Bacterial head (or spear) rot of broccoli: pathogenicity and cultivar susceptibility

by

Denise Darling

PhD Thesis
The University of Edinburgh
1998

Abstract

Bacterial head rot of broccoli is an opportunistic infection caused predominantly by *Pseudomonas fluorescens*. Whilst crop losses of between 30 and 100% have been reported, little is known of the disease aetiology. The research conducted in this thesis covered two main areas: Broccoli cultivar susceptibility to disease and bacterial pathogenicity.

Cultivar susceptibility: Two *in vitro* pathogenicity tests to distinguish disease susceptibility levels were developed. The first test, inoculating seedling hypocotyls with a needle using bacteria removed from an agar colony, produced low disease levels and variability between replicates and therefore was rejected. The second test, based on excised heads from mature plants, successfully distinguished between 10 cultivars with the results corresponding to previously published data for resistance (with one exception). Head morphology, in relation to disease susceptibility, was also studied. Differences in morphology may predispose certain cultivars to disease by influencing the duration free water remains on the head and the availability of sites for infection. Head shape (doming), head size, bud number, bud prominence and stomatal number were assessed. Whilst these factors differed significantly between cultivars, two sets of factors correlated positively at a significant level: Head size and disease susceptibility, and head doming and disease resistance.

Pathogenicity: Biosurfactants, produced by pathogenic bacterial strains, have been implicated in the development of head rot on unwounded heads, by aiding bacterial establishment and spread. Their role in disease, as virulence or pathogenicity factors, was assessed using a mutant approach.

Initially, five biosurfactant detection tests were evaluated to allow the mutants produced to be tested for loss of production. The most suitable proved to be monitoring changes in the

surface tension of a water droplet following the addition of bacterial cells removed from a colony.

Thirty-five mutants of a *Pseudomonas fluorescens* strain, defective in biosurfactant production (surf $^-$), were produced by Tn5 mutagenesis. Southern blotting demonstrated that 17 of the 35 mutants had single Tn5 insertions, while the rest had more than one insert. Twenty-two different *Sal*I profiles were found in the 35 mutants indicating that 13 mutants shared their Tn5-inserted restriction pattern with at least one other strain. Of the 17 with only one Tn5 insertion, five were reduced in their ability to produce disease symptoms (vir $^-$), whilst the other 12 showed no change. Although vir $^-$ mutants were also detected amongst the 18 mutants with multiple insertions, biosurfactant-loss may not be the only cause of reduced virulence, because other gene functions may have been affected. Growth rate and the production of three extracellular products (pectic and proteolytic enzymes, and fluorescent pigment) were unaffected in these 17 mutants. Therefore, because some surf $^-$ mutants were unaffected in their ability to cause disease, it was concluded that biosurfactant production in *P. fluorescens* is neither a virulence nor a pathogenicity factor. Biosurfactants are probably produced, in this bacterium, to assist initial establishment and colonisation of the plant surface. Further work is required to elucidate the nature of the mutated genes to define the precise role of biosurfactants in the host/pathogen interaction.

Declaration

I hereby declare that all of the results presented in this thesis have been generated by myself alone, and have not been submitted previously for a degree. Work by other authors has been specifically acknowledged via references.

Denise Darling
September 1998

Dedication

It would be an honour to dedicate this thesis to my mum, Maureen. Not only has she been a devoted parent, with endless patience and love, but also a great friend.

Acknowledgements

Firstly, I would like to thank my supervisor Dr Rob Harling, for his help and advice throughout this project, and my second supervisor, Dr Neil McRoberts, for proof reading Chapter 4 and helping with the set up of the head morphology field trial.

I am indebted to Dr Antonius Suwanto for sharing his expertise with Tn5 and providing the plasmid vector, pMH1701. Thanks also goes to Dr Simon (Universität Bielefeld, Germany) for providing the plasmid vector PSUP2021, which was used in the mutagenesis study, and to Dr Maurice Gallagher (Institute of Cell and Molecular Biology, University of Edinburgh) for kindly advising me on which Tn5 vector to use.

I am grateful to Robert Redpath and the technical staff at the Scottish Agricultural College for their help with the maintenance of the plant material used in this work.

Moreover, my deepest thanks goes to my Mum and Dad for supporting me throughout, and to Stuart for brightening every day with his love and support during our postgraduate studies.

The Ministry of Agriculture, Fisheries and Food (MAFF), London, funded this research.

Table of Content

List of Tables

Chapter 2

Chapter 3

Chapter 5

Chapter 6

Chapter 9

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Plates

Chapter 1

Chapter 2

Chapter 3

Chapter 5

List of abbreviations

ANOVA	Analysis of variance
Apase	Acid phosphatase
ATP	Adenosine triphosphate
avr	Avirulence
bp	Base pair
ca	Circa
Ca^{2+}	Calcium ions
cf	Compare with
cfu	Colony forming units
cm	Centimetre
cm^2	Centimetre squared
CMC	Critical Micelle Concentration
CO_2	Carbon dioxide
CTAB	Cetyl-trimethylammonium bromide
CVP	Crystal Violet Pectate
df	Degrees of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
Dr	Doctor
dsp	Disease specific
E.carotovora	*Erwinia carotovora*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
e.g.	For example
EMBL	European Molecular Biology Laboratory
EPS	Extracellular polysaccharide
et al.	And others
EtBr	Ethidium bromide
F	ANOVA value
Fcrit	ANOVA significance value
fw	Fresh weight
g	Gramme
h	Hours
H^+	Hydrogen ions
H_2O_2	Hydrogen peroxide
ha	Hectare
HPLC	High Performance Liquid Chromatography
HR	Hypersensitive response
hrp	Hypersensitive response gene
Hz	Hertz
i.e.	That is
INA	Ice nucleating activity
ina+	Ice nucleating activity phenotype
IPM	Integrated pest management
IS50	Insertion Sequence
k	Kilo
K^+	Potassium ions
Kan^r	Kanamycin resistance
kb	Kilobase
KB	King's B Broth
KBA	King's B Agar
kg	Kilogramme
LB	Luria Broth
LSD	Least Significant Difference
LPS	Lipopolysaccharide
M	DNA size marker
MDH	Malate dehydrogenase
mg	Milligramme
Mg^{2+}	Magnesium ions
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Millilitre
MM	Minimal Medium
mM	Millimolar
mm	millimetre
N	Disease susceptibility not established
NA	Nutrient agar
Na^{2+}	Sodium ions
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NB	Nutrient broth
N.B.	Note well
ng	Nanogramme
nm	Nanometre
NPK	Nitrogen, Phosphate, Potassium Fertiliser
NS	No significant difference found
N5024	*Pseudomonas fluorescens* isolate N5024
N5027	*Pseudomonas fluorescens* isolate N5027
OD	Optical density
OD_{500nm}	Optical density at 500nm wavelength
p	Probability
P.A.F.	Pseudomonas agar F
P. Crop	Premium Crop
Pers. comm.	Personal communication
Pers. obs.	Personal observation
P.fluorescens	*Pseudomonas fluorescens*
pg	Picogramme
pH =	Acidity/alkalinity value
ppm	Parts per million
P. putida	*Pseudomonas putida*
P.s.s	*Pseudomonas syringae* pv. *syringae*
pv.	Pathovar

P5067	*Erwinia carotovora* ssp *atroseptica* isolate P5067
P5064	*Pseudomonas fluorescens* isolate P5064
P5038	*Pseudomonas fluorescens* isolate P5038
P5049	*Pseudomonas fluorescens* isolate P5049
P1065	*Erwinia carotovora* ssp *carotovora* isolate P1065
r	Correlation value
R	Resistant to disease
R gene	Resistance gene
Ri	Root inducing
RNA	Ribonucleic acid
rpm	Rotations per minute
S	Susceptible to disease
SABS	Sodium alkylbenzene sulphonate
SDS	Sodium dodecyl sulphate
SEM	Standard Error of the Mean
$spec^R$	Spectinomycin resistant
spp.	Species
SSC	Standard saline citrate buffer
ssp.	Subspecies
$strep^R$	Streptomycin resistant
$surf^-$	Non-surfactant producer
TAE	Tris/Acetate/EDTA Buffer
TBE	Tris/Borate/EDTA Buffer
TE	Tris/EDTA Buffer
Ti	Tumour inducing
TLC	Thin Layer Chromatography
TMV	Tobacco Mosaic Virus
Tn5	Transposon Tn5
tox^-	Mutant which can no longer produce toxins
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane-hydrochloric acid
UV	Ultraviolet light
V	Volts
vir^-	Non-virulent (avirulent)
vir^+	Virulent strain
vs	Versus
W	Wild-type strain
w/v	Weight per volume
Zn^{2+}	Zinc ions
@	At
β	Beta
µg	Microgrammes: 10^{-6} g
µl	Microlitre
µM	Micrometre
θ	Contact angle
+ ve	Positive
-ve	Negative
%	Percentage
°C	Degrees Centigrade
<	Less than
>	Greater than
^{14}C	Radiolabelled carbon
λ	Lambda
°	Degrees
£	Pound Sterling
±	Plus or minus
’’	Inch
x	Multiplied by

Chapter 1: Bacterial plant disease and head rot of broccoli - Introduction to the field of study

1.1 Bacterial diseases of vegetable crops

Diseases of economically important crops such as broccoli (*Brassica oleracea* var. *italica*) can lead to a significant reduction in yield. Whilst it is difficult to accurately assess the degree of loss due to bacterial plant diseases (which are generally of lower economic importance than fungal or viral plant diseases), significant levels of damage can occur (Billing, 1987). Pathogenic bacteria are responsible for a variety of plant diseases including blights (fruit or blossom death) and cankers (stem lesions); vascular wilts (xylem vessels are invaded by necrogenic pathogens resulting in wilting); leaf spots; overgrowths; scabs, and soft rots (tissue maceration and collapse) (Billing, 1987). Unfortunately, however, bacterial diseases of plants are often difficult to control, requiring a combination of control measures to combat them (Agrios, 1988).

1.2 Bacterial pathogenicity

To determine whether a bacterial strain is the causal organism for a previously unreported disease Koch's postulates must be considered since plant pathogenic bacteria are found widely as epiphytes and therefore plant association does not imply causality of disease (Sigee, 1993). Koch's postulates decree that the bacterium associated with a disease must firstly be isolated and grown in pure culture (with its colony morphology being noted). If it can be then be shown to induce disease symptoms when inoculated on to healthy plant tissue of the same species, and to possess the same colony morphology when isolated in pure culture again, it can be deemed the causal organism (Agrios, 1988).

Almost all bacterial plant pathogens are rod shaped, gram negative organisms (with the exception of *Streptomyces* which is filamentous). Gram negative bacteria possess a thin peptidoglycan layer (unlike gram positive species, of which *Clavibacter* and *Bacillus* are the only plant pathogens) and a lipid rich layer, separated by the periplasm which contains amino acids, sugar binding proteins and hydrolytic enzymes (Billing, 1987). In addition, most pathogens are motile, having either polar (*Pseudomonas* and *Xanthomonas*) or peritrichous (*Erwinia* and *Agrobacterium*) flagella, which are important for chemotaxis and entry in to plant tissue (Billing, 1987).

Pathogenicity can be defined as the fundamental ability of a pathogen to cause disease (Sigee, 1993) and involves the pathogen adhering to and penetrating the plant tissues (which are composed mainly of cellulose, pectic polymers and pectins) to obtain nutrients, whilst neutralising the plant's defense reaction. Virulence can be defined as the degree to which that pathogen affects the health of the plant (Sigee, 1993).

The initial stages of pathogenesis involve the pathogen attaching to and invading the plant tissue through natural openings, such as stomata or wounds. This is usually a passive process (Goto, 1992). Recognition then occurs between the pathogen and host cell, which can result in either a compatible (disease) or incompatible (no disease) reaction. During incompatible reactions the hypersensitive response (HR) may occur, which is a plant defense reaction where localised cell death occurs to stem the spread of infection, whilst in compatible

reactions mild degeneration of the host cell membrane releases nutrients or stimulants that aid bacterial growth without causing structural damage to the plasma membrane (Goto, 1992). As bacterial growth progresses, watersoaking develops, creating conditions for accelerated bacterial growth (Goto, 1992).

The ability of a pathogen to bring about disease depends on the pathogenicity (*hrp*) and virulence (disease specific genes - *dsp*) genes it possesses. *Hrp* genes are so named because they are involved in generating the hypersensitive response (in non-host plants) and pathogenicity (in host plants), while virulence (or *dsp*) genes influence pathogenicity but not the HR (Goto, 1992). The products of some of these genes are chemical substances, known as pathogenicity and virulence factors, which either affect certain components/metabolic mechanisms within the plant, or serve to increase disease severity, respectively. Pathogenicity and virulence factors include enzymes, toxins, growth regulators, siderophores, ice nucleating activity and polysaccharides. The various factors are briefly discussed below.

Enzymes are the most important pathogenicity and virulence factors in soft rot diseases. Pectinases or pectolytic enzymes macerate plant tissue by degrading the pectic substances within the middle lamella of plant cell walls (Lund, 1982). This results in damage to the cell wall, leading to osmotic fragility and loss of turgor. It is thought that cell death results from weakening of the primary cell wall, which cannot then support the osmotically fragile protoplast, causing it to burst (providing the pathogen with carbon for energy and nitrogen for nutrition). Other examples of cell wall degrading enzymes include: Cutinases, which degrade cutin

(the main component of the cuticular layer); cellulases, which both degrade the cellulose that comprises the skeletal substance of cell walls (facilitating the penetration and spread of the pathogen) and release soluble sugars to provide nutrients; galactanases; xylanases, and arabanases. Once inside the plant cell, proteinases degrade protein substances, amylases dissolve starch, whilst phospholipids or lipases degrade lipids. Only a small group of micro-organisms can degrade lignin, which is found in the middle lamella and in fibres which strengthen the plant. In addition to the damage induced by bacterial pathogenicity factors, when plant proteins are reduced to amino compounds they may release ammonia resulting in additional tissue damage (this also aids bacterial pectic lyases which require a high pH to operate).

Toxins (which act directly on the protoplast) are non-host specific virulence factors that seriously damage or kill living host protoplasts by affecting cell permeability or inhibiting enzymes. Examples of toxins produced by *Pseudomonas syringae* include syringomycin, which causes necrosis and chlorosis, and coronatine and phaseolotoxin which both cause chlorosis.

Siderophores are iron-chelating water-soluble pigments produced by many bacterial pathogens and plant associated bacteria (Billing, 1987) and have been reported to be responsible for disease in *Erwinia chrysanthemii* by chelating iron during the iron-limited conditions which prevail *in planta* (Mourgues *et al.*, 1998). Furthermore, some bacteria, such as *Pseudomonas syringae*, have ice-nucleating

proteins present in their cell walls, which can cause death by ice crystal formation (frost damage) in soft tissue at higher temperatures than normal.

Plant pathogens may also produce plant growth regulators, such as auxin, indole acetic acid, gibberellins, cytokinins, ethylene, abscisic acid and polysaccharides, which can cause a hormonal imbalance in the plant and induce abnormal growth. This results in diseases which exhibit symptoms such as stunting, overgrowths and excessive root branching.

In addition to the specific pathogenicity and virulence factors described above, bacteria also produce polysaccharides, which may be present either in capsule form or as water soluble polymers, that surround the bacterial cell membrane making their surface hydrophilic. Moreover, they increase the surface area available for cell activity; prevent rapid diffusion; attract and concentrate nutrients; hold extracellular enzymes, and absorb and bind water. However, whilst extracellular polysaccharides (EPS) promote watersoaking (which aids bacterial multiplication), help in adhesion and protect the cell against inhibitors, lipopolysaccharides (LPS) are the only polysaccharides known to influence plant disease directly by interfering with translocation causing vascular wilt diseases.

However, it is not only the bacterial pathogenicity/virulence factors that determine the outcome of disease as environmental conditions such as temperature, moisture (free water and humidity), light, soil nutrients, and soil pH, are crucial for the development of plant diseases. This is because they influence the spread and fitness of the pathogen, and the resistance of the host, and can therefore determine

whether a compatible host-pathogen interaction occurs. Indeed, opportunistic pathogens, such as *Pseudomonas marginalis*, *Bacillus polymyxa* and *Clostridium* species, can be found. These are weak pathogens (which generally occur as saprophytes on plant tissue) that can only cause disease under certain environmental conditions (Sigee, 1993). Broccoli head rot is an example of an opportunistic infection.

The most important environmental factors include temperature, which governs the intensity of the plant/pathogen interaction (bacterial pathogens generally require a temperature of between 25 and 30°C), and water, which acts as a pathogen vector (Goodman *et al.*, 1986) and is essential for the multiplication and spread of bacteria. This is due to water dissolving the polysaccharide matrix in which bacterial cells are embedded, releasing them to readily gain access into natural openings or wounds. During rainfall bacteria emerge from diseased tissues into surface water, providing a source of inoculum. For example, young lesions of citrus canker discharge bacterial cells at concentrations of 10^5 to 10^7 cells/ml throughout the period of rainfall (Goto, 1992). While bacteria are immersed in water, they are protected from the deleterious effects of desiccation and can potentially be transported aerially, on plant surfaces, or on the ground to reach new sites of infection (Goto, 1992). Pathogen dispersal can also occur by irrigation water, soil micro-organisms, seeds and planting material, insects and farming practices (Goto, 1992).

1.3 Plant defense

Plants are defended structurally from disease by their thick cuticle and wax layer, which form a water repellent surface to help prevent pathogens attacking and multiplying. In addition, the structure of the epidermal cell walls, the size, location and shape of stomata, and the presence of tissues comprised of thick-walled cells all hinder the pathogen's advance. Phenolic compounds, tannins and hydrolytic enzymes (e.g. glucanase and chitinase) may also be produced and provide resistance to pathogens by breaking down the pathogen's cell wall components. These factors are all present in mature plants prior to the arrival of a pathogen, but as levels of production vary between species, some plants are more susceptible to a particular pathogen than others.

However, once a pathogen is established, the plant actively responds with additional structural defense products. For example, cork layers or an abscission layer (a gap between two layers of cells, due to the middle lamella being dissolved) may form, isolating the site of infection from healthy tissue; the outer layer of parenchyma cells may swell to form amorphous, fibrillar material which traps bacteria; cell walls may thicken and become infused with phenolic substances to resist pathogen penetration, and callose papillae may be deposited on inner cell walls. However, the effectiveness of such defenses are limited compared with biochemical resistance factors.

Biochemical resistance can be considered the most effective type of plant defense because even in the absence of structural defenses disease can be completely

checked. Toxic (antimicrobial) compounds, such as phenolics (e.g. chlorogenic and caffeic acids), the oxidation products of phenolic compounds, and phytoalexins (of which most are phenolic compounds) are synthesised in healthy cells adjacent to localised damage in response to chemical signals from damaged cells (Agrios, 1988). Various types of phytoalexins have been isolated and are usually genus specific. For example, the Legume family produces isoflavanoid compounds (Wyman and Van Etten, 1978) whereas *Solanaceous* plants synthesise terpinoids (Tomiyama *et al.*, 1968). Phytoalexins (e.g. pistatin from pea and rishitin in potato) are produced in appreciable amounts in response to elicitors, such as glucan, glycoproteins and polysaccharides. However, pathogens may produce suppressors of phytoalexins, such as toxins, glucans or glycoproteins, which prevent phytoalexin production in susceptible host-pathogen interactions (Agrios, 1988). Interestingly, it has been established that certain phenolic compounds are produced at a faster rate in resistant cultivars (Agrios, 1988). Furthermore, phenol-oxidising enzymes (e.g. polyphenoloxidases and peroxidase) oxidise phenolic compounds to quinones, which are often more toxic to microorganisms than the original phenols. In addition, pathogen attack may induce altered enzyme synthesis in the plant which can lead to increased resistance (e.g. phenylalanine ammonia lyase synthesis which is a key enzyme in the production of the basic molecule required for phenolics, including phytoalexins and lignin).

As mentioned previously, during incompatible host-pathogen interactions, a hypersensitive reaction (HR) may occur, which causes localised plant cell death around the site of invasion and stimulates phytoalexin production resulting in the spread of the pathogen being stemmed. It requires the involvement of hypersensitivity and pathogenicity (*hrp*) genes which have been cloned in species such as *Erwinia amylovora*, *Pseudomonas solanacearum, Pseudomonas syringae* and *Xanthomonas campestris*, are highly conserved in many different pathovars of the same species or different species of the same genera (Goto, 1992). Moreover, the expression of *hrp* genes is influenced by nutritional conditions, and the development of a HR is dependent on environmental factors, the age of the plant, bacterial metabolic state, inoculum level and bacteria-cell contact (Sigee, 1993). It should be noted that opportunistic pathogens do not induce a HR.

Induced resistance, where exposure to a pathogen leads to resistance developing to all pathogens, may also occur. This requires *de novo* enzyme synthesis in the plant and appears to follow induction of the HR and involves translocation of signal molecules such as salicylic acid (Sigee, 1993).

The genetic control of resistance may be regulated by a gene-for-gene system as proposed by Flor, in 1955, for the fungal pathogen *Melampsora lini* (the causal organism of rust disease in flax) (cited in Sigee, 1993). It states that virulence in the pathogen is inherited as a recessive characteristic and resistance in the host as a dominant characteristic. For each gene conferring resistance of the host, there is a corresponding gene in the pathogen that confers the avirulence of the pathogen.

Incompatibility results from the active interaction of the pathogen's dominant avirulence (A), and the plant's dominant resistance (R), genes. The absence of the gene product from either results in a compatible situation. This theory has since been confirmed in the diseases caused by plant pathogenic bacteria such as *P. syringae* pvs. g*lycinea* and *tomato*, *Xanthomonas campestris* pvs. *malvacearum, oryzae* and *vesicatoria* (Goto, 1992), and fungal diseases such as potato late blight, and the mildews of wheat and barley (Sigee, 1993).

1.4 Control of bacterial disease

There are several forms of control available to combat plant diseases: Chemical, where an infection is prevented or cured with the use of specific compounds; physical, where the plant is protected from the pathogen (e.g. the use of polythene mulches); biological, where a micro-organism antagonist is used to prevent pathogen attack; cultural, where inoculum is reduced by crop rotation and finally, regulatory, where a pathogen is excluded from an area via legislation. The control of soft rots of vegetables is based almost exclusively on preventative and cultural practices, but care must also be taken to avoid inflicting wounding on the crop.

However, ultimately the use of resistant varieties is the cheapest, easiest, safest and most effective control.

1.5 Broccoli (*Brassicae oleracea* var *italica*)

Broccoli or calabrese (*Brassicae oleracea* var *italica*) takes its name from the Calabria region of Italy, where it is thought to have originated (Anon, 1984). It is an annual plant with an edible primary head (or spear), consisting of a stem holding a head of compact green flower buds (Anon, 1984) (Plate 1.1). Secondary heads may be produced as side shoots in the axils of the leaves. Spears for fresh markets should be firm, with well packed flower buds and no trace of yellow petals or opening flowers. The latest available figures state that production in Scotland increased from 217.5ha in 1982 to 1004ha in 1992, accounting for 20% of the total UK production in 1991 (Anon, 1993). Broccoli popularity has continued to increase among consumers due in part to its increasingly well documented antioxidant health properties.

Plate 1.1: Mature broccoli (*Brassica oleracea* var. *italica*) plant, showing a healthy head

The Scottish climate provides ideal conditions for broccoli production, allowing longer harvest intervals and planning of successional harvest throughout the season. Heads are programmed to mature from June through to December, with earlier production in May and June being possible if polythene covers are used in the field. Soil type is relatively unimportant, as plants grow well provided there is good drainage and structure to the soil. Although broccoli can tolerate more acidic conditions than other *Brassicas*, the pH should ideally be maintained at 6.5 via liming. Plants should be spaced at a distance of 50cm between rows and 23cm in rows.

The main broccoli pests include cabbage root fly (*Delia brassicae*) whose larvae feed on roots, with severe infestations causing stunting, discolouration of leaves and possibly wilting and death of the plant. To combat cabbage root fly insecticide must be applied at sowing time. The flea beetle (*Phyllotreta*) larvae also feed on roots whilst adult feeding makes holes in leaves and stems of seedlings. The cabbage aphid (*Brevicoryne brassicae*) causes leaves to curl and turn yellow and heavy aphid contamination may spoil heads. Cabbage caterpillars can also cause damage by feeding on leaves and heads.

The main diseases of broccoli include: Clubroot, caused by *Plasmodiophora brassicae*, which induces swelling, then rotting of roots; damping off, due to *Pythium* species; wirestem, induced by *Rhizoctonia solani*; downy mildew, caused by *Peronospora parasitica* (causing yellowing of upper surfaces); dark leaf spot, caused by *Alternaria brassicae* and *Alternaria brassicicola*; ring spot, due to

Mycosphaerella brassicicola and white blister, due to *Albugo candida*. Of the bacterial diseases, black rot of crucifers, caused by *Xanthomonas campestris* pv. *campestris*, results in the development of large yellow to brown lesions along leaf margins, and head rot (or spear rot) is an opportunistic bacterial disease caused predominantly by *Pseudomonas* spp. (Wimalajeewa, 1985; Hildebrand, 1986, Robertson *et al.*, 1993).

1.6 Head rot of broccoli

Bacterial head rot of broccoli leads to crop losses which represents a £9.5 million annual loss for the UK broccoli industry (Campbell *et al.*, 1995). This makes it the most important disease of broccoli, and indeed the most important limiting factor to production. First reported in Britain by Dowson and Dillon-Weston in 1937 head rot has since been described in Australia (Wimalajeewa, 1985; Wimalajeewa *et al.*, 1987), Canada (Hildebrand, 1986; 1989; Hildebrand *et al.* 1990), and the U.S.A. (Canaday *et al.*, 1987; 1991; Canaday and Wyatt, 1992). Losses of between 30% and 100% have been recorded in America (Hildebrand, 1989), while in Australia loss has been estimated at 35% in the field with an additional 10% occurring in storage (Wimalajeewa *et al.*, 1987).

Disease symptoms start with areas of watersoaking on the head which deteriorate into a brown/black soft-rot under suitable environmental conditions (Plate 1.2).

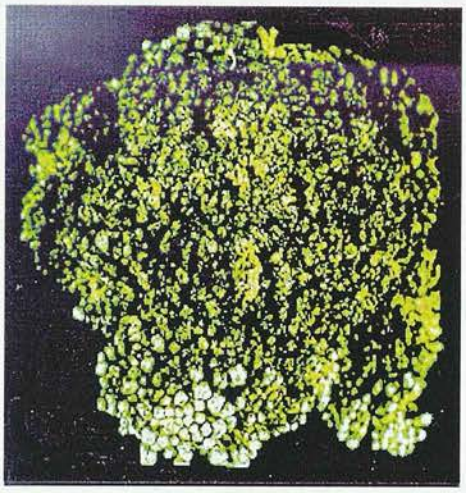


Plate 1.2: A broccoli head showing severe soft rot symptoms

Disease incidence and severity increase when head maturity coincides with periods of persistent wet weather (Canaday and Wyatt, 1992) and high humidity (Canaday *et al.*, 1991). Although the role of water and temperature in dispersal, colonisation and multiplication of plant pathogenic bacteria in general is well documented (Goodman *et al.*, 1986), little epidemiological data currently exist for head rot. Higher disease levels are typically found in autumn due to high daytime temperatures followed by low night-time temperatures, which causes long periods of dew formation, and hence free water availability, on the heads (Campbell *et al.*, 1995). Ludy *et al.* (1997) demonstrated the importance of free water for disease initiation when they reported that decreasing the frequency of overhead irrigation, from every two to every eight days, reduced the incidence of head rot.

Furthermore, field observations indicate that 48h at 100% humidity (coinciding with head maturation) leads to disease development (A. Ewan, personal communication cited in Simpson, 1993). However, there are conflicting reports over the optimum temperature for disease induction. Canaday *et al.* (1991) reported that warm wet weather promotes disease, whilst both Wimalajeewa *et al.* (1987) and Robertson *et al.* (1993) found cool wet weather was most conducive to disease development.

Other factors have been identified which affect disease incidence. For example, Canaday *et al.* (1991) noted that disease incidence correlated inversely with the number of days from transplanting to harvest (indicating that the fastest maturing cultivars are the most susceptible to disease), and also that larger heads are significantly more prone to infection. Also, Canaday and Wyatt (1992) demonstrated that under disease conducive conditions, nitrogen application (in the form of ammonium nitrate) increased disease levels in the susceptible cultivar Premium Crop but not in resistant Shogun, and in addition that the length of time required for head maturation decreased with increasing nitrogen application (Canaday and Wyatt, 1992).

1.7 Head rot pathogens

As mentioned previously, most reports cite the fluorescent *Pseudomonas* species, *P. marginalis* or *P. fluorescens* as the causal organisms (Hildebrand, 1986; Wimalajeewa *et al.*, 1987; Robertson *et al.*, 1993; Campbell *et al.*, 1995). *P. fluorescens* is normally a non-pathogenic epiphyte, but can become an

opportunistic pathogen under appropriate environmental conditions (e.g. it has been identified as the principal soft-rot pathogen of endive (*Cichorium endivia* var. *latifolia*) (Morris and Nguyen-the, 1996)). Although many strains are capable of inducing disease, the bacteria responsible vary in their virulence towards broccoli tissue (Hildebrand, 1986; 1989; Wimalajeewa *et al.*, 1987; Robertson *et al.*, 1993), and Hildebrand (1989) found that the pathogenic and saprophytic fluorescent Pseudomonads he isolated from diseased heads could be classified into four distinct groups (11 subgroups).

Whilst *Erwinia carotovora* has frequently been isolated from diseased heads, the majority of isolates can only induce disease on wounded heads (Dowson and Dillon-Weston, 1937; Wimalajeewa, 1985; Canaday *et al.*, 1987; Hildebrand, 1989). However, in Scotland, *Erwinia carotovora ssp. carotovora* and *E. carotovora ssp. atroseptica* have been isolated from diseased heads and shown to be pathogenic on non-wounded heads (Robertson *et al.*, 1993). The Scottish isolates of *Erwinia* are reported to be a serologically distinctive group (Campbell *et al.*, 1995).

The majority of pathogenic Pseudomonads on broccoli are capable of producing both pectolytic enzymes and biosurfactants (Hildebrand, 1989; Robertson *et al.*, 1993). Harling *et al.* (1994) isolated 72 pathogenic and non-pathogenic fluorescent isolates and recorded that the majority of the 13 pathogenic strains produced pectolytic enzymes and biosurfactants. The role of pectolytic enzymes in soft rot disease has been well documented (Liao *et al.*, 1993; Agrios, 1988), but

biosurfactants have been less well explored. Biosurfactants reduce the surface tension of water, leading to watersoaking and nutrient leakage. Hildebrand (1989) reported that only biosurfactant producing, pectolytic, strains of *Pseudomonas* could attack the waxy plant surface and initiate disease development on unwounded tissue. However, exceptions to this rule have been found in Scotland, whereby isolates lacking either one of these characteristics can still cause disease (Campbell *et al.*, 1995). For example, *Erwinia carotovora* produces pectolytic enzymes but not biosurfactants. This throws the involvement of biosurfactants in disease into question. However, the disease process appears to involve complex bacterial interactions, since Hildebrand (1989) demonstrated that whilst individual isolates lacking either pectolytic enzymes or biosurfactants did not produce disease, co-inoculating complementary isolates (such as pectolytic enzyme producing *Pseudomonas viridiflava* or *Erwinia* species with non-pectolytic, surfactant-producing strains of *Pseudomonas fluorescens*) caused extensive decay (Hildebrand, 1989). Therefore it may be that residual bacteria are influential in the development of disease by providing complementary pathogenicity factors.

Laycock *et al.* (1991) identified the peptidolipid biosurfactant viscosin from *Pseudomonas fluorescens* biovar II, which was isolated from diseased heads. Viscosin did not alter the wax structure, but acted as a wetting agent, thereby allowing bacterial enzymes to attack the plant surface and disrupt cell membranes, altering permeability (Laycock *et al.*, 1991). It was reported that viscosin may be associated with pathogenicity by enabling bacterial spread and penetration of plant

surfaces. Although there are few reports of the involvement in general of bacterial biosurfactants in pathogenicity, the phytotoxic effects of industrial surfactants used in the agricultural industry have been well documented (e.g. Knoche *et al.*, 1992; Bussotti *et al.*, 1997). The subject of biosurfactants in disease will be described in more detail in Chapters 4 and 6.

It is not known whether other bacterial pathogenicity factors, such as cellulases, lyases and toxins are involved in the disease aetiology of head rot.

Our understanding of bacterial growth on broccoli tissue is also limited. Robertson and Brokenshire (1992) found that approximately 10^6 total pectolytic, and 10^6 fluorescent *Pseudomonas* bacteria were present on watersoaked broccoli heads, whilst heads with full soft rot symptoms possessed 1.5×10^8 total pectolytic, and 3.6×10^7 fluorescent *Pseudomonas* bacteria. However, there are no reports on whether pathogenic bacteria multiply more successfully than non-pathogenic, or if competition occurs between isolates. Indeed, pathogenic strains may multiply more rapidly on susceptible broccoli cultivars compared with resistant ones. Research is required into identifying the factors which favour bacterial growth *in planta* and will be assessed in Chapter 3.

Stomata have been implicated as the entry site for the bacterial pathogens (Hildebrand, 1989) and it has been suggested that biosurfactant-producing strains kill the guard cells around stomata (Hildebrand, 1989; Gladstone, 1997). It is not known whether stomatal distribution or number varies between resistant and susceptible cultivars: Variation in these factors may potentially predispose certain

cultivars to disease. Notably, Simpson (1993) reported high stomatal numbers in the susceptible cultivar, Corvet, but more quantitative work is required, and this is presented in Chapter 3.

1.8 Cultivar resistance and other control methods for head rot

Field trials have indicated that broccoli cultivars vary in their susceptibility to head rot, although none are completely resistant. Shogun, Green Defender and Samurai are regarded as among the most resistant cultivars, while Skiff and Premium Crop are held particularly susceptible to disease (Canaday *et al.*, 1991; Robertson *et al.*, 1993).

Currently there are no completely effective head rot control methods available. Between 50% and 80% disease control has been achieved when applying the copper compounds, copper oxychloride (Cuprokylt) or copper hydroxide (Kocide 101), in a two spray procedure: Firstly at the button stage of head formation (heads 1.5cm) and then seven days later (Robertson *et al.,* 1993). Canaday *et al.* (1987) reported that spraying maneb, copper, maneb plus copper, mancozeb, benomyl, chlorothalonil, anilazine, sulphur, dilute sulfuric acid, sodium hypochlorite or calcium propionate all failed to adequately control disease, whilst Neuvel *et al.* (1996) noted that a weekly application of the stone meal Silkaben or calcium chloride during head formation reduced disease by 50% and 33% respectively. Even the antibiotic Kasugamycin, which is active against many bacterial and fungal diseases (including crucifer black rot), and the generally more effective mixture of kasugamycin and copper oxichloride (Kasumin), produced

unsatisfactory disease prevention (Campbell *et al.*, 1995). Applying bacterial antagonists or a water surfactant were also ineffective in reducing disease incidence or severity (Canaday *et al.*, 1987). In a more environmentally friendly experiment, Campbell *et al.* (1995) attempted to control disease by covering the soil with polythene mulches, which theoretically work by reducing the amount of soil borne inoculum transferred to the plant tissue by rain splash. However, only a 50% reduction in disease was achieved. The authors state that because mulching reduces weed competition, retains soil moisture and solarises the roots, these factors may enhance the plant's ability to withstand disease (although no difference was found in the nitrogen or mineral content of mulched and unmulched tissue, so this may not have occurred in their field trial (Campbell *et al.*, 1995)). Disease incidence may also be reduced slightly by decreasing the nitrogen input (Canaday and Wyatt, 1992) and minimising overhead irrigation (Ludy *et al.*, 1989) which both lead to less favourable conditions for pathogen spread and penetration.

Currently, growers must compromise between either planting a resistant cultivar or planting one which exhibits the desirable marketable characteristics. Desirable characteristics include large firm heads, tightly packed buds and a deep green colour (Anon, 1984). Fortunately there are resistant cultivars, such as Shogun, Samurai, and Marathon, which do possess good agronomic characters and are therefore popular commercially (Robertson *et al.*, 1993), but this is very limiting in terms of consumer choice. Once the factors governing disease resistance are fully established, a wider variety of cultivars could be bred with improved disease

resistance. Unfortunately, at present, little information is available on factors involved in host resistance.

There have been no definitive reports regarding the influence of structural or biochemical defense factors in the resistance of broccoli to disease. Plant defense products protect the plant either by inhibiting the invading microbe, or by strengthening the plant cell against attack. *Brassicas* produce the constitutive compounds glucosinolates (a group of sulphur containing glycosides), which are hydrolysed by the endogenous enzyme myroinase to indole or isothiocyanate derivatives after tissue damage. These compounds have been shown to be toxic to fungi (Mithen *et al.*, 1986, 1987) and may play a role in disease resistance responses generally. In addition, phytoalexins, callose, cutins and lignins are all well studied plant defense products, but whether they are activated in head rot has not been studied.

There are few reports on the production of phytoalexins by *Brassicas*, although Takasugi *et al.* (1986) identified three sulphur containing compounds in Chinese cabbage (*Brassica campestris* L ssp. *pekinensis*) following inoculation with *Pseudomonas cichorii* or *Erwinia carotovora*. These were termed methoxybrassinin, brassinin and cyclobrassinin. In addition, Conn *et al.* (1988) reported qualitative and quantitative differences in the elicitation of phytoalexins in several *Brassicae* following inoculation with *Alternaria brassicae*, the causal organism of dark leaf spot in broccoli. Preliminary work by Simpson (1994) indicated that inoculating broccoli leaves and heads of the resistant cultivar

Shogun, and the susceptible cultivar Corvet, with two pathogenic (*E. carotovora* ssp. *carotovora* P1065 and *P. fluorescens* P5038), and two non-pathogenic (*P. fluorescens* N5004 and non-fluorescent isolate N5104) bacteria, stimulated the production of antibacterial compounds, in both cultivars. The compounds were not found in healthy tissue and it was speculated that these products may be phytoalexins. Simpson (1994) stated that the inhibitory extracts may be as a result of self inhibition effects by the bacteria, due to *in vitro* growing conditions (Wilkinson, 1975, cited in Simpson, 1994). Fluctuations in inhibitory activity were observed, with inhibition declining with age suggesting that compounds were unstable under storage. The greatest inhibition was also found with *Erwinia carotovora* spp *carotovora*, contradicting the literature which states that phytoalexin production is normally associated with incompatible interactions (Fett, 1984; Fett and Jones, 1984; Billing, 1987). However, follow up work by Heggie (1995) found that these compounds increased in response to infection and inhibited invading organisms, but the results did not prove that they accumulated to inhibitory concentrations in the region of the pathogen (considered a requirement of phytoalexins). Therefore, although further work is required for full understanding, these preliminary results strongly indicate that phytoalexins may be involved in resistance to head rot.

Simpson (1994) also carried out preliminary studies on the involvement of callose and lignin in disease resistance. It was found that localised callose deposition occurred in leaves of the cultivars Corvet (susceptible) and Shogun (resistant)

which had been inoculated with the two pathogenic and two non-pathogenic bacterial isolates described previously. No reaction was found with control samples. Lignin deposition was not found in any material.

In addition to specific defense products, head morphology may also be important in predisposing certain cultivars to disease. Characteristics such as head doming, bud size and tightness (compactness), bud prominence and head weight/diameter ratio vary considerably between broccoli cultivars, and these may affect the duration with which free water remains on the head, in turn influencing the success of bacterial multiplication and penetration of host tissue. Field resistant cultivars such as Shogun and Samurai have domed heads, while susceptible Corvet and Skiff, have relatively flat heads.

Canaday (1989) related head tightness (compactness) and doming with disease incidence, in 13 cultivars, and found a regression correlation of 0.935 ($P<0.05$) (for both characters against the percentage of diseased heads), strongly supporting the involvement of morphology in disease susceptibility. He suggested that the model predicted 85% of the variability in disease incidence when applied to 12 other cultivars. Robertson and Brokenshire (1992) studied the role of head doming in disease, and suggested a relationship between domed heads and resistant cultivars. Results were inconclusive with flatter shaped heads because although some cultivars were susceptible, others were only moderately susceptible. They explained this as being due to head morphology playing only a partial role in the complex process governing disease susceptibility.

Stomata are the sites of infection (Hildebrand, 1989), and high stomatal numbers have been found in the susceptible cultivar Corvet (Simpson, 1993). However, further quantitative work is necessary with resistant cultivars to establish a causal relationship with stomatal number.

1.9 Aims of this thesis

With the present knowledge about broccoli head rot limited, a reliable pathogenicity test was the initial priority in this study, and two tests were examined for their applicability: Firstly, a seedling test, which involves inoculating seedling hypocotyls with a sterile needle which had been dipped into a bacterial colony growing on an agar plate, and secondly, an excised head test, where mature heads were inoculated by covering the buds with cotton lint soaked in a bacterial suspension. Both methods were assessed on a range of cultivars and both had their protocols adjusted to determine whether improvements could be made. Then the growth characteristics of various pathogenic bacteria were then assessed to ascertain their optimal growth conditions. Subsequently, one pathogenic and one non-pathogenic bacterial strain were inoculated on to broccoli heads to monitor growth *in planta*, and to determine whether competition occurs between pathogenic and non-pathogenic bacteria and if growth occurs more quickly on susceptible, as opposed to resistant, heads.

A field trial was then conducted to determine whether cultivar head morphology influences disease susceptibility to test the hypothesis that certain characters may predispose cultivars to infection by influencing the factors which enhance bacterial

multiplication and survival, such as the duration with which free water remains on the head.

Finally, the role of biosurfactants in disease development was investigated using a genetic mutant approach. Initially, biosurfactant production was examined, then biosurfactant-minus mutants of a pathogenic strain of *Pseudomonas fluorescens* were produced via Tn5 mutagenesis. These mutants were characterised for altered pathogenicity, growth, pectic enzyme, fluorescent pigment and protease production, and genetically characterised by determining the Tn5 insertion site using Southern blotting.

Chapter 2: Development of a test to determine pathogenicity and cultivar susceptibility

2.1 Introduction

With head rot costing the UK broccoli industry an estimated £9.5 million annually (Campbell *et al.*, 1995), a reliable *in vitro* pathogenicity test is required to carry out much needed research. Pathogenicity testing involves inoculating plant tissue with a micro-organism of interest to determine whether it can induce disease. A good pathogenicity test should be sensitive to low inoculum concentration; accurate enough to distinguish pathogenic from non-pathogenic strains, and reliable enough to produce the same results on different occasions. The inoculum can be applied to either wounded or intact tissue, often at a concentration of 1×10^8 cfu/ml. This bacterial number is usually considerably higher than the threshold concentration required for disease initiation, but it ensures that there are sufficient numbers present for even weak pathogens to induce symptoms (although care must be taken to confirm that no host tissue response is detected when inoculating non-pathogenic isolates).

Although we are ultimately interested in the disease process as it occurs in the field, unfortunately field-based experimentation is expensive to conduct, time consuming and labour intensive. In addition, environmental conditions cannot be controlled. *In vitro* testing, therefore, provides a more rapid, and cost effective alternative to field experiments, in terms of labour, materials and space required. Most importantly however, is that laboratory based testing can be closely controlled, making it suitable for detailed host/pathogen interaction studies. For example, isolate pathogenicity, cultivar susceptibility, the influence of certain environmental factors on disease (i.e.

temperature and humidity), and the effect of disease development on plant tissue can be readily analysed. Notably, if cultivar susceptibility *in vitro* reflects field ratings, then these tests could be adopted to screen new cultivars in breeding programmes. In this chapter the *in vitro* pathogenicity tests currently available for head rot are assessed and improvements suggested.

2.1.1 Considerations for the use of *in vitro* pathogenicity testing in plant disease research

Laboratory-based pathogenicity tests are attempts at modelling the disease process as it occurs in the field, and therefore must aim to mimic the 'natural' disease environment where possible to reduce the likelihood of laboratory artefacts, which may be caused by a number of factors. For example, unless mature plants are used, the tissue type may differ in its genetic control of resistance; environmental variables, which may influence disease, may not be present; the influence of host morphology on the disease process is often eliminated from the disease process; wounding often occurs during inoculation and therefore the initial stage of pathogen invasion into plant tissue is by-passed; inoculated pathogens have less competition than they would in the field and finally, once tissue is excised from the plant, it ceases to grow and consequently may respond differently to the invading micro-organism.

Other than the potential problem of artefacts, laboratory-based testing is subject to several other shortcomings, which must be considered during the planning of an experiment prior to its execution. These include: (1) the latent period of disease (before symptoms occur) which means that results may not be known for several days; (2) the danger of introducing pathogenic contaminants (Sigee, 1993) which

may bias results, and (3) the requirement for plant material of a suitable developmental age for testing i.e. if mature tissue is desired, which takes several weeks to develop, it may be wise to produce a constant supply of plant material to ensure that sufficient tissue is available when required. If so, this may be costly to produce and requires space and constant maintenance (although it is generally still less expensive than conducting field trials).

In addition, residual bacteria which are present on field grown plant heads and leaves may affect pathogenicity test results by providing a pathogenicity or virulence factor lacking in the inoculated isolate, with the result that the two strains, in combination, can bring about disease. This was demonstrated by Hildebrand (1989) who described how pectolytic bacteria could only rot broccoli tissue if they were co-inoculated with biosurfactant producers which were capable of attacking the waxy plant tissue. Therefore the influence of residual bacteria on *in vitro* results is an important consideration. Robertson and Brokenshire (1992) found that broccoli heads showing bacterial watersoaking symptoms contained approximately 10^6 pectolytic *Pseudomonas* bacteria, while those with soft rot symptoms had 1.5×10^8 pectolytic *Pseudomonas*-type bacteria. Producing test material in the glasshouse, rather than the field, will reduce the number of residual bacteria present during testing, but it is not known how effective this is. To address this problem, the number of residual bacteria present on glasshouse grown heads was determined during the course of this work.

Other issues to bear in mind when performing pathogenicity testing include the bacterial suspension's growth stage at inoculation. For example, Klotz (1993) described how the growth stage of *Pseudomonas syringae* pv. *syringae* may influence virulence or pathogenicity test results by influencing the bacterium's sensitivity to oxidative stress, heat and selected antibiotics. Bacterial stress responses have been well documented previously in enteric bacteria (Farr and Kogoma, 1991; Klotz, 1993). In *Escherichia coli* during stationary growth the synthesis rate of 20 specific proteins increases (Farr and Kogoma, 1991). Among the compounds that accumulate are molecules involved in alternative nutrient utilisation (Csonka, 1989) and those which increase resistance against hydrogen peroxide (H_2O_2). These compounds may have an effect on the ability of bacteria to cause disease. In particular, a high antioxidative capacity is crucial in phytopathogenic bacteria, during tissue colonisation, because plant tissues respond to contact initially by raising the production of active oxygen species, which consists primarily of H_2O_2. Exogenous H_2O_2 penetrates target cells and damages DNA, RNA and protein structures. This, and the fact that bacterial growth is affected by the composition of the nutrient environment, means that all testing should be carried out using bacteria of the same age which have been isolated from the same media source prior to each experiment.

However, it is not only the bacterium's fitness which may affect pathogenicity test results, as the plant's growth history is also important for the outcome of the plant-bacterium interaction (in terms of the plant's nutritional status, humidity, light conditions) (Goodman *et al.*, 1986; Agrios, 1988). Interestingly, Yang *et al.* (1997) described how younger rice leaves, and those positioned lower down the plant, were

more susceptible to infection by the fungus *Pyricularia grisea*, than older, more highly positioned, leaves.

However, with careful planning and uniform production of host and inoculum material, reliable pathogenicity test results can be obtained. In addition, the verification of Koch's postulates - where the suspected pathogen is isolated from diseased tissue, cultured on agar to obtain a pure colony, then re-inoculated on to plant material before it can be determined pathogenic or non-pathogenic (Agrios, 1988) - may prevent errors when identifying the causal organism responsible for a newly discovered disease. Notably, where possible, positive and negative controls should be carried out alongside all experimental inoculations. This involves employing the same protocol implemented with the experimental samples, except that the inoculum source is replaced with water or a known non-pathogenic isolate for the negative control, and a known pathogenic isolate for the positive control. If the positive control produces disease and the negative control does not, the protocol can be assumed to be working reliably and accurately.

However, to overcome some of the problems associated with *in vitro* testing, such as the influence of residual bacteria and the time required to produce material, tissue culture techniques are becoming increasingly popular. This involves, whilst under aseptic conditions, using plant cells and tissues to rapidly propagate material for testing. Tissue culture has many benefits over conventional laboratory testing techniques in that it: (1) requires the minimum of space; (2) reduces the possibility of contamination; and (3) is a dynamic growing system, and wounding is not necessary.

Examples of tissue culture techniques being used in the field of plant pathology include Jin *et al.* (1996) who examined the phytotoxicity of *Fusarium solani* (the causal organism of sudden death syndrome of soybean) culture filtrate on soybean calli, and Platt and Bollen (1993) who monitored host tissue responses to disease development on potato tissue culture plantlets. In addition, Scheck *et al.* (1997) found using lilac (*Syringa vulgaris* 'Sensation') tissue culture plantlets with *P. syringae* pv *syringae*, rather than the deciduous woody plant host, rapidly reduced the bioassay time required to identify pathogenic strains. However, even tissue culture can lead to conditions being to a large degree artificial because the plant tissue may be physiologically and genetically very different from intact plants (Ingram, 1977). For example, defence barriers such as cuticles may not be present in cultured cells. Furthermore, Leifert *et al.* (1994) illustrated the problems that may arise with tissue culture analyses, by describing how non-pathogenic strains in the field can become pathogenic towards plant tissue cultures and conversely, that field pathogens remain latent *in vitro.* They explained that this was due to differences in plant metabolism affecting pathogenesis. Consequently, tissue culture experimentation results should be treated with suitable caution and ideally only be used as a basis for further work (Ingram, 1977). Indeed, it is perhaps more suitable for studying the effect of specific pathogenicity factors on plant tissue once the disease aetiology has been thoroughly characterised. Tissue culture would therefore not be advisable for head rot research given the current low level of knowledge.

Other techniques available to study plant disease include immunological and molecular approaches. For example, immunological techniques using monoclonal antibodies can rapidly detect the presence of known pathogens such as *Erwinia carotovora* ssp. *atroseptica* and *Xanthomonas campestris* pv. *campestris* (Duncan and Torrance, 1992), and molecular biology techniques provide a more direct route to study the disease process at the DNA level with relative ease. This in turn allows the genetic control of particular pathogenicity factors or the role of specific genes in the disease process to be investigated. For example, the genetic sequences of many pathogenicity and virulence factors, such as the phytotoxins produced by *P. syringae* pv. *syringae* (Mo *et al.*, 1995) or the pectic enzyme genes of *Erwinia carotovora* ssp. *atroseptica* (Lindeberg and Collmer, 1992), have now been established which allows their role in disease to be studied in depth. Scheck *et al.* (1997) used DNA probes homologous with the *syr*B and *syr*D genes sequences involved in syringomycin production, to detect pathogenic strains of *P. syringae* pv. *syringae*. Such techniques can be employed *in vitro* and *in planta*, and can be conducted without the requirement for host tissue. However, as for tissue culture testing, these techniques are perhaps more appropriate for the analysis of disease where the basic characteristics have been well established. New, or less well characterised diseases, must be approached at a more eliminatory level, following Koch's postulates, and ensuring that the pathogenicity test successfully models the natural disease aetiology.

However, whilst all laboratory-based tests are to some extent artificial, if designed carefully, they should enable the main disease characteristics to be reflected and provide a useful research tool.

2.1.2 Broccoli head rot pathogenicity testing

Presently, the most successful head rot test involves inoculating mature broccoli heads with cotton lint soaked in 1 x 10^8 cfu/ml bacterial suspension. Results are obtained within 5 days, with pathogenic samples causing an extensive soft rot. This methodology was derived from Hildebrand (1989) who tested whole plants, with the heads enclosed in polythene bags to maintain humidity. Campbell *et al.* (1995) improved on this methodology by excising the mature heads and incubating them in plastic tissue culture pots, significantly reducing the space required during experimentation. A split temperature regime of 20°C/10°C was also implemented to encourage dew formation. It was suggested that dew droplets, which develop following a reduction in incubation temperature, enhance bacterial multiplication (Campbell *et al.*, 1995). Unfortunately, head sterilisation is not possible with this technique, as soaking heads in sterile dH_2O or surface sterilants was found to lead to the development of rotting (Simpson, 1993). To compensate for this, heads are grown under glasshouse conditions to reduce the level of natural saprophytic bacteria on the heads, prior to inoculation. In addition, positive and negative controls are inoculated with a known pathogen and distilled water, respectively. This technique successfully identifies pathogenic isolates according to Koch's postulates (Harling *et al.*, 1994), but it is not yet known whether it can be used to distinguish disease susceptibilities of different cultivars. Notably, mature broccoli plants still have to be grown prior to testing, a process which takes 12-14 weeks.

2.1.3 An alternative methodology for studying broccoli head rot

Daniels *et al.* (1984) reduced the production time, material and space required to study crucifer black rot in the laboratory by testing seedling material. Turnip (*Brassica campestris)* seedlings were inoculated with *Xanthomonas campestris* pv. *campestris* mutants, with 95% of the seedlings showing the characteristic disease symptoms (the remaining seedlings showed localised discolouration) (Daniels *et al.*, 1984).

In Daniels' (1984) work, testing was carried out by removing bacteria from an agar plate colony with a sterile needle, then stabbing it into the seedling's hypocotyl. This was found to be less time consuming than using a spray or syringe, and introduced approximately 5×10^6 colony forming units (cfu) into each seedling. A more accurate level of the bacterial numbers inoculated was gauged by dipping the needle into a colony at a specific depth each time. They concluded that the threshold inoculum concentration required for successful infection was 10^2 - 10^3 cfu/seedling. However, other *Brassica* seedlings, including those of brussel sprouts, cabbage, broccoli, cauliflower and Chinese cabbage, all failed to produce either reproducible results, rapid germination, or disease expression. In addition, initial results testing plant pathogenic *Pseudomonas* species, including *P. syringae* and *P. tabaci,* on seedling material gave unreliable symptom production. Moreover, when Robertson and Brokenshire (1992) used seedlings to test for broccoli susceptibility to the pathogen *Peronospora parasitica*, which causes downy mildew, the results did not correlate with the field disease susceptibility ratings of mature heads, indicating that developing a seedling protocol for head rot may be problematic.

However, Simpson (1993) had some success when inoculating broccoli seedlings of the cultivars Shogun and Skiff, with *Erwinia carotovora* ssp. *atroseptica* isolates P1065 and P5067, but more comprehensive testing is certainly required. If a seedling test can be successfully developed for head rot, it would have many advantages over the excised head pathogenicity test in that: (1) plant material can be produced quickly; (2) it requires very little incubation space and most importantly, (3) the seedling tissue can be produced axenically, eliminating doubts over contamination.

2.1.4 Aims

Two laboratory-based pathogenicity tests were evaluated in this study: The excised head pathogenicity test of Campbell *et al.* (1995) and the seedling test of Daniels *et al.* (1984) (adapted for broccoli/*Pseudomonas*). The aims were twofold. Firstly, to determine whether the disease susceptibilities of broccoli cultivars could successfully be distinguished by the two test methods and accurately shown to reflect those of field data (Robertson *et al.*, 1993) and secondly, to determine whether the seedling test could accurately identify pathogenic strains, as shown previously for the excised head test (Harling *et al.* 1994).

In addition, the inoculation protocol of both tests was manipulated to identify the most successful procedure for disease induction.

Initially, however, experiments were conducted to quantify the number of residual bacteria present on glasshouse grown heads, to determine how many are present during the excised head pathogenicity test, and to establish the effect bacterial inoculum concentration has on symptom development in the excised head test, to determine the threshold level of bacteria required for disease development.

2.2 Materials and methods

2.2.1 Quantification of the number of residual bacteria present on glasshouse grown broccoli heads

Residual bacteria present on the excised heads to be used in the laboratory-based pathogenicity test were quantified from the broccoli cultivars Premium Crop and Dixie, which are susceptible and resistant to disease respectively (Robertson *et al.*, 1993). Five heads were used per cultivar. Plants were grown to maturity in 7'' pots (approximately 12-14 weeks), under glasshouse conditions (25°C, with a 16h photoperiod provided by 400W overhead Truelite lamps (Osram)), then the heads were removed and measured. One gram of bud tissue (flowering apparatus and supporting stem) was removed with a sterile scalpel immediately below the sepals and added to 9ml of $^1/_4$ strength sterile Ringer's solution (Oxoid) in a Seward's Stomacher bag (Fisons Scientific). This was sonicated at 40kHz in a water bath (Decon, Fisons Scientific), for 5 minutes, to displace the bacteria and then the suspension was serially diluted in $^1/_4$ strength Ringer's solution. A 100µl aliquot of each dilution was plated on to King's B agar (KBA) plates (King *et al.*, 1954) and Crystal Violet Pectate (CVP) agar (Cuppels and Kelman, 1974), to allow the total number of fluorescent and pectolytic colonies, respectively, to be recorded. KBA enhances fluorescein (a fluorescent pigment) production, by providing protease peptone and cations such as magnesium, which causes the colonies of producers to fluoresce under UV light, whilst CVP agar provides a rich pectate source which can be degraded by pectolytic enzymes resulting in pitting of the agar. The plates were then incubated for 48h, at 25°C (±2°C).

2.2.2 Effect of the bacterial inoculum concentration on disease symptom development in the excised head pathogenicity test

To establish the sensitivity of the *in vitro* excised head pathogenicity test (Campbell *et al.*, 1995) to pathogenic bacteria (i.e. the minimum inoculum concentration required to induce disease symptoms), four inoculum concentrations (1 x 10^2, 10^4, 10^6 and 10^8 cfu/ml) were tested on the susceptible cultivar Skiff (the standard inoculum concentration is 2ml of a 1 x 10^8 cfu/ml suspension (Campbell *et al.*, 1995)). Pathogenic isolates *Erwinia carotovora* ssp. *atroseptica* P5067 and *Pseudomonas fluorescens* P5038 were used.

Glasshouse grown heads were excised at maturity and inoculated by covering the buds with two, $2cm^2$, pieces of cotton lint (Boots Ltd) (soaked in a suspension of bacteria) which were placed on top of each other. The suspension was prepared by diluting a loopful of bacteria, from a 24h KBA culture, in 50ml of sterile distilled water. This was then quantified on a spectrophotometer (an OD_{500nm} value of 0.1 represents 1 x 10^8 cfu/ml (Chapter 3, Figure 3.2), and then diluted appropriately to obtain the four test concentrations. The inoculated heads were incubated in sealed 'Magenta GA7' vessels (Sigma chemicals), containing 20ml of sterile water to maintain humidity. The broccoli heads were kept upright by sponge collars (Plate 2.1). The pots were incubated in a Fison's cabinet at a split temperature require (±2°C) of 20°C (day)/ 10°C (night), for 5 days, with a 16 hour photoperiod. Four replicates were used for each treatment.

Plate 2.1: The excised broccoli head pathogenicity test, showing the inoculated head situated in a Magenta Vessel (Sigma), supported by a sponge collar

A five point scoring scale was used to assess the severity of disease symptoms: 0 = no symptoms, 1 = watersoaking (loss of waxy bloom from florets), 2 = watersoaking and tissue browning but no rotting, 3 = tissue browning and softening, 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arc sine (Schefler, 1969); thus categories 0 - 4 became 0% (0), 33% (1), 50% (2), 67% (3) and 100% (4) respectively. Results were analysed in a single factor ANOVA.

2.2.3 Differentiating between cultivar susceptibility to disease with the excised head pathogenicity test

To determine whether the laboratory-based excised head pathogenicity test could reliably distinguish susceptibility amongst broccoli cultivars, in accordance with those found in the field (i.e. Robertson *et al.*, 1993), ten cultivars were inoculated

with pathogenic *Erwinia carotovora* ssp. *atroseptica* isolate P5067. The protocol was as described in Section 2.2.2, using an inoculum concentration of 1 x 10^4 cfu/ml to allow for the greatest possible differentiation in cultivar disease susceptibility (whilst this concentration is considerably lower than the 1 x 10^8 cfu/ml concentration usually employed in this test (Section 2.2.4), it was found to still induce disease development, (Section 2.3.2)). Fifteen replicates were used for each cultivar.

The susceptibility level of eight of the broccoli cultivars had been previously determined in field trials (Robertson *et al.*, 1993): Skiff, Premium Crop and Corvet (susceptible) and Shogun, Samurai, Marathon, Greenbelt and Dixie (resistant). It should be noted that cultivars described as resistant in the field still showed limited disease development, but considerably less than susceptible cultivars. The cultivars, Trixie and Headline, had not been previously tested. Results were recorded against the five point scoring scale described in Section 2.2.2.

2.2.4 Effect of inoculation method on disease symptom development in the excised head pathogenicity test

The inoculation method used in the excised head pathogenicity test was modified to determine how this would affect disease symptom development. Pathogenic *Erwinia carotovora* ssp. *atroseptica* isolate P5067 was inoculated at a concentration of 1 x 10^8 cfu/ml on susceptible broccoli cultivar Skiff (as described in Section 2.2.2, with the exception of the inoculation method). Four replicates were used for each method. The following inoculation methods were tested: (1) 200µl (2.0 x 10^7 cfu) of bacterial suspension was pipetted directly on to the buds, then the inoculated area was covered with sterile water-soaked lint. This was to determine whether bacteria

would colonise more successfully if they were directly inoculated on to the plant tissue rather than on to lint. (2) 200µl (2.0×10^7 cfu) of bacterial suspension was pipetted on to lint which had been soaked in sterile water. The lint was then placed on top of the buds. This method mimics the standard inoculation protocol of Campbell *et al.* (1995), except that only $^1/_{10}$th of the volume of suspension was applied (2.0×10^7 cfu, rather than the 2×10^8 cfu/ml suspension). This will therefore provide a comparison based on the inoculum concentration. (3) 200µl (2.0×10^7 cfu) of suspension was pipetted on to dry lint which was then placed over the buds. The dry lint would determine whether water in the suspension alone could sustain bacterial pathogenesis. (4) One individual bud was stab inoculated with a sterile needle, which had been dipped into a bacterial colony on a 24h KBA culture. The bud was then covered with water-soaked lint. This would determine how readily the infection progressed to other buds on the head following wounding. Finally, (5) a constant supply of bacterial suspension was maintained around several buds by cutting the narrow end off of a 1ml pipette tip and using it as a collar around several buds. The bottom of the tip was then sealed with re-useable adhesive gum (Blu-Tack, Bostik), and 0.5ml of a 1×10^8 cfu/ml suspension (5×10^7 cfu) was added to the inside of the tip, surrounding the buds. This was to determine whether constant moisture would lead to greater disease development. All five procedures were compared with the standard inoculum method described in Section 2.2.2 (2×10^8 cfu).

2.2.5 Use of broccoli seedlings to determine bacterial pathogenicity and cultivar susceptibility to disease

The following method was adapted from Daniels *et al.* (1984) to establish if the head rot pathogens could successfully induce disease in seedlings, rather than heads, and whether broccoli cultivar susceptibility to disease could be determined reliably. Initially, broccoli seeds were surface sterilised in a 1% available chlorine solution for 30 minutes, rinsed in distilled H_2O three times, then placed on to 1% Davis water agar (Oxoid) plates to germinate. After 1-2 days, the seedlings were transferred to individual replidish (Sterilin) wells containing 2.5ml plant tissue culture agar (Murashige and Skoog, 1962) and incubated at 20°C (±2°C), 16h photoperiod, until they reached a height of 3-5cm (ca. 4 days). Seedling hypocotyls were then stab-inoculated using a sterile needle which had been dipped into a 24h KBA bacterial colony. The replidishes were placed in sealed plant propagators containing 40ml of sterile water to maintain humidity. Potential micro-organism contamination was minimised by wiping the propagators with 90% ethanol prior to use. In total, six bacterial strains were used for the inoculations, as shown in Table 2.1. Of the six cultivars tested, three were previously determined as susceptible (Skiff, Corvet, Premium Crop) and three as resistant (Shogun, Samurai and Marathon) in field trials (Robertson *et al.*, 1993). Twenty-five replicate seedlings were inoculated for each isolate/cultivar combination. The control involved dipping the needle into KBA only. Inoculated seedlings were incubated for five days under the same conditions employed prior to inoculation.

A four point scoring scale was used to assess the results as follows: 0 = healthy, 1 = brown discolouration at inoculation point, 2 = brown discolouration with some

limited tissue softening, 3 = spreading soft rot. Data were transformed using arc sine (Schefler, 1969), giving transformed values of 0% (0), 33.3% (1), 66.7% (2) and 100% (3).

Table 2.1: Bacterial strains used in the seedling pathogenicity test

Isolate number	Species	Pathogenicity rating[a]
P5067	*Erwinia carotovora* ssp. *atroseptica*	Pathogenic
P1065	*Erwinia carotovora* ssp. *carotovora*	Pathogenic
P5038	*Pseudomonas fluorescens* (Group IVb)	Pathogenic
P5049	*Pseudomonas fluorescens* (Group IVb)	Pathogenic
N5024	*Pseudomonas fluorescens* (Group Vb)	Non-pathogenic
N5027	*Pseudomonas fluorescens* (Group Vb)	Non-pathogenic

[a]Pathogenicity ratings taken from Robertson *et al.*, 1993.

2.2.6 Effect of modifying the seedling pathogenicity test's inoculation protocol on disease development

To determine whether the seedling pathogenicity test could be improved, various inoculation alterations were applied to the inoculation protocol described in Section 2.2.5 (procedure 5 in this experiment). The alterations were assessed on susceptible cultivar Skiff with pathogenic isolates *Pseudomonas fluorescens* P5049 and *Erwinia carotovora* ssp. *atroseptica*, P5067. The following modifications were tested: (1) a split temperature of 20°C (±2°C) (day)/10°C (±2°C) (night) with the hypocotyl stab protocol described previously, as this had improved results in the mature head test (Campbell *et al.*, 1995); (2) the cotyledon, rather than the hypocotyl, was stab inoculated; (3) the cotyledons were smeared lightly over their upper and lower surface with bacteria from one colony (1mm in diameter, cfu/ml not established) taken from a 24h KBA plate, and (4) the hypocotyls were smeared with bacteria (as described for procedure 3). The alterations were all compared to the hypocotyl stab method carried out at 20°C (Section 2.2.5/procedure 5). Four replicates were carried out for each procedure and data were transformed with arc sine (Schefler, 1969).

2.3 Results

2.3.1 Quantification of the number of residual bacteria present on glasshouse grown broccoli heads

Table 2.2 demonstrates that whilst the number of residual bacteria isolated was relatively low, Premium Crop contained a slightly higher colony forming number than Dixie, but not at a statistically significant level. The average size of the heads was fairly small, at 4.2cm, with those of Premium Crop being significantly smaller than those of Dixie. Therefore this may indicate that the heads of Premium Crop were not as mature as those of Dixie, which may have affected the number of residual bacteria found, but as more bacteria were isolated from Premium Crop this does not appear to be the case. Reassuringly, no pectolytic bacteria were detected on glasshouse grown plants. Forty percent of bacteria from Premium Crop were fluorescent, whilst only 24% of the bacteria on Dixie (resistant) fluoresced.

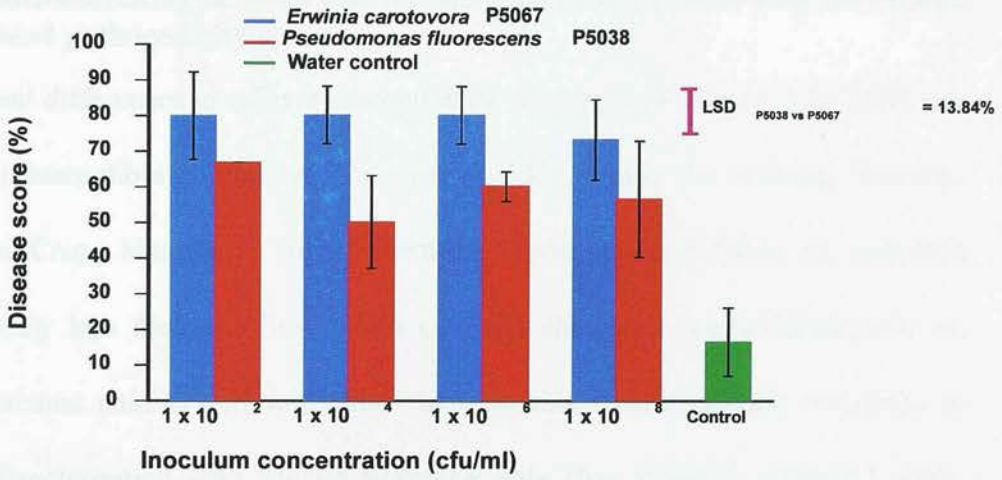
Table 2.2: Number of residual bacteria present on healthy glasshouse grown heads of broccoli cultivars Premium Crop and Dixie

Cultivar	Head Size (cm)	Mean CFU /g fw tissue	Mean fluorescent CFU	Mean pectolytic CFU
Premium Crop (susceptible)	3.5	3.58×10^{3}	1.42×10^{3}	-
Dixie (resistant)	4.9	1.32×10^{3}	3.2×10^{2}	-
Mean	4.2	2.45×10^{3}	8.7×10^{2}	-
LSD ($p < 0.05$)	0.82	NS	NS	NS

CFU/g fw tissue = Colony Forming Units / Gram of Fresh Weight tissue
NS = No significant difference

2.3.2 Effect of the bacterial inoculum concentration on disease symptom development in the excised head pathogenicity test

Figure 2.1 shows the disease scores obtained at each inoculum concentration tested.



Data transformed with arc sine (Schefler, 1969) 0 = no disease; 100% = Black soft rot
$LSD_{Erwinia\ carotovora}$ ssp. *atroseptica* inoculations = not significant
$LSD_{Pseudomonas\ fluorescens}$ inoculations = not significant
$LSD_{P\ fluorescens}$ vs *Erwinia carotovora* inoculations = 13.84% @ $p < 0.05$

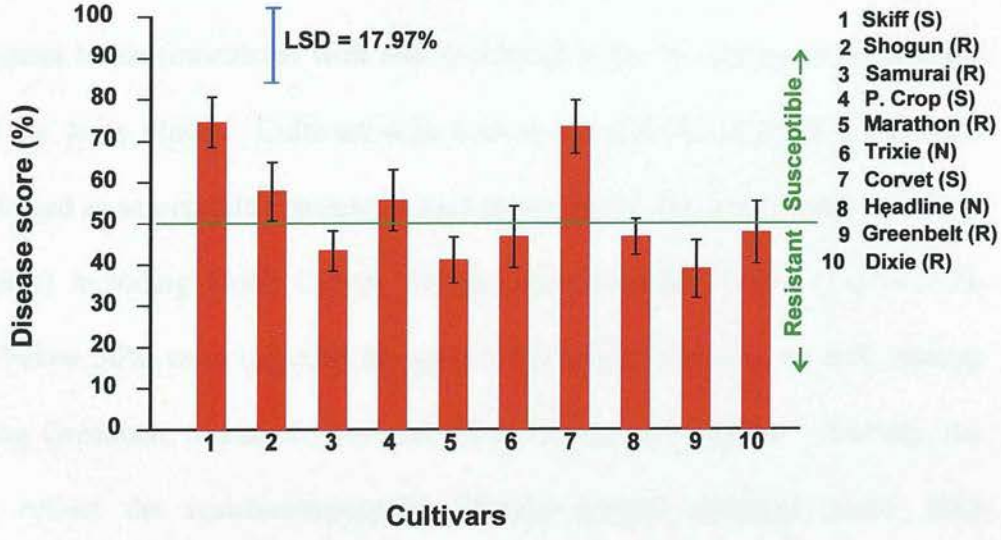
Figure 2.1 Effect of inoculum concentration on disease symptom development in the excised head pathogenicity test, carried out on cultivar Skiff (with Standard Error of the Mean (SEM) bars)

As indicated in Figure 2.1, no significant difference was found between the four inoculum concentrations tested with either bacterial strain. However, the level of disease generated by the two strains was significantly different (LSD = 13.84% @ $p<0.05$): The *Erwinia carotovora* P5067 inoculations produced soft rotting of the tissue, while *P. fluorescens* P5038 inoculations produced predominately tissue browning and softening, with only occasional rotting. Also, the disease scores produced by the *P. fluorescens* strain were also more variable between concentrations, than those of the *Erwinia* strain which produced more uniform SEM values. Reassuringly the water controls produced no disease symptoms (mean

16.65%). Therefore, the threshold level of bacteria required to induce head rot must lie below two millilitres of a 1 x 10^2 cfu/ml.

2.3.3 Differentiating between cultivar susceptibility to disease with the excised head pathogenicity test

Significant differences in cultivar susceptibility were evident (Figure 2.2): Skiff was the most susceptible cultivar with a score of 74%, whilst the cultivars Samurai, Premium Crop, Marathon, Trixie, Headline, Greenbelt and Dixie all sustained significantly less disease. Susceptible cultivars showed a severe black soft rot, while resistant cultivars showed either watersoaking (more resistant cultivars), or brown discolouration with limited softening only (less resistant cultivars) (Plate 2.2).



Data transformed with arc sine (Schefler, 1969). *LSD value = 17.97% @ p< 0.05*
S = susceptible to disease in the field (51% or more i.e. tissue softening and rotting),
R = resistant to disease in the field (50% or less i.e. no soft rotting),
N = susceptibility not established (Robertson *et al.*, 1993).

Figure 2.2: Disease development induced by *Erwinia carotovora* ssp *atroseptica* P5067 in the excised head pathogenicity test (with SEM bars)

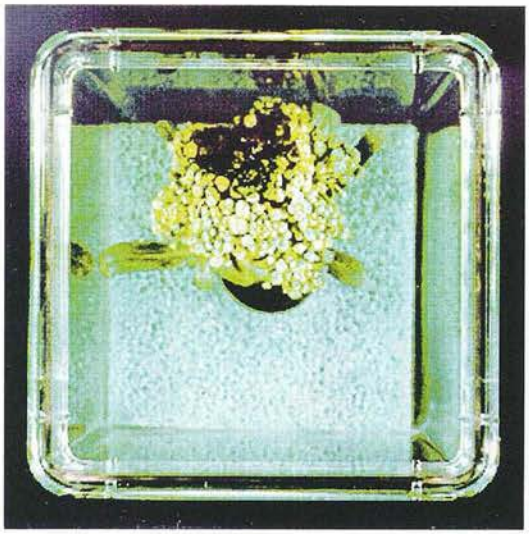
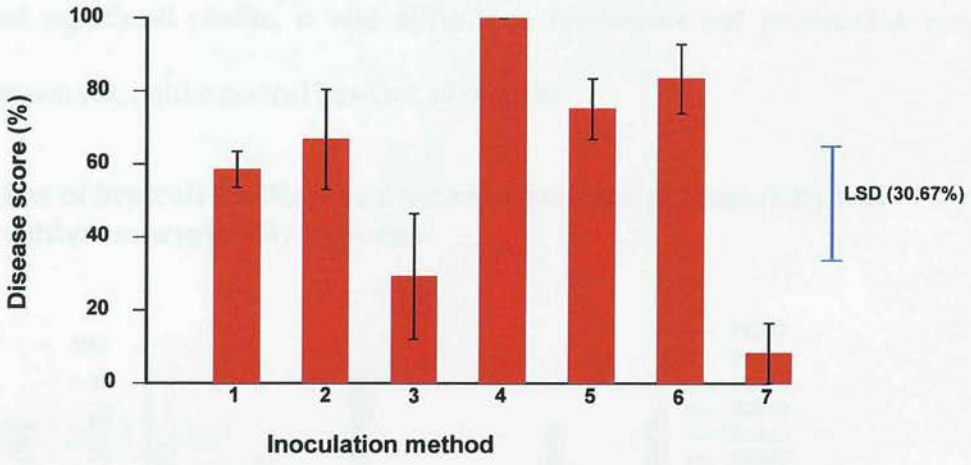


Plate 2.2: Severe black soft rot on broccoli cultivar Skiff after being subjected to the excised head pathogenicity test

The control heads (inoculated with water) showed either no symptoms, or a slight loss of the waxy bloom. Cultivars with a mean transformed score of above 50% were classed as susceptible (because disease scores above this level included soft rot symptoms) including Skiff, Corvet, Shogun and Premium Crop (Figure 2.2). Those below 50% were regarded as resistant (because there was no soft rotting) including Greenbelt, Marathon, Samurai, Trixie, Dixie and Headline. Notably, the results reflect the resistant/susceptible disease ratings observed under field conditions (Robertson *et al.*, 1993), with the exception of Shogun, which was reported as more resistant to disease in the field.

2.3.4 Effect of inoculation method on disease symptom development in the excised head pathogenicity test

The results of the six different inoculation methods, on symptom expression in the excised head test, can be seen in Figure 2.3.



1 = 2 x 10^7 cfu applied to buds directly
2 = 2 x 10^7 cfu applied to wet lint
3 = 2 x 10^7 cfu applied to dry lint
4 = bud stab inoculation
5 = collar containing inoculum
6 = 2 x 10^8 cfu applied to wet lint (standard protocol)
7 = water control (with standard protocol)

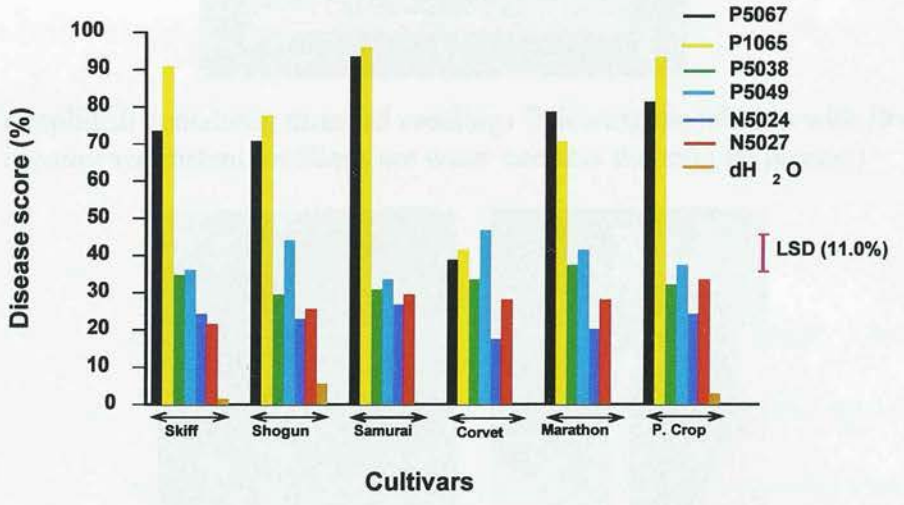
0% = No disease symptoms, 100% = Severe black rot
Data arc sine transformed (Schefler, 1969)
LSD value = 30.67%

Figure 2.3: Disease scores induced by six different inoculation methods in the excised head test, using *Erwinia carotovora* ssp. *atroseptica* P5067 on broccoli cultivar Skiff (with SEM bars)

Stab inoculating one bud (procedure 4) produced the highest disease score, with all replicates producing full soft rot. The standard protocol (procedure 6) of soaking the lint in 2ml of a 1 x 10^8 cfu/ml suspension produced the second highest score, followed by procedure 5 (supplying constant inoculum to the buds). There was no statistically significant difference between these three methods. However, adding 2 x 10^7 cfu to the wet lint (procedure 2); pipetting directly on to the buds (procedure 1), and pipetting the suspension on to dry lint (procedure 3) gave statistically less

disease than the most successful method (stab inoculation). All six methods were significantly different to the water control. As expected, applying bacterial inoculum on to dry lint produced the least disease symptoms of all, and the greatest variability. Although maintaining a constant supply of inoculum around the buds produced significant results, it was difficult to implement and produced a very runny brown rot, unlike normal head rot symptoms.

2.3.5 Use of broccoli seedlings to determine bacterial pathogenicity and cultivar susceptibility to disease



Data transformed with arc sine (Schefler, 1969) 0 = no disease; 100% = spreading soft rot
LSD = 11.0% ($p < 0.05$)

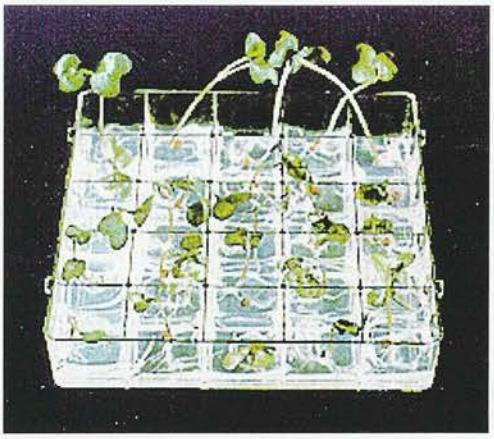
Bacterial isolates

Pathogenic	Non-pathogenic
P5067 - *Erwinia carotovora* ssp. *atroseptica*	N5024 - *Pseudomonas fluorescens* Group Vb
P1065 - *Erwinia carotovora* ssp. *carotovora*	N5027 - *Pseudomonas fluorescens* Group Vb
P5038 - *Pseudomonas fluorescens* Group IVb	
P5049 - *Pseudomonas fluorescens* Group IVb	

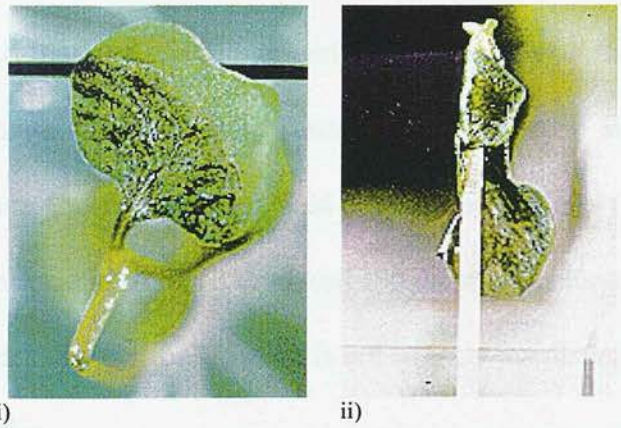
Broccoli cultivars (susceptibilities are according to Robertson *et al.*, 1993)
Skiff (susceptible); Corvet (susceptible); Premium Crop (susceptible);
Shogun (resistant); Samurai (resistant); Marathon (resistant)

Figure 2.4: Results of the seedling pathogenicity test showing the disease levels produced by six bacterial isolates on six broccoli cultivars

Figure 2.4 highlights that significantly different disease levels were found among the six bacterial strains. Only the *Erwinia* species caused a soft rot of the hypocotyl and cotyledons, in all cultivars except Corvet (Plate 2.3).



a) a replidish containing diseased seedlings following inoculation with *Erwinia carotovora* (distant seedlings are water controls showing no disease)



i) ii)

b) a close up of two seedlings, showing i) the soft rot spreading over the cotyledon surface, and ii) the collapse of the cotyledons

Plate 2.3: Disease symptoms produced by *Erwinia carotovora* isolate inoculation of broccoli seedling material a) seedlings situated in a replidish after inoculation, showing complete tissue collapse (distant seedlings are water controls showing no disease); b) two seedlings showing the spread of disease symptoms over the cotyledon surface

Erwinia carotovora ssp. *atroseptica* P1065 was the most pathogenic isolate with a disease score of 82%. The *Pseudomonas* species, capable of producing disease on excised heads, could not rot seedling tissue of any cultivars, causing localised browning only. Symptoms did not spread to the cotyledons. The mean Pseudomonad disease score was 36%. Non pathogenic strains also caused occasional tissue browning (25%) as did the control (water) inoculations (1.6%), however, all six isolates produced significantly higher scores than the mean water control value.

The cultivars were all found to differ significantly in their susceptibility to disease. Cultivars Premium Crop and Samurai were classed as susceptible, with disease levels of 50.2% and 51.5% respectively (mean score for all isolates inoculated), but the remaining cultivars were classed as resistant. Corvet was the most resistant cultivar while Samurai was the most susceptible. However, whilst Marathon, Shogun and Premium Crop reflect their field susceptibility ratings (Robertson *et al.* 1993), Corvet, Samurai and Skiff all produced scores which contradicted their field ratings. This indicates that the seedling test is not suitable for distinguishing cultivar disease susceptibilities.

Interestingly, all cultivars were most susceptible to *Erwinia* P1065, except Corvet which showed greater disease scores following inoculation with the *Pseudomonas fluorescens* isolate P5038. In addition, although Samurai had the highest disease score after inoculation with the *Erwinia* spp., it had the lowest disease score following inoculation with the pathogenic *Pseudomonas* species i.e. the results were

extremely variable. Therefore the disease symptoms and variation induced appears to be specific to the particular cultivar/pathogenic isolate combination, rather than any one cultivar or bacterial isolate.

Table 2.3 shows the Standard Error of the Mean (SEM) values obtained for each bacterial/cultivar interaction tested.

Table 2.3 : Standard error of the mean values obtained from the seedling pathogenicity test results presented in Figure 2.4

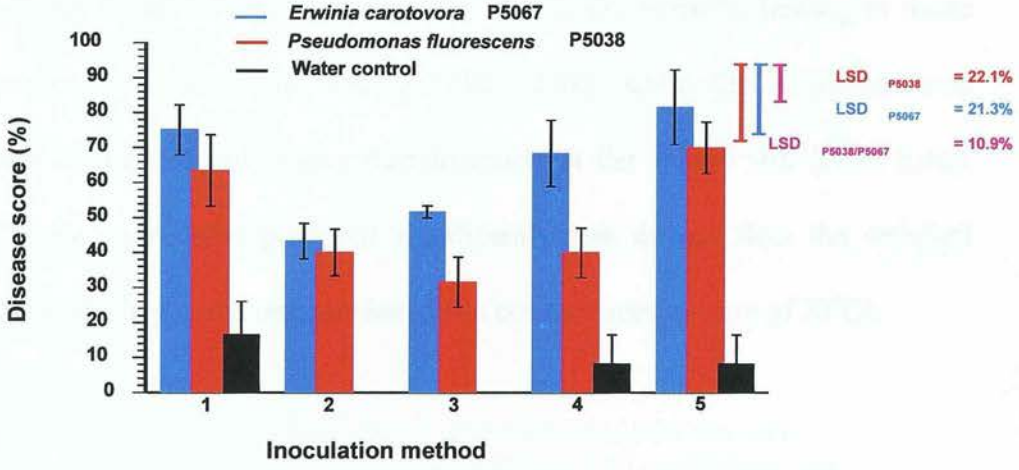
	Cultivar					
Isolate	**Skiff**	**Shogun**	**Samurai**	**Corvet**	**Marathon**	**P.Crop**
P5067	11.54	9.68	6.67	**11.98**[t]	10.8	10.94
P1065	10.56	11.36[t]	5.86	11.08	**15.07**[t]	9.42
P5038	2.67	4.42	3.69	3.85	**7.02**	4.68
P5049	**12.72**[t]	9.21	9.43[t]	11.55	9.65	11.10[t]
N5024	6.10	6.34	5.44	**6.79**	6.66	6.10
N5027	**6.52**	5.81	5.86	4.98	6.298	3.85
water control	2.66	**4.98**	0.00	0.00	0.00	3.69

SEM values where $p<0.05$
Values in bold = Highest SEM value for that bacterial isolate
t = Highest SEM value obtained for that broccoli cultivar

From Table 2.3 it can be seen that variation in the disease scores obtained was considerable, particularly following the inoculation of either *Erwinia* strain, where replicates produced disease scores ranging from healthy (0%) to full rot (100%) (results not shown). However, three of the four pathogenic isolates, namely P5067, P1065 and P5049, all produced considerable variation. In addition, the most variation occurred in the field susceptible cultivars Skiff and Corvet, and the field resistant cultivars Shogun and Marathon.

2.3.6 Effect of modifying the seedling pathogenicity test's inoculation protocol on disease development

Figure 2.5 shows the disease levels induced, in the seedling tissue, following the use of five different inoculation procedures.



Data arc sine transformed (Schefler, 1969) 0% - no disease, 100% = full soft rot

1 = Hypocotyl stab (split temperature of 20/10°C)
2 = Cotyledon stab (constant 20 °C)
3 = Cotyledon smear (constant 20 °C)
4 = Hypocotyl smear (constant 20 °C)
5 = Hypocotyl stab (constant 20 °C)

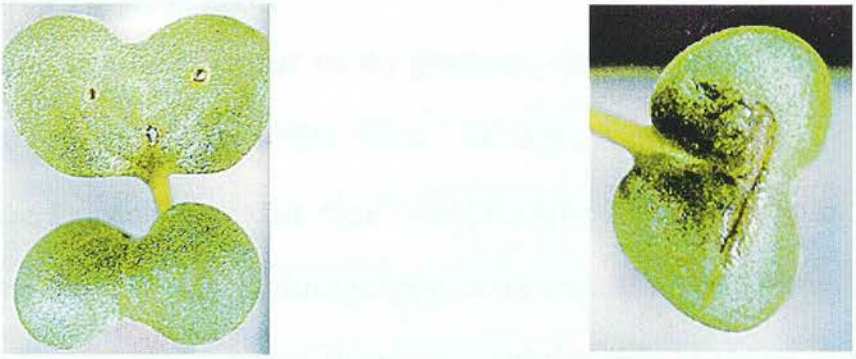
LSD for bacteria inoculated

$LSD_{Pseudomonas\ fluorescens}$	= 22.1% @ p<0.05
$LSD_{Erwinia\ carotovora}$	= 21.3% @ p<0.05
$LSD_{Erwinia\ vs\ P.\ fluorescens}$	= 10.9% @ p<0.05

Figure 2.5: Disease levels induced by five seedling inoculation procedures, carried out using *Erwinia carotovora* P5067 and *Pseudomonas fluorescens* P5038 on cultivar Skiff (with SEM bars)

The hypocotyl stab method (procedure 5), produced the most disease for both pathogenic isolates, and therefore none of the other four inoculation methods have been able to improve this pathogenicity tests results (Figure 2.5). The hypocotyl stab incubated at a split temperature of 20°C (16h)/10°C (8h) (procedure 1) produced the second highest disease score and although there was no statistically significant difference between the disease levels of these two procedures, the split

temperature regime generated symptoms more rapidly (results not shown). The hypocotyl smear method (procedure 4) produced variable disease symptoms, and significantly less disease than the hypocotyl stab method following inoculation with *Pseudomonas fluorescens* P5049, but not with *Erwinia carotovora* ssp. *atroseptica*. The cotyledon smear inoculation (procedure 3) was also variable, leading to tissue discolouration and occasional rotting (Plate 2.4a), while the cotyledon stab (procedure 2) induced only tissue discolouration at the wound site (Plate 2.4b). Both of these procedures produced significantly less disease than the standard protocol (i.e. the hypocotyl stab incubated at a constant temperature of 20°C).



(a) bacteria stabbed into cotyledon (b) bacteria smeared over the cotyledon

Plate 2.4: Disease symptoms induced after inoculation of broccoli cotyledons with *Erwinia carotovora* P5067 taken from an agar colony (a) by stab inoculating with bacteria, and (b) after smearing with bacteria over the cotyledon surface

The two bacterial isolates used for inoculation produced significantly different levels of disease, with *Erwinia carotovora* ssp. *atroseptica* being more pathogenic on seedling tissue than the *Pseudomonas fluorescens* strain (which also produced less variable results). However, the most successful inoculation methods induced the highest disease levels irrespective of which isolate was used and, conversely, the least successful methods induced the lowest disease levels.

2.4 Discussion

2.4.1 Quantification of the number of residual bacteria present on glasshouse grown broccoli heads

The number of bacteria present on the glasshouse-grown heads was approximately 2.5 x 10^3 cfu/gram fresh weight tissue. Of these, between 24% - 40% were fluorescent Pseudomonads, but none were pectolytic. This is encouraging when using these heads in head rot pathogenicity testing because residual pectolytic strains may complement non-pectolytic strains in inoculation tests to induce disease, and therefore the results generated would be inaccurate. As Robertson *et al.* (1993) isolated 10^6 pectolytic bacteria from field-grown (watersoaked) heads, this demonstrates the high numbers of pectolytic bacteria which can reside on broccoli tissue prior to the development of rotting. In addition, this emphasises the substantial reduction in bacterial number obtained here by producing heads under glass. Presumably the remainder of bacteria isolated in this study were non-fluorescent saprophytes. Unfortunately, no data were available for comparison concerning the number of bacteria isolated from healthy heads grown in the field.

The susceptible cultivar Premium Crop harboured more bacteria than resistant Dixie, but the difference was not statistically significant. Therefore bacteria are colonising both resistant and susceptible broccoli tissues equally. However, heads from Premium Crop were significantly smaller than those from Dixie, which contradicts field data which demonstrated that on average field grown Premium Crop heads were significantly larger than those of Dixie (Table 3.4, Chapter 3). Therefore, either Premium Crop heads require more time to fully mature than those of Dixie, (which

may lead to a more profound difference in bacterial number being detected between the two cultivars), or producing these cultivars under glass leads to the growth of uncharacteristic head sizes. Notably, head size is usually smaller under glass than in the field because the high temperatures induce premature bolting and so heads must be excised at the smaller size of approximately 4-6cm. Unfortunately, as the glasshouses were used for production of many different plant species, it was not possible to cool them to the optimum temperature for broccoli. However, this does not explain why Premium Crop heads were uncharacteristically smaller than those of Dixie. As the heads are routinely used in pathogenicity tests at this size, the results have successfully fulfilled our initial objective to ascertain how many residual bacteria where present on glasshouse grown heads.

Although the number of residual bacteria found was low, and none were pectolytic, the possibility of residual strains influencing pathogenicity test results, by providing complementary disease factors lacking in the bacterium inoculated, cannot be ruled out. As surface sterilising heads is not possible (because even soaking the heads in sterile distilled water leads to disease development (Robertson *et al.*, 1993)), this would only be achieved by carrying out pathogenicity testing on axenically produced broccoli heads, which would be unrealistic. However, because many bacterial strains prove to be non-pathogenic in the excised head test, and the negative (water) controls do not rot, this reduces doubt over the test's reliability.

2.4.2 Effect of the bacterial inoculum concentration on disease symptom development in the excised head pathogenicity test

The pathogenicity test was sensitive down to a bacterial inoculum concentration of 1 x 10^2 cfu/ml, with no significant difference being found between the disease level induced by all four concentrations inoculated (ranging from 1 x 10^2 to 1 x 10^8 cfu/ml). Therefore this test has been shown to be extremely sensitive to head rot pathogens, and as the negative control produced no disease symptoms, the test can be considered reliable. As the *Erwinia* strain was significantly more pathogenic to broccoli tissue than the *P. fluorescens* isolate (which induced tissue discolouration and softening predominately), this supports the results of Harling *et al.* (1994) who claimed that this test could successfully differentiate between isolate aggressiveness. Although disease was produced at all four inoculum concentrations, for both strains, a concentration of 1 x 10^8 cfu/ml will continued to be implemented during testing to ensure that disease developed with strains less pathogenic than the ones tested here.

2.4.3 Differentiating between cultivar susceptibility to disease with the excised head pathogenicity test

In addition to being extremely sensitive to testing for head rot pathogens, the excised head pathogenicity test was also shown to differentiate clearly between cultivar resistance levels following the inoculation of an *Erwinia carotovora* ssp. *atroseptica* strain. Greenbelt proved to be the most resistant cultivar, while Skiff was the most susceptible. The cultivars' susceptibility to disease was found to reflect the field data of Robertson *et al.* (1993), with the exception of Shogun. Shogun was classed as resistant in the field by Robertson *et al.* (1993), but was found to be susceptible in this laboratory test, with a disease score of 58%. Interestingly, growers are now

reporting that Shogun is becoming increasingly susceptible to disease (Harling, per. communication) so it may be that the field data for this cultivar are no longer accurate. Alternatively, the increased sensitivity of Shogun may arise from the test plants' growth environment. All material was produced under glass which leads to the cultivars' physical characteristics being slightly different from those produced in the field. For example, heads tend to be smaller, paler in colour and bolt prematurely due to the high temperatures. Such factors may well lead to a change in the plants' ability to protect itself from pathogens because as Goodman *et al.* (1986) stated, the plant's growth environment and the nutrients available to it, affect the outcome of the plant-pathogen interaction. If this had occurred in the case of Shogun, all cultivars would have been expected to be affected, but possibly certain cultivars are more influenced by their environment than others. Another possibility is that Shoguns' susceptibility may simply be an artefact of this *in vitro* pathogenicity test, demonstrating that it is not capable of accurately determining cultivar susceptibility, but as the other seven cultivars reflected their field susceptibility levels, this seems more implausible. Further field work is required to establish Shogun's current susceptibility.

Headline and Trixie were not tested by Robertson *et al.* (1993) so it is not known whether their disease susceptibilities reflect field observations. It would now be beneficial to test further broccoli cultivars both in the field, and with this excised head test, to ascertain whether compatible results are obtained.

This test is straightforward to execute, residual bacteria can be minimised by producing heads under glass, and it is sensitive to low inoculum concentrations. In addition, both isolate pathogenicity (Harling *et al.*, 1994) and cultivar susceptibility (in the majority of cultivars) can be differentiated successfully. Therefore it is proposed that this pathogenicity test is a suitable model for head rot research and, after further research, it may even have the potential to be used as a screen for cultivar resistance in breeding programmes.

2.4.4 Effect of inoculation method on disease symptom development in the excised head pathogenicity test

Five inoculation methods were compared with the standard protocol described in the previous section.

Full soft rot developed in all replicates after stab inoculating one bud with a needle which had been dipped into a bacterial colony (procedure 4). Although this technique would assure the most reliable results, it introduces tissue wounding which by-passes the initial stage of pathogen entry into the host. As some bacteria lack the genes necessary to successfully enter plant tissue, and are therefore only pathogenic on wounded tissue, this method may lead to bacteria being wrongly categorised. However, it would remain a useful method for studying bacterial pathogenesis in strains which are already known to be pathogenic. The standard inoculation protocol of Campbell *et al.* (1995 - also described in Section 2.4.3) (procedure 6), which involved soaking the lint in 2×10^8 cfu of bacterial suspension, induced the second highest level of disease. The same protocol tested with only 2×10^7 cfu of bacteria (procedure 2), produced slightly lower levels of disease than procedure 6 but not at a

significant level (although the level of disease generated in procedure 2 was significantly lower than the most successful stab inoculation method (procedure 4)). This supports the results found in Section 2.3.3 which demonstrated that the excised head test was sensitive to both low and high pathogen concentrations. However, as the results produced here with a lower bacterial concentration were also more variable (Figure 2.3), it demonstrates the improved reliability achieved through testing with a high bacterial concentration. Maintaining a constant supply of inoculum around the buds (procedure 5) also produced a high level of disease but was extremely difficult to implement. Three inoculation methods, namely inoculating 2×10^7 cfu bacteria onto, wet lint (procedure 2); directly on to the buds (procedure 1), or onto dry lint (procedure 3), produced significantly less disease than the bud stab method. However, inoculating wet lint, or the buds directly, still induced disease scores of over 50%, which represents successful disease initiation, albeit at a low level. Inoculating on to dry lint did not induce disease (so the water present in the inoculation suspension alone cannot sustain pathogenesis) and therefore this method has failed to model the disease process and would be unsuitable for head rot research. Therefore the quantity of water appears to be more influential than the inoculation concentration in determining pathogenesis, although constant moisture does not enhance results compared to the standard inoculation procedure (procedure 6). Tissue wounding is the most productive inoculation method in that successful disease development is assured.

Since this research was carried out, Gladstone (1997) has found that the standard method of soaking the lint in a 1×10^8 cfu/ml suspension can be enhanced further by

using smaller pieces of lint. Circles of 1cm in diameter were used and abundant uniform rotting was obtained. It is possible that when larger pieces of lint are used, the bacteria in the centre of the lint are deprived of oxygen, or become subjected to unfavourably high temperatures, and fail to multiply. Alternatively, the smaller sized lint may adhere more closely to the buds. The quality of lint used has also been observed to improve results (pers. observation), with lint containing a high percentage of cotton (creating a more 'fluffy' texture) producing the most successful and reliable results, presumably due to it retaining moisture more effectively.

Therefore, to summarise, soaking the lint in at least 2ml of a 1 x 10^8 cfu/ml bacterial suspension and using 1cm^2 (preferably circular) pieces of cotton-rich lint appears to be the most suitable inoculation method. In addition, incubating excised heads in plastic pots to maintain humidity, at a day/night temperature of 20°C/10°C with a 16h photoperiod, ensures results will be accurate and sensitive.

2.4.5 Use of broccoli seedlings to determine bacterial pathogenicity and cultivar susceptibility to disease

The seedling pathogenicity test failed to identify all pathogenic isolates - only the aggressive *Erwinia* strains successfully rotted the seedling tissue, although the results were variable between replicates. The pathogenic Pseudomonads did not produce a disease score above 50%, rendering them being erroneously classed as non-pathogenic which they are clearly not on broccoli heads. As the *Erwinia* strains were shown to be more aggressive than the Pseudomonads, this reflects the trend found on mature broccoli heads (Section 2.2.2), but unlike the seedling test the Pseudomonads can still induce full disease symptoms on the heads. Daniels *et al.*, (1984) found stab

inoculating *Pseudomonas* spp. on to seedlings produced unreliable symptoms. Therefore, the expression of host resistance and/or pathogen virulence is clearly different for young vs mature host tissue in the case of *Pseudomonas*. The combination of structural and biochemical defence products produced by a particular host can vary depending on factors such as age of the plant and the particular tissue attacked (Agrios, 1988) and therefore this may explain the low levels of disease induced here. Inoculation of older seedlings may prove more reliable. For example, the high concentrations of phenolic compounds and tannins present in the cells of young fruits have been held responsible for their resistance to *Botrytis*, with maturing tissue becoming steadily less resistant to disease (Agrios, 1988). Indeed, there are many factors which may be responsible for the poor disease development observed, including the nutritional condition of the seedlings and the testing environment, so perhaps another nutrient source, and a change in incubation temperature or humidity, would be more conducive to disease development.

The non-pathogenic isolates produced variable results with a mean disease score of 25% (with occasional tissue browning and softening being observed). In addition, three cultivars (Skiff, Shogun, and Premium Crop) contained replicates which showed tissue browning symptoms following control inoculation, which should not have occurred because the tissue was produced axenically and so no bacteria should have been present to stimulate a host response reaction. Possibly contamination occurred during inoculation with bacteria entering, either from the needle, or the surrounding environment. Alternatively, the plant tissue may have come into contact with and reacted to, the nutrient agar used to stab the needle into before inoculation,

or indeed, the inoculation needle may have induced a response (if so this may have influenced all of the results).

In addition to the poor disease expression induced by the pathogenic isolates, only three out of the six cultivars showed the same disease susceptibility as they did in the field for mature tissue (Robertson *et al.*, (1993). For example, Samurai was erroneously found to be susceptible and Corvet resistant to the pathogens tested. Possibly Samurai develops more resistance as it matures than other cultivars, thereby improving its resistance to disease. In the case of Corvet, head morphology may be an influential factor in this cultivars predisposition to disease. Corvet has a fairly flat head shape which may enable water droplets to collect, and bacteria to multiply (Chapter 3, Section 3.3.5), but as this morphology is absent from seedling testing it may have influenced the results.

Disease symptoms between sub-samples were variable even with the most pathogenic isolates, with the replicates of one isolate producing disease scores which ranged from full rot to no symptoms. Variability may result from the inoculum concentration being imprecise, as the inoculation needle may have picked up different amounts of bacteria each time, and this was not quantified. Possibly the threshold of bacteria required to successfully rot seedling tissue was not always met. Daniels *et al.* (1984) stated that 10^2-10^3 cfu/seedling are required to induce disease on turnip seedlings when inoculating with *Xanthomonas campestris* pv *campestris*. However, they found that 5×10^6 cfu were introduced by the needle which is considerably greater than the number of bacteria required to initiate disease. Therefore, even though the

threshold concentration required by head rot pathogens will differ from the system studied by Daniels *et al.* (1984), it would be expected that sufficient bacteria were inoculated into the seedlings. If not, it may not be worthwhile employing a test method which requires such a high bacterial inoculum concentration to induce disease, especially when the excised head test only requires 2ml of a 1 x 10^2 cfu/ml suspension to successfully mimic the disease process (as shown in Section 2.3.2).

Furthermore, the bacteria do not need to overcome the host tissue's defences in this seedling test because wounding occurs, in contrast with the excised head test. This factor can be assumed to lower the threshold concentration of bacteria required to initiate disease. However, from the low level of disease obtained this was apparently not so, which is particularly surprising.

Possibly seedling tissue is just not suitable for use as a head rot pathogenicity test. Daniels *et al.* (1984) noted that no other *Brassica* species gave such reproducible, rapid germination, growth and disease expression as the turnip cultivar 'Just Right', and Robertson and Brokenshire (1992) also failed to develop a *Brassica* seedling method to successfully model downy mildew. Yessad *et al.* (1992) found testing strains of *P. syringae* pv. *syringae* on microcuttings of pear produced a more susceptible response than using seedlings, which they proposed was due to the continuous growth affecting sensitivity. Microcuttings may therefore be worthwhile pursuing for head rot research.

Therefore to summarise, the seedling pathogenicity test is unsuitable for distinguishing cultivar susceptibility, and fails to detect some pathogenic isolates in its

present form. Although this test has the advantages that material can be produced quickly, it requires little space and seeds can be surface sterilised before treatment, it would require considerable research to ensure reliability. However, it may prove useful for studying host-pathogen interactions at the cellular level with virulent pathogens.

2.4.6 Effect of modifying the seedling pathogenicity test's inoculation protocol on disease development

Applying alternative inoculation methods to the seedling pathogenicity test failed to improve the disease levels obtained. Both cotyledon inoculations (procedures 2 and 3) produced extremely poor results, after both stab and smear inoculations, with only tissue discolouration developing. The hypocotyl stab inoculations (procedures 1 and 5) induced the most disease, whilst smearing the inoculum onto the hypocotyl surface (procedure 4) led to variable results with a significantly lower disease score than that produced by the hypocotyl stab protocol after inoculation with the *Pseudomonas* strain, but not the *Erwinia* strain. Incubating the stab inoculated hypocotyls at 20°C/10°C led to the faster development of symptoms. Therefore, although smear inoculating bacteria on to either the cotyledons or hypocotyls of seedlings (procedures 3 and 4) prevents any wounding of the tissue, it unfortunately was found to be at the expense of sensitivity, as both these methods produced significantly less disease than the hypocotyl stab method. Therefore these results support those of the seedling pathogenicity trial, in that it is the tissue type rather than the experimental protocol which is responsible for the low disease levels induced.

2.4.7 Conclusions drawn from the work in this chapter

The excised head pathogenicity test successfully allows bacterial isolate pathogenicity and cultivar susceptibility to be assessed reliably in a five day period. The successful method may be enhanced even further by reducing the size of lint used to inoculate the bacterial suspension and by selecting the lint used carefully. This test therefore has potential for use as a head rot research tool and may even have wider implications for testing pathogenicity in breeding programmes, to select more resistant cultivars.

On the other hand, the seedling pathogenicity test failed to distinguish accurately both the pathogenicity of *Pseudomonas* spp. (which is expressed on mature heads), and the relative susceptibility of cultivars, most probably because of the young age of the host material being incompatible with the majority of pathogenic strains. However, due to the advantages a successful seedling pathogenicity test would offer, in terms of the reduction of space and materials, it is worthwhile investigating the feasibility of improving this methodology for head rot research. Possibly older seedlings may provide more accurate and reliable results, or the use of microcuttings may improve symptom development. Alternatively testing broccoli leaf material, or even another host plant altogether (as long as the bacteria are found to be pathogenic, and non-pathogenic, on both tissue types) may be the most successful route to take.

Neither of the pathogenicity tests assessed allows for the effect factors such as head morphology or environmental changes may have on disease development, but no one test can fully duplicate the disease process as it occurs in the field. As Daniels *et al.* (1984) stated, when testing for pathogenicity mutants, it is important to use several

different techniques to ensure that the full range of mutant types are detected. This means that ideally a further pathogenicity test should be developed to complement the results of the excised head test. However, until this is developed the excised head pathogenicity test should provide a very useful head rot research tool.

Chapter 3: Factors influencing bacterial pathogenicity and cultivar susceptibility to head rot disease

3.1 Introduction

Literature dealing with bacterial pathogenicity and cultivar resistance in general was reviewed in Chapter 1; aspects relating to head rot will be summarised here.

Field trials have indicated that broccoli cultivars vary in their susceptibility to head rot, although none are completely resistant. Shogun, Green Defender and Samurai are regarded as among the most resistant cultivars, while Skiff and Premium Crop are held particularly susceptible to disease (Canaday *et al.*, 1991; Robertson *et al.*, 1993). In addition, the fluorescent *Pseudomonas* spp. and *Erwinia* spp. responsible for inducing disease vary in their virulence towards broccoli (Hildebrand, 1986; 1989; Wimalajeewa *et al.*, 1987; Robertson *et al.*, 1993). However, little is known about the factors responsible for controlling susceptibility to head rot, or bacterial pathogenicity, and therefore these issues will be addressed in this chapter.

3.1.1 Head rot disease incidence

It is known that persistent wet weather, coinciding with head maturity, increases disease incidence and severity (Canaday *et al.*, 1987), and Ludy *et al.* (1997) demonstrated the importance of free water in disease development when they reduced the frequency of overhead irrigation and obtained a decrease in the incidence of head rot. However, while some researchers claim that cool temperatures are optimal for disease development (Robertson *et al.*, 1993; Wimalajeewa *et al.*, 1987), others have reported that warm temperatures are more conducive (Canaday *et al.*, 1991).

In addition, Canaday *et al.* (1991) noted that the fastest maturing cultivars were the most susceptible to disease, and that larger heads were significantly more prone to infection, but the reason for this susceptibility is unknown. Applying nitrogen to the crop was found to increase the level of disease (Robertson *et al.*, 1993; Canaday and Wyatt, 1992), and to reduce the length of time required for head maturation (Canaday and Wyatt, 1992).

Our understanding of bacterial growth during infection on broccoli is limited. Robertson and Brokenshire (1992) found approximately 1.5 x 10^8 pectolytic, and 3.6 x 10^7 fluorescent, *Pseudomonas* bacteria on heads showing soft rot symptoms. However, there are no reports on whether pathogenic bacteria multiply more successfully *in planta* than non-pathogenic ones, or indeed, if cultivar susceptibility influences bacterial multiplication. Both of these factors may influence the development of disease.

Presently there are no completely effective head rot control methods available. Spraying copper compounds, and the use of polythene mulches, achieved approximately 50% success (Robertson *et al.*, 1993; Canaday *et al.*, 1987), whilst bacterial antagonists, a water surfactant (Canaday *et al.*, 1987), and even the antibiotics, Kasugamycin and Kasumin (Campbell *et al.*, 1995), all produced unsatisfactory results. Decreasing the nitrogen input (Canaday and Wyatt, 1992) and minimising overhead irrigation (Ludy *et al.*, 1989) may also help to reduce the disease incidence by producing less favourable conditions for pathogen spread and infection, but such conditions cannot guarantee a reduction in disease.

Fortunately there are several highly resistant cultivars, such as Shogun and Samurai, which also possess good agronomic characters (i.e. large firm heads, tightly packed buds, a deep green colour, etc. (Anon, 1984)) and therefore are popular commercially (Robertson *et al.*, 1993), but this limits consumer choice. However, once the factors governing disease resistance are fully established, cultivars could be bred to possess highly marketable traits coupled with strong disease resistance.

3.1.2 Factors influencing broccoli cultivar resistance to disease

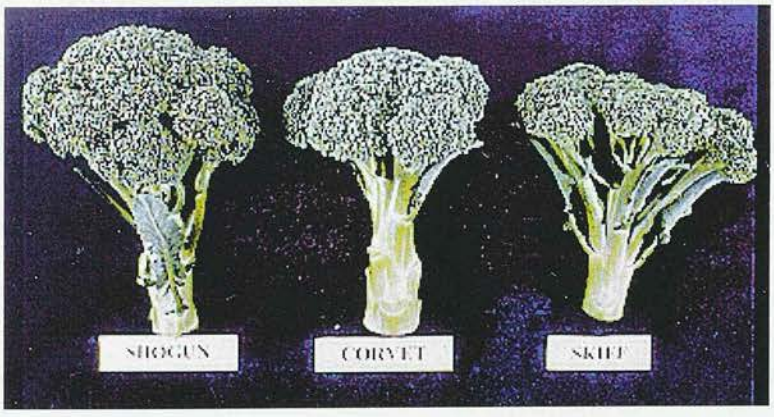
There have been no extensive reports regarding the influence of structural or biochemical defence factors, such as glucosinolates, phytoalexins, callose, cutins and lignins, in the resistance of broccoli to head rot disease.

Phytoalexins are produced in *Brassicas* following inoculation with *Pseudomonas cichorii*, *Erwinia carotovora* (Takasugi *et al.*, 1986) and *Alternaria brassicae* (Conn *et al.*, 1988), so it seems feasible that phytoalexins may be induced in response to head rot pathogens. Preliminary work by Simpson (1994) indicated that inoculating the leaves and heads of the broccoli cultivars Shogun and Corvet, with both pathogenic and non-pathogenic bacteria, stimulated the production of antibacterial compounds. The compounds were speculated to be phytoalexins because they increased in response to infection and inhibited invading organisms (Heggie, 1995), and although further work is required, these results strongly indicate that phytoalexin induction may be involved in cultivar resistance to head rot.

Further preliminary studies by Simpson (1994) suggested that localised callose deposition occurs in the leaves of the cultivars Corvet and Shogun, following

inoculation with both pathogenic and non-pathogenic bacterial isolates. Lignin was found not to be a factor in head rot defence (Simpson, 1994).

In addition to specific active defence products, head morphology, which varies considerably between cultivars, may predispose certain cultivars to disease by affecting the duration with which free water remains on the head. This then has implications for bacterial multiplication and penetration of host tissue. For example, field resistant cultivars such as Shogun and Samurai have fairly domed heads which would allow water to run off, thereby reducing the duration with which free water was present on the head, while cultivars with flat heads, such as susceptible Skiff, would allow free water to remain for considerably longer (Plate 3.1).



i) Shogun ii) Corvet, and iii) Skiff

Plate 3.1: Differences in head doming between three broccoli cultivars

Canaday (1989) found a regression correlation of 0.935 ($P<0.05$) between head tightness (compactness), doming and the percentage of diseased heads, which strongly supports the involvement of morphology in disease susceptibility. He suggested that the model predicted 85% of the variability in disease incidence when applied to 12 other broccoli cultivars. However, although Robertson and Brokenshire (1992) also found a relationship between domed heads and resistant cultivars, results were inconclusive with flatter shaped heads because although some cultivars were susceptible, others were only moderately susceptible. They claimed that this was due to head morphology being only partially involved in the complex process governing disease susceptibility, and suggested that more highly domed cultivars should now be tested. Therefore in light of these conflicting results the role of head morphology in disease susceptibility will be analysed in this chapter.

Stomata, for example, have been implicated as the entry site for bacterial pathogens (Hildebrand, 1989) and it has been suggested that biosurfactant-producing strains are responsible for the induction of necrotic lesions on the guard cells around stomata (Hildebrand, 1989; Gladstone, 1997). However, it is not known whether stomatal distribution, or number, varies between resistant and susceptible cultivars, as this may potentially predispose certain cultivars to disease. Simpson (1993) reported high stomatal numbers in the susceptible cultivar, Corvet, but more quantitative work is required.

3.1.3 Aims

This chapter aims to address the areas of bacterial pathogenicity and cultivar resistance to disease in broccoli head rot.

Pathogenicity: Initially a calibration curve was plotted of bacterial growth as cells against absorbance (OD_{500nm}) over time, to allow a cell count to be determined by absorbance in later experiments. Subsequently, the growth of three head rot pathogens was studied, at 20°C (the temperature used in the excised head test (Section 2.2.2, Chapter 2)), to acquire more information about the growth characteristics of these specific strains. In addition, the optimum growth temperature was established for one *Erwinia* and one *Pseudomonas* strain. Although optimum growth temperatures are widely published for particular bacterial species (Bergey, 1974), the individual strains responsible for head rot may vary from the species norm, so it is important to establish their specific characteristics. Finally, bacterial growth was studied *in planta* on one resistant and one susceptible cultivar, using spontaneous antibiotic-resistant mutants generated from one pathogenic, and one non-pathogenic bacterial strain. This was to determine two main factors: (1) whether competition occurs between bacterial strains i.e. whether pathogenic strains can multiply more successfully than non-pathogenic ones, and (2) whether bacteria multiply faster on susceptible, rather than resistant, cultivars.

Cultivar susceptibility: To determine the importance of head morphology in the disease process, head size, bud number, bud prominence, and head doming were assessed in ten field grown cultivars and compared with cultivar disease susceptibility ratings obtained both, in the field (Robertson *et al.*, 1993), and in the laboratory (Section 2.3.3, Chapter 2). In a separate study, stomatal number was quantified in nine glasshouse grown cultivars and related to the field (Robertson *et al.*, 1993) and laboratory (Chapter 2) disease susceptibility values.

3.2 Materials and methods

3.2.1 Spectrophotometric quantification of bacterial cell number

Four isolates of pathogenic bacteria were tested to determine the number of bacterial colony forming units corresponding to each OD_{500nm} value over time: *Erwinia carotovora* ssp. *atroseptica* isolates P5067 and P1065 and *P. fluorescens* Group IVb isolates P5038 and P5049. All had been previously isolated from diseased tissue (Robertson *et al.*, 1993; Campbell *et al.*, 1995). Each isolate was sub-cultured from a 24h KBA plate into 200ml of KB broth, in a 500ml flask. Flasks were incubated at 25°C (±2°C) with shaking at 200rpm. Immediately upon inoculation and then subsequently every 3h, the OD_{500nm} was recorded as an indication of bacterial growth. In addition, a 100µl aliquot was serially diluted and plated on to KBA to allow recording of the colony forming units present. Three replicate plates were used for each count. This would enable an estimate of bacterial colony forming units present in future bacterial KB broth suspensions to be quantified rapidly. In addition, three replicate flasks were carried out for each isolate to ensure consistency of results.

3.2.2 Estimating the optimum growth temperature of two pathogenic bacteria

The optimum growth temperature was calculated for two bacterial isolates: *Erwinia carotovora* ssp. *atroseptica* P5067 and *Pseudomonas fluorescens* Group IVb P5038. Three replicate flasks containing 100ml of NB (Oxoid) were inoculated with 0.1ml (1 x 10^8 cells) of an overnight NB suspension. Flasks were incubated at either 20 °C, 25 °C, or 30°C (±2°C) with constant shaking at 200rpm. The OD_{500nm} was recorded after 12h and 16h incubation, providing a 4h interval for growth determination.

3.2.3 Bacterial growth rate at 20°C

Bacterial growth was studied over a 120h period at the incubation temperature employed in the excised head pathogenicity test (Section 2.2.2, Chapter 2). Three pathogenic isolates, *Erwinia carotovora* ssp *atroseptica* P5067 and *Pseudomonas fluorescens* Group IVb isolates P5038 and P5049 were studied. Three replicate flasks containing 100ml of NB (Oxoid) were inoculated with 0.1ml (1×10^8 cells) of suspension from an overnight NB culture and incubated in a Fisons Cabinet at 20°C (±2°C). The flasks were shaken at 200rpm throughout and the OD_{500nm} recorded immediately after inoculation, and subsequently every 4h.

3.2.4 Bacterial growth *in planta*

Spontaneous antibiotic-resistant mutants were isolated from two bacterial strains: Pathogenic *Pseudomonas fluorescens* Group IVb P5038, and non-pathogenic *Pseudomonas fluorescens* N5027. The mutants were then inoculated on to both a resistant, and a susceptible, broccoli cultivar to allow *in planta* bacterial growth to be monitored.

a) Mutant production

To generate spontaneous antibiotic-resistant mutants, a large loopful of bacteria was removed from a 24h NA plate colony and inoculated into a 250ml flask containing 100ml of NB supplemented with either 500µg/ml of streptomycin sulphate (Sigma) or 300µg/ml spectinomycin dihydrochloride (Sigma). Isolate P5038 was inoculated into broth containing streptomycin sulphate, and N5027 was inoculated into broth containing spectinomycin dihydrochloride. The antibiotic concentration was set at three times the manufacturer's suggested working concentration to ensure that any

mutants produced were highly resistant to the antibiotic. Flasks were shaken at 200rpm, at 25°C (±2 °C), until antibiotic-resistant cells developed (between 24 and 48h). When the broth had become opaque (indicating that spontaneous mutants were present), a 100µl aliquot was plated on to NA containing the relevant antibiotic to allow colonies to form. Individual colonies were then streaked on to NA plates twice, before being finally plated on to NA containing antibiotics to confirm their antibiotic-resistant phenotype. Mutant colonies were stored on NA, without antibiotics, to prevent them developing antibiotic dependence (Meynell and Meynell, 1970).

b) Mutant characterisation

To ensure that the mutants were not altered phenotypically in terms of growth rate or pathogenicity from their wild-types, growth rate was studied over a 4h period in KB broth, as described in Section 3.2.2, at 25°C. Three replicate flasks were used for each isolate. Pathogenicity was compared on susceptible cultivar Skiff, as described in Chapter 2 (Section 2.2.2), using an inoculum concentration of 1×10^8 cells/ml. Four replicates were carried out for each isolate.

c) Inoculation of the mutants on to plant tissue

The two antibiotic-resistant mutants were inoculated on to broccoli heads by diluting a loopful of bacteria from 24h NA, without antibiotics, in dH_2O to give a final concentration of 3.3×10^7 cells/ml (calculated according to OD_{500nm} value - Section 3.3.1). Three millilitres of the suspension were then pipetted on to two $2cm^2$ pieces of cotton lint to give a total bacterial count of 1×10^8 cells. The two mutant isolates (one pathogenic and one non-pathogenic) were inoculated separately, and then

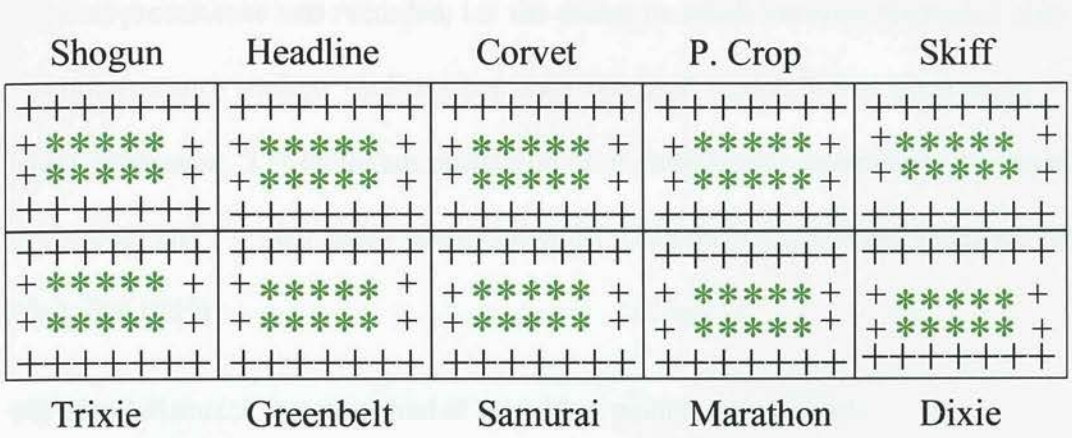
together (using 1.5ml of each suspension - i.e. 4.9×10^7cells). The two pieces of lint were placed together on top of the buds of either Corvet (susceptible) or Shogun (resistant), as described for the excised head pathogenicity test (Chapter 2, Section 2.2.2). Heads were incubated for either 1, 3 or 6 days prior to the re-isolation of mutants from the heads. Four replicates were carried out for each treatment.

d) Mutant isolation from plant tissue following incubation on excised broccoli heads

To assess mutant growth on plant tissue, buds were excised with a sterile scalpel and the bacteria were isolated as described in Chapter 2 (Section 2.2.1). The resulting bacterial suspension was serially diluted and a 0.1ml aliquot of each dilution was plated on to: (1) NA, (2) NA plus streptomycin at 500µg/ml, and (3) NA plus spectinomycin at 300µg/ml. Plates were incubated at 25°C (±2°C) for 2 days, to allow for colony formation.

3.2.5 Determining whether broccoli head morphology influences cultivar susceptibility to disease

Five resistant (Shogun, Samurai, Marathon, Greenbelt, and Dixie), three susceptible (Skiff, Premium Crop and Corvet) and two cultivars of unknown susceptibility (Trixie, Headline) were transplanted from module trays, at 4 weeks old, to field plots at Diamond Field, Auchincruive, Ayrshire (soil type is a sandy clay loam). Ten test plants were sown for each cultivar, at a distance of 40cm apart within rows, and 50cm between the rows. Each cultivar plot consisted of two rows of five plants (Figure 3.1).



+ = Guard plant
* = Trial plant

Figure 3.1: Plan of the broccoli head morphology field trial showing the cultivar positions

Guard plants, of the same broccoli cultivar, surrounded each plot and the trial was protected by wire throughout the trial to reduce wildlife damage. A general fertilizer of 16:16:16 NPK (nitrogen:phosphate:potassium) was applied at a rate of 200kg/hectare two days after transplantation. Mature heads were excised for measurement eight to ten weeks after transplanting (depending on maturity).

The following morphological characters were assessed on each head (ten heads per cultivar):

(1) Degree of head doming, i.e. whether inflorescences were triangular or flat in longitudinal section (As illustrated in Plate 3.1). A five point scale was used; 0 = flat head, 1 = slight doming, 2 = moderate doming, 3 = rounded dome, 4 = peaked dome. Data were transformed using arc sine (Schefler, 1969).

(2) Bud number was counted in a $1cm^2$ area, at three regions of the head: One central and two peripheral.

(3) Bud prominence was recorded, i.e. the extent to which the buds protruded above the inflorescence surface. A five point scale was used where; 0 = no protrusion, 1 = slight protrusion, 2 = moderate protrusion, 3 = considerable protrusion, 4 = highly protruded, and 5 = very highly protruded buds. Data were transformed using arc sine (Schefler 1969).

(4) Head diameter was measured at the widest point in centimetres.

The data for all morphological characters were analysed with ANOVA and were then compared with the disease susceptibility ratings obtained in the field (Robertson *et al.*, 1993) and in the laboratory (Section 2.2.3, Chapter 2), initially by pair-wise correlation equations and then subsequently as a unit in a linear discriminant analysis (Genstat 5, Rothamsted Experimental Station, 1995). This analysis finds the linear combination of variables which best separates the cultivars by their response to disease (resistant or susceptible).

3.2.6 Determining whether cultivar stomatal number influences susceptibility to disease

Stomatal number was quantified in nine glasshouse grown cultivars, including six resistant cultivars (Samurai, Dixie, Trixie, Shogun, Marathon, Greenbelt) and three susceptible ones (Premium Crop, Skiff and Headline) (Robertson *et al.*, 1993). For each cultivar, one sepal was excised from two different mature buds (repeated on three broccoli heads) giving a total of six sepals for each cultivar. Sepals were cleared in methanol for 2h, washed three times in distilled H_2O, then stained in 10% safranin for 10 minutes. After washing again, the sepals were placed in 20% glycerol for 5 minutes, before being mounted (with the outer surface upwards) in glycerol and

viewed under a light microscope (x400 magnification). The number of stomata were counted in five separate fields of view for each sepal, giving a total of 30 counts for each cultivar. The number obtained was then multiplied by 4.29 (D. Smith, pers. communication) to convert into stomatal number per mm^2. The data were then compared with disease susceptibility data from the field (Robertson *et al.*, 1993) and the laboratory (obtained as described in Chapter 2).

3.3 RESULTS

3.3.1 Spectrophotometric quantification of bacterial cell number

Figure 3.2 presents the number of colony forming units obtained over time from KB broth suspensions of four pathogenic bacteria in relation to the corresponding OD_{500nm} value.

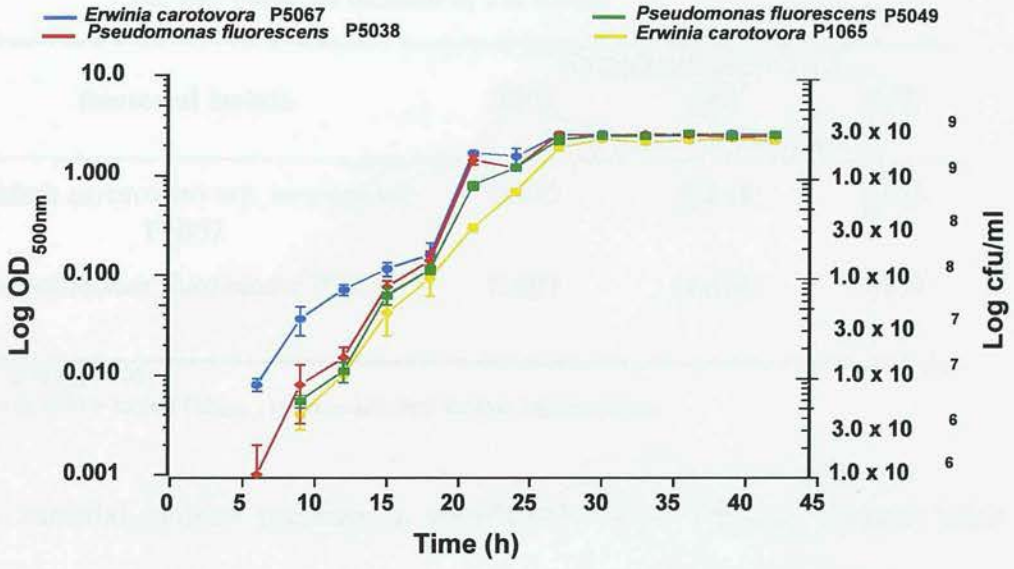


Figure 3.2: Mean number of bacterial colony forming units present at each OD_{500nm} value recorded over time for four pathogenic isolates (with SEM bars)

From Figure 3.2 it can be seen that the four bacterial strains showed similar growth curves, especially during stationary phase, although *E. carotovora* P5067 was initially faster. The first OD_{500nm} value registering on the spectrophotometer for all isolates was 0.003, which represents an average of 3.5×10^6 cfu/ml. All four suspensions entered stationary phase at an OD_{500nm} value of 2.3, representing 2.5×10^9 cfu/ml. Of interest for the excised head pathogenicity test (Chapter 2, Section 2.2.2) is the OD_{500nm} of 0.1 which roughly represents 1×10^8 cfu/ml (for all four isolates), because this is the inoculum concentration used.

3.3.2 Estimating the optimum growth temperature of two pathogenic bacteria

Table 3.1 presents the growth of two bacterial strains, obtained at three incubation temperatures, as expressed by the increase in OD_{500nm} value produced over 3h.

Table 3.1: Effect of temperature on the OD_{500nm} increase obtained over 3h, for two bacterial isolates in KB broth

Bacterial isolate	Temperature (± 2°C)		
	20°C	25°C	30°C
	OD increase obtained		
Erwinia carotovora ssp. *atroseptica* P5067	0.035	**0.113**	0.044
Pseudomonas fluorescens P5038	0.087	**0.228**	0.171

LSD = 0.019 ($p<0.05$)
Values in bold = largest OD_{500nm} increase obtained for that bacterial strain

Both bacterial isolates produced a significantly larger OD_{500nm} increase when incubated at 25°C than the other two temperatures. In addition, the *P. fluorescens* strain produced significantly larger OD_{500nm} increases than the *Erwinia carotovora* strain, at all three temperatures tested, which may indicate that *P. fluorescens* has a higher growth rate.

3.3.3 Bacterial growth rate at 20°C

Figure 3.3 shows the growth obtained in NB for three pathogenic isolates.

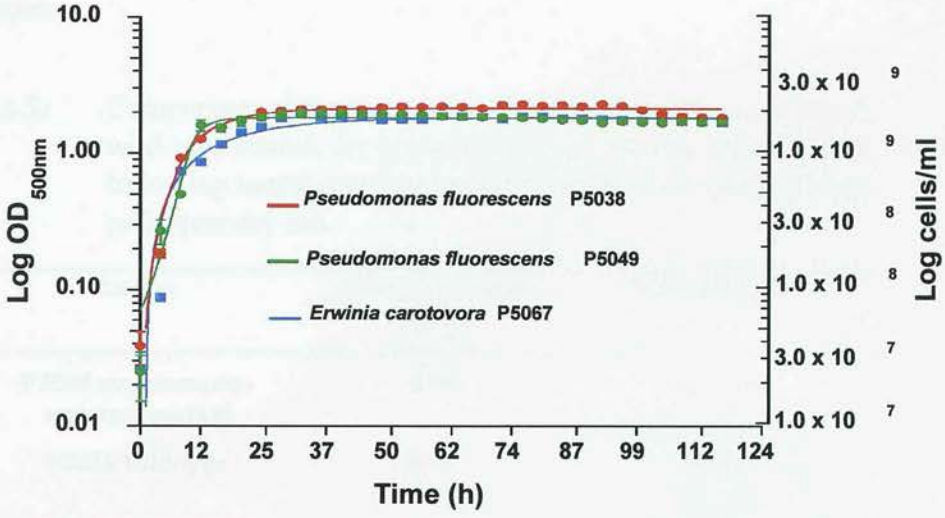


Figure 3.3: Growth of *Erwinia carotovora* ssp. *atroseptica* P5067 and *Pseudomonas fluorescens* isolates P5038 and P5049, in nutrient broth at 20°C (with SEM bars)

From Figure 3.3 it can be seen that all three pathogenic isolates have multiplied at a similar rate over time and have entered stationary phase by 25h at approximately 2.0 x 10^9 cells/ml.

3.3.4 Bacterial growth *in planta*

a) Mutant production

Spontaneously, *Pseudomonas fluorescens* P5038 became resistant to streptomycin sulphate, whilst *Pseudomonas fluorescens* N5027 became resistant to spectinomycin dihydrochloride.

b) Mutant characterisation

Table 3.2 presents the results of the phenotypic comparison (growth rate and pathogenicity) carried out between the spontaneous mutants and their respective wild-types.

Table 3.2: Comparison of the two antibiotic-resistant mutants with their wild-type strains, for growth (OD_{500nm} increase over 4h from 12-16h following inoculation) and pathogenicity in the excised heads pathogenicity test.

Isolate	OD_{500nm} increase over 4h	Pathogenicity[a]
P5038 streptomycin-resistant mutant	0.54	62.7%
P5038 wild-type	0.56	67.0%
N5027 spectinomycin-resistant mutant	0.50	33.0%
N5027 wild-type	0.58	24.7%

a = Pathogenicity in the excised head rot test where 0% = no disease, 100% = full soft rot
$LSD_{Pathogenicity}$ = 13.98%
LSD_{Growth} = No significant difference detected (ANOVA F value = 0.66, F crit = 3.49)

From Table 3.2 it can be seen that no significant difference was found between the growth rates of the wild-type vs the mutant (shown by OD_{500nm} increase), indicating that the mutants are growing at an equivalent rate to their wild-types. Wild type P5038 and its mutant strain produced significantly more disease in the excised head pathogenicity test than wild-type N5027 or its mutant strain, but there was no significant difference between the disease levels of the wild-types and their respective mutants. These results indicate that both mutant strains have not altered phenotypically from their wild-types in terms of growth in culture and pathogenicity on excised broccoli heads.

c) Mutant isolation from plant tissue following incubation on excised broccoli heads

After six days incubation, heads inoculated with pathogenic P5038 (wild-type and mutant) showed only limited disease symptom development (tissue discolouration and softening, but little rot). Heads inoculated with the non-pathogenic strains showed only occasional browning.

Table 3.3: Mean number of bacterial colonies (cells) obtained from broccoli heads after 1, 3, and 6 days incubation following inoculation with antibiotic resistant mutants.

	P. fluorescens strains	P5038 (strep^R)		N5027 (spec^R)		P5038 (strep^R)/ N5027 (spec^R)	
		Cultivar tested					
Day	Isolation medium	Shogun	Corvet	Shogun	Corvet	Shogun	Corvet
1	NA + Spectinomycin	0	0	5.0×10^7	5×10^7	0	0
	NA + Streptomycin	5×10^6	2.5×10^5	0	0	2.5×10^5	2.5×10^5
	NA	1.2×10^6	0	2.2×10^6	2.8×10^{11}	1.0×10^7	7.5×10^{10}
3	NA + Spectinomycin	0	0	2.8×10^7	2.5×10^{11}	1.0×10^7	0
	NA + Streptomycin	4.5×10^6	1.8×10^7	0	0	2.6×10^8	5.0×10^6
	NA	1.0×10^{10}	2.6×10^8	1×10^{11}	5.0×10^{11}	6.0×10^9	5.0×10^{10}
6	NA + Spectinomycin	0	0	3.5×10^9	1.3×10^{10}	6.8×10^9	7.5×10^9
	NA + Streptomycin	3.0×10^{10}	3.0×10^9	0	0	4.0×10^9	3.4×10^{10}
	NA	4.0×10^{10}	2.7×10^{10}	3.6×10^{10}	3×10^{12}	6.4×10^{10}	1.2×10^{11}

$LSD_{Day} = 2.4 \times 10^{10}$ cells
$LSD_{Plates} = 2.4 \times 10^{10}$ cells
$LSD_{Isolates}$ = NS (ANOVA F value = 1.2, F crit = 3.0)
$LSD_{Cultivars}$ = NS (ANOVA F value = 2.7, F crit = 3.9)

The results presented in Table 3.3 indicate that the bacterial cells isolated from broccoli tissue increased steadily, over the 6 day period. However, no significant difference was observed between the number of bacterial colonies isolated from either the pathogenic and non-pathogenic strain; the resistant and susceptible heads; the heads inoculated with only one mutant strain against those inoculated with both mutants together, or the NA plates containing either streptomycin or spectinomycin (but both of these plates contained fewer colonies than the NA plates). As a control,

mutants were also plated on to plates containing the antibiotic that they were not resistant to. No growth was obtained, indicating that all of the colonies formed on the antibiotic plates were resistant only to their particular antibiotic and that there was no cross-contamination.

However, the experimental results were extremely variable (as shown by the high LSD values), with some replicates producing no bacterial colonies whilst others produced over 10^6 cells, and this was found for all inoculum/cultivar combinations. To determine whether this variability may have been due to the mutants losing their antibiotic resistance, a loopful of each mutant strain was removed from a NA plate colony, inoculated in to 100ml of nutrient broth, and incubated for 3h, with shaking at 200rpm. A 100µl aliquot was then plated on to (1) NA, and (2) NA containing the antibiotic that it was resistant to. Two replicates were used for each mutant/medium combination. Both mutants produced similar colony numbers on the two agar types, so loss of resistance does not appear to be responsible for the experimental variability obtained.

3.3.5 Determining whether broccoli head morphology influences cultivar susceptibility to disease

Table 3.4 shows the results of the analysis of five broccoli head characters carried out on ten cultivars which varied in disease susceptibility.

Table 3.4: Scores obtained for five head morphological characters in ten broccoli cultivars which vary in their susceptibility to disease

	Cultivars										
	P.Crop	Skiff	Corvet	Samurai	Shogun	Marathon	Dixie	Greenbelt	Headline	Trixie	LSD ($p < 0.05$)
Susceptibility[3]	S	S	S	R	R	R	R	R	N	N	
Head characteristic											
bud prominence[1t] (%)	33.3	46.7	48.3	40.0	31.7	28.3	41.7	46.7	**78.3**	33.3	10.6%
head diameter (cm)	**10.6**	9.1	10.4	6.4	6.2	6.8	7.8	7.4	7.2	7.9	1.8
no. of buds /cm^2 at side of head	24.5	51.0	40.1	64.4	**77.2**	77.1	40.4	42.7	41.4	42.8	10.44
no. of buds /cm^2 centre head	25.3	71.6	54.6	85.2	**96.7**	90.8	48.0	50.8	48.8	55.2	19.10
head doming[2t] (%)	33.3	51.7	44.1	63.3	**83.3**	68.3	68.3	75	71.7	76.7	15.1%

[1]Bud Prominence 0% = no prominence; 100% = