67:** 139-144.

Moore LW. 1979. Practical use and success of *Agrobacterium radiobacter* strain 84 for crown gall control. pp. 553-568 in: *Soil-Borne Plant Pathogens*. B Schippers and W Gams, eds. Academic Press, London.

Morris RO. 1986. Genes specifying auxin and cytokinin biosynthesis in phytopathogenes. *Annual Review of Plant Physiology* **37:** 509-538.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15:** 473-497.

Murphy PJ and Roberts WP. 1979. A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. *The Journal of General Microbiology* **114:** 207-213.

Murphy PJ, Tate ME and Kerr A. 1981. Substituents at N^6 and C-5' control selective uptake and toxicity of the adenine-nucleotide bacteriocin, agrocin 84, in agrobacteria. *European Journal of Biochemistry* **115:** 539-543.

Nelson EB and Craft CM. 1991. Metabolism of *Pythium* sporangium germinating stimulants by *Enterobacter* cloacae and another soil bacteria. *Phytopathology*,(Abstract) **81:** 1178.

Nester EW and Gordon MP. 1991. Molecular strategies in the interaction between *Agrobacterium* and its hosts. in: *Molecular strategies of pathogens and host plants*.. pp 3-28. Patil SS, Ouchi S, Mills D and Vance C, eds. Springer-Verlag.

Nester EW, Gordon MP, Amasino RM and Yanofsky MF. 1984. Crown gall: a molecular and physiological analysis, *Annual Review of Plant Physiology*, **35**:387-413.

New PB and Kerr A. 1972. Biological control of crown gall: field measurements and glasshouse experiments. *The Journal of Applied Bacteriology* **35:** 279-287.

Ooms G. 1991. Genetic engineering of plants and cultures. pp. 223-257 in: *Plant Biotechnology*. Fowler MW, Warren GS and Moo-Young, eds. Pergamon Press.

Ophel K and Kerr A. 1987. Ecology of *Agrobacterium*: plasmids and biovars, pp. 3-5 In: *Molecular Genetics of Plant-Microbe Interactions*. DPS Verma and N Brisson, eds. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Ophel K and Kerr A. 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systematic Bacteriology* **40:** 236-241.

Ophel KM. 1987. *Agrobacterium*: Plasmids and biovars. *PhD thesis*, University of Adelaide, Adelaide, South Australia.

Pan SQ, Charles T, Jin S, WU ZL and Nester EW. 1993. Performed dimeric state of the sensor protein *Vir*A is involved in plant *Agrobacterium* signal transduction. *Proceedings of the National Academy of Sciences USA* **90(21):** 9939-9943.

Panagopoulos CG, Psallidas PG and Alivizatos AS. 1979. Evidence of a breakdown in the effectiveness of biological control of crown gall, pp. 569-578 in: *Soil-Borne Plant Pathogens*. B Schippers and W Gams, eds. Academic Press, London.

Pang SZ and Sanford JC. 1988. *Agrobacterium* mediated gene transfer in papaya. *Journal of American Society for Horticultural Science* **113:** 287-291.

Parke D, Ornston NL and Nester EW. 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **169:** 5336-5338.

Parke JL. 1991. Root colonization by indigenous and introduced microorganisms. pp. 33-42. In: *The Rhizosphere and plant growth.*. Keister DL and Cregan PB, eds. Kluwer Academic Publishers.

Penalver R, Vicedo B, Salcedo CI and Lopez M. 1994. *Agrobacterium radiobacter* Strains K84, K1026 and K84 Ag^- produce an antibiotic-like substance, active *in vitro* against *A. tumefaciens* and phytopathogenic *Erwinia* and *Pseudomonas spp*. *Biocontrol Science And Technology* **4:** 259-267.

Pu X-A and Goodman RN. 1992. Inhibition of necrogenesis by *Agrobacterium tumefaciens* on grape explants. *Physiological Molecular Plant Pathology* **41:** 241-254.

Pu X-A and Goodman RN. 1993. Attachment of Agrobacteria to grape cells. *Appllied and Environmental Microbiology* **59(8):** 2572-2577.

Pu X-A and Goodman RN. 1993. Tumor formation by *Agrobacterium tumefaciens* is suppressed by *Agrobacterium radiobacter* HLB-2 on grape plants. *American Journal of Enology and Viticulture* **44:** 249-254.

Regensburg-Tunik AJG and Hooykaas PJJ. 1993. Transgenic N. glauca plants expressing bacterial virulence gene *vir*F are converted into hosts for nopaline strains of *Agrobacterium tumefaciens*. *Nature* **363:** 69-71.

Riley M. 1993. Functions of the gene products of *Escherichia coli. Microbiology Review* **57:** 862-952.

Roberts WP, Tate ME and Kerr A. 1977. Agrocin 84 is a 6-N-phosphoramidate of an adenine nucleotide analogue. *Nature* **265:** 379-381.

Robertson Crews, J. L., Colby, S. and Matthysse, A.G. 1990. *Agrobacterium rhizogenes* mutants that fail to bind to plant cells, *Journal of Bacteriology.* **172**:6182-6188.

Robertson JL, Holliday T and Matthysse AG. 1988. Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment. *Journal of Bacteriology* **170:** 1408-1411.

Robinette D and Matthysse AG. 1990. Inhibition by *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* of development of the hypersensitive response elicited by *Pseudomonas syringae* pv. *phaseolicola. Journal of Bacteriology* **172:** 5742-5749.

Roitsch T, Jin S and Nester EW. 1994. The binding of the site transcriptional activator *Vir*G from *Agrobacterium* comprises both conserved and specific nonconserved sequences. *FEBS Letters* **338(2):** 127-132.

Romantschuk M. 1992. Attachment of plant pathogenic bacteria to plant surfaces. *Annual Review of Phytopathology* **30:** 225-243.

Rong L, Karcher SJ, O'neal K, Hawes MC, Yerkes CD, Jayaswal RK. Hallberg CA and Gelvin SB. 1990. *picA*, a novel plant-inducible locus on the *Agrobacterium tumefaciens* chromosome. *Journal of Bacteriology* **172:** 5828-5836.

Rüger HJ and Höfle MG. 1992. Marine star-shaped aggregate-forming bacteria: *Agrobacterium atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp. nov.; nom. rev.; *Agrobacterium gelatinovorum* sp. nov.; nom. rev.;

Agrobacterium stellulatum sp. nov.; nom. rev. *International Journal of Systematic Bacteriology* **42:** 133-143.

Ryder MH and Jones DA. 1990. Biological control of crown gall. pp. 45-63 in: *Biological Control of Soil-Borne Plant Pathogens*. D Hornby, ed. CAB International, Wallingford, UK.

Ryder MH, Slota JE, Scarim A and Farrand SK. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. *Journal of Bacteriology* **169:** 4184-4189.

Sawada H, Ieki H, Oyaizu H and Matsumoto S. 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium*and for *Agrobacterium radiobacter* and *Agrobacteriumr hizogenes*. *International Journal of Systematic Bacteriology* **43:** 694-702.

Schenk RU and Hilderbrant AC. 1972. Medium and techniques for induction and growth of monocotyledonous and docotyledonous plant cell cultures. *Canadian Journal of Botany* **50:** 199-204.

Schröder G, Waffenschmidt S, Weiler EW and Schroder J. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. *European Journal of Biochemistry* **138:** 387-392.

Sciaky D, Montoya AL and Chilton M-D. 1978. Fingerprints of *Agrobacterium* Ti plasmids. *Plasmid* **1:** 238-253.

Scott ES, Wicks TJ and Lee TC. 1992. The development of an assay for resistant to *Phytophthora cambivora* in almond rootstocks using shoots excised from tissue cultures. *Plant Pathology* **41:** 639-645.

Sharma NK and Skidmore DS. 1988. *In vitro* expression of partial resistance to *Phytophthora palmivora* by shoot cultures of papaya. *Plant Cell tissue and Organ Culture* **14:** 187-196.

Shaw CH, Ashby AM, Loake GJ and Watson MD. 1988. One small step: the missing link in crown gall. *Oxford Surveys of Plant Molecular and Cell Biology* **5:** 177-183.

Shim J-S, Farrand SK and Kerr A. 1987. Biological control of crown gall: construction and testing of new biocontrol agents. *Phytopathology* **77:** 463-466.

Simon R. 1984. High frequency mobilization of Gram-Negative bacterial replicons by the *in vitro* constructed Tn5-Mob tranposon. *Molecular and General Genetics* **196**:413-420.

Slota JE and Farrand SK. 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. *Plasmid* **8:** 175-186.

Smith VA and Hindley J. 1978. Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells. *Nature* 276: 498-500.

Stachel SE and Nester EW. 1986. The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO Journal* **5:** 1445-1454.

Stachel SE, Messens E, Van Montagu M and Zambryski P. 1985. Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. *Nature* **318:** 624-629.

Stachel SE, Timmerman B and Zambryski P. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* **322:** 706-712.

Stockwell VO, Kawalek MD Moore LW and Loper JE. 1990. Plasmid transfer between *Agrobacterium radiobacter* K84 and *A. tumefaciens* in crown gall . *Phytopathology* **80(10):** 1001. (Abstract.)

Stonier T. 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. *Journal of Bacteriology* **79:** 889-898.

Tanimoto E, Douglas C and Halperin. 1979. Factors affecting crown gall tumorigenesis in tubor slices of Jerusalem artichoke (*Helianthus tuberosus* L.). *Plant Physiology* **63:** 698-994.

Tate ME, Donner SC, Jones DA, Fajardo NN, McClure NC, Ahmadi AR, Rosewarne GR, Clare BG and Kerr A. 1995. Agrocin 434 & all that: Another nucleoside product of agrobacteria and its possible involvement in biological control. Presented at the International Conference on *Agrobacterium and beyond.* Paris , France. CNRS,Gif Sur Yvette, France.

Tate ME, Murphy PJ, Roberts WP and Kerr A. 1979. Adenine N^6-substituent of agrocin 84 determines its bacteriocin-like specificity. *Nature* **280:** 697-699.

Tate ME. 1968. Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). *Analytical Biochemistry* **23:** 141-149.

Thomashow LS, Reeves S and Thomashow MF. 1984. Crown gall oncogenesis: evidence that a T-DNA gene from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. *Proceedings of the National Academy of Sciences USA* **81:** 5071-5075.

Thomashow MF, Hugly S, Buchholz WG and Thomashow LS. 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. *Science* **231:** 616-618.

Thomashow MF, Hugly S, Buchholz WG and Thomashow LS. 1988. Molecular basis for the auxin-independent pheotype of crown-gall tumor tissues. *Science* **231:** 616-618.

Thomashow MF, Karlinsey JE, Marks JR and Hurlbert RE. 1987. Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. *Journal of Bacteriology* **169:** 3209-3216.

Thomashow MF, Nutter R, Montoya AC, Gordon MP and Nester EW. 1980. Integration and organization of the Ti plasmid sequences in crown gall tumors. *Cell* **19:** 729-739.

Tinland, B., Huss, B., Paulus, F., Bonnard, G. and Otten, L. 1989. *Agrobacterium tumefaciens* 6b genes are strain specific and affect the activity of auxin as well as cytokinin genes, *Molcular and General Genetics* **219:**217-224.

Utkhede RS and Smith EM. 1993. Evaluation of biological and chemical treatments for control of crown gall on young apple trees in the Kootenay Valley of British Columbia. *Journal of Phytopathology* **137:** 265-271.

Van Larebeke N, Genetello C, Hernalsteens JP, De Picker A, Zaenen I, Messens E, van Montagu M and Schell J. 1977. Transfer of Ti plasmids between *Agrobacterium* strains by mobilization with the conjugative plasmid RP4. *Molecular and General Genetics* **152:** 119-124.

Van Larebeke N, Genetello C, Schell J, Schilperoort RA, Hermans AK, Hernalsteens JP and van Montagu M. 1975. Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. *Nature* **255:** 742-743.

Van Zyl FGH, Strijdom BW and Staphorst JL. 1986. Susceptibility of *Agrobacterium tumefaciens* strains to two agrocin-producing *Agrobacterium* strains. *Applied and Environmental Microbiology* **52:** 234-238.

Vicedo B, Lopez MJ, Asins MJ, Llop P and Lopez MM. 1995. Transfer of pTi, pAtK84b and pAgK84 plasmids between *Agrobacterium tumefaciens* and strain K84 of *A. radiobacter* in a tumour. Molecular characterization of transferred plasmids. Presented at the International Conference on *Agrobacterium and beyond*. Paris , France. CNRS,Gif Sur Yvette, France.

Vicedo B, Peñalver R, Asins MJ and López MM. 1993. Biological control of *Agrobacterium tumefaciens*, colonization, and pAgK84 transfer with *Agrobacterium radiobacter* K84 and the Tra⁻ mutant strain K1026. *Applied and Environmental Microbiology* **59:** 309-315.

Wagner VT and Matthyse AG. 1992. Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells, *Journal of Bacteriol* **174:** 5999-6003.

Wang C-L, Farrand SK and Hwang I. 1994. Organization and expression of the genes on pAgK84 that encode production of agrocin 84. *Molecular Plant-Microbe Interactions* **7:** 472-481.

Warren GS. 1991. Cell culture and recombinant DNA technology in plant pathology. pp.283-292 in: *Plant Biotechnology*. Fowler MW, Warren GS and Moo-Young, eds. Pergamon Press.

Watson B, Currier TC, Gordon MP, Chilton MD and Nester EW. 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **123:** 255-264.

Webster J, Dos Santos M and Thomson JA. 1986. Agrocin-producing *Agrobacterium tumefaciens* strain active against grapevine isolates. *Applied and Environmental Microbiology* **52:** 217-219.

Whatley MH, Margot JB, Schell J, Lippincott BB and Lippincott JA. 1978. Plasmid and chromosomal determination of *Agrobacterium* adherence specificity. *Journal of General Microbilogy* **107:** 395-398.

Willems A and Collins MD. 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *International Journal of Systematic Bacteriology* **43:** 305-313.

Winans S. 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiological Reviews* **56:** 12-31.

Winans SC, Mantis NJ, Chen CY, Chang CH and Han DC. 1994. Host recognition by the VirA, VirG two-component regulatory proteins of *Agrobacterium tumefaciens*. *Research in Microbiology* **145(5-6):** 461-473.

Yanagi M and Yamasato K. 1993. Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiology Letter* **107:** 115-120.

Yanofsky MF, Lowe B, Montoya A, Rubin R, Krul W, Gordon MP. and Nester, EW. 1985. Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens, Molcular and General Genetics* **201**: 237-246.

Zambryski PC. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology* **43:** 465-490.

Zhan X, Jones DA and Kerr A. 1990. The pTiC58 *tzs* gene promotes high-efficiency root induction by agropine strain 1855 of *Agrobacterium rhizogenes*. *Plant Molecular Biology* **14:** 785-792.

APPENDICES

APPENDIX A: Bacterial Media and Antibiotics

1. Bergersen's Media (Bergersen, 1961)

Agar	1.5 g/75 ml water (autoclave).
5x Salts*	20 ml
10% Manitol (atoclave)	5 ml
Thiamine (filter seterilized)	0.1 ml (from stock 25 mg/100 ml)
Biotin	0.1 ml (from stock 1 mg/ml).

* 5x Salts (per litre)

Na_2HPO_4	0.89 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2$	0.2 g
$FeCl_3$	0.1 g
$(NH_4)2SO_4$	1 g

Adjust to pH 7.0 with KOH, then autoclave.

2. Luria Bertani (LB) broth, per liter

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

3. MMG medium, per liter

Sodium glutamate	1 g
Tri-sodium citrate	0.5 g
$(NH_4)_2SO_4$	1 g
K_2HPO_4	1.74 g
KH_2PO_4	5.44 g

Dissolve the solids in about 954 ml distilled water and autoclave. When cool, add the following filter-sterilized solutions:

0.5% $Fe(NO_3)_3$	1 ml
0.02% biotin	1 ml
1% thiamine	1 ml
Doy's trace elements *	1 ml
10% $MgSO_4$	2 ml
10% glucose	50 ml

* Doy's trace elements

$MnSO_4 \cdot H_2O$	1 g
H_3BO_3	300 mg
$ZnSO_4 \cdot 7H_2O$	300 mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
$CuSO_4$	25 mg
$CoCl_2 \cdot 6H_2O$	25 mg

Dissolve solids and dilute to 100 ml with distilled water. Filter sterilize.

4. Nutrient broth, per liter

Nutrient broth (Oxoid)	13 g

5. Stonier's medium, per liter (Stonier, 1960).

Sodium glutamate	2 g
Tri-potassium citrate	10 g
$CaSO_4$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
NH_4NO_3	2.7 g
NaH_2PO_4	0.3 g
K_2HPO_4	0.88 g
Fe-EDTA *	2.5 ml
$MnCl_2$ *	0.1 mg

$ZnCl_2$ *	0.5 mg
Biotin	200 µg
Agar	15 g

* Stock solutions:

Fe-EDTA – 278 mg $FeSO_4$ + 372 mg Na_2EDTA per 100 ml

$ZnCl_2$ – 0.05 g per 100 ml

$MnCl_2$ – 0.01 g per 100 ml

6. TY medium, (Beringer, 1974), per liter

Tryptone	5 g
Yeast extract	3 g
$CaCl_2 \cdot 6H_2O$	1.3 g
Agar	15 g

7. YM medium, per liter

Mannitol	10 g
Yeast extract	1 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.01 g
$CaCl_2$	0.2 g
Agar	15 g

8. Soft buffered agar overlay (Stonier. 1960), per 100 ml

0.2 M NaH_2PO_4	3.9 ml
0.2 M Na_2HPO_4	6.1 ml
Purified agar	0.7 g

9. Selective medium for biovar 1, *A. tumefaciens* , (Brisbane and Kerr, 1983), per liter

Arabitol (L-)	3.04 g
Crystal violet (0.1%)	2 ml
K_2HPO_4	1.04 g
$MgSO_4.7H_2O$	0.25 g
KH_2PO_4	0.54 g
NH_4NO_3	0.16 g
Sodium taurocholate	0.29 g
Agar	15 g

autoclave, cool, then per 100 ml add:

Sodium Selenite	1 ml
Cyclohexamide (actidione) (2%)	1 ml

10. Selective Medium for *A. rhizogenes* (biovar 2) (Brisbane and Kerr, 1983), per liter

Erythritol	3.05 g
Malachite green (0.1%)	5 ml
K_2HPO_4	1.04 g
$MgSO_4.7H_2O$	0.25 g
KH_2PO_4	0.54 g
NH_4NO_3	0.16 g
Sodium taurocholate	0.29 g
Yeast extract (1% solution)	1 ml
Agar	15 g

autoclave, cool, then per 100 ml add:

Sodium Selenite	1 ml
Cyclohexamide (actidione) (2%)	1 ml

Antibiotics:

Compound*	**Abbreviation**	**Solvent**	**Stock** (mg/ml)	**Concentration in media** (μg/ml)
Chloramphenicol	Chl	EtOH (100%)	40	30
Kanamycin	Km	H_2O	50	200
Rifampicin	Rif	H_2O	10	25
Streptomycin	Str	H_2O	40	250
Tetracycline	Tc	EtOH (50%)	10	10

* All antibiotics were supplied by Sigma Chemical Co.

Appendix B: Tissue Culture media

MSO Medium

MS (Murashige & Skoog, 1962) salts plus B5 vitamins (Schenk & Hildebrandt, 1972):

Macronutrients	**mg/ml**
$CaCl_2$	440
$FeSO_4.7H_2O$	27.8
KH_2PO_4	170
KNO_3	1900
$MgSO_4.7H_2O$	370
Na_2EDTA	37.3
NH_4NO_3	1650
Micronutrients	
$CoCl_2.6H_2O$	0.025
$CuSO_4.6H_2O$	0.025
H_3BO_3	6.2
KI	0.83
$MnSO_4.4H_2O$	0.25
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.6
Carbohydrate	
Sucrose	30 g/l
Vitamins	
Myo-inositol	100 mg/ml
Nicotonic acid	1 mg/ml
Pyridoxine	1 mg/ml
Thiamine HCl	10 mg/ml
Agar	8 g/l
pH	5.8

APPENDIX C

Miscellaneous Buffers and Solutions

Buffered Saline, per 100 ml

NaCl	0.85 g
0.2 M NaH_2PO_4	3.9 ml
0.2 M Na_2HPO_4	6.1 ml
pH 7.3	

Standard Markers

	per ml
2-deoxyadenosine	5 mg
Orange G	1 mg
Xylene cyanol	1 mg
Fructose	10 mg
Nitrobenzene sulfonate	4 mg
Sodium azide (preservative)	1 mg

HVPE Buffers

Citrate Buffer (0.05 M; pH 6.0)

Dissolve 10.5 g citric acid (FW=210.14) and 5.7 g NaOH (FW=40) per liter solution.

Formic-Acetic Acid Buffer (0.75 M HCOOH/1.03 M CH_3COOH; pH 1.75)

Dissolve 28.4 ml 98% HCOOH and 59.2 ml glacial CH_3COOH per liter solution.

Appendix D

List of presentation /publications.

Preliminary descriptions of this work have been presented at the following conferences:

McClure NC, Ahmadi AR and Clare BG. 1994. The role of agrocin 434 produced by *Agrobacterium* strain K84 and derivatives in the biological control of *Agrobacterium* biovar 2 pathogens. pp. 125-127 In: *Improving Plant Productivity with Rhizosphere Bacteria.* MH Ryder, PM Stephens and GD Bowen, eds. CSIRO Division of Soils, Adelaide.

McClure NC, Ahmadi AR and Clare BG. 1994. Factors involved in the biological control of *Agrobacterium* pathogens by *A. rhizogenes* K84. p. A60. In: *Abstracta of the Australian Society for Microbiology Annual Scientific Meeting*, September 1994, Melbourne, Vicoria.

Tate ME, Donner SC, Jones DA, Fajardo NN, McClure NC, Ahmadi AR, Rosewarne GR, Clare BG and Kerr A. 1995. Agrocin 434 & all that: Another nucleoside product of agrobacteria and its possible involvement in biological control. In: Abstracts of International Conference on *Agrobacterium and beyond.* Paris , France. CNRS,Gif Sur Yvette, France, September 1995.

Ahmadi AR, McClure NC and Clare BG. 1995. Factors involved in the biological control of crown gall disease by derivatives of *Agrobacterium rhizogenes* K84. In: Abstracts of International Conference on *Agrobacterium and beyond.* Paris , France. CNRS,Gif Sur Yvette, France, September 1995.

McClure, N. C., Ahmadi, A. R. & 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 M. H. Ryder, P. M. Stephens & G. D. Bowen (Eds.), *Improving plant productivity with rhizosphere bacteria: proceedings of the Third International Workshop on Plant Growth-Promoting Rhizobacteria, Adelaide, South Australia, March, 7-11, 1994*, (pp. 125-127). S. Aust., CSIRO Division of Soils.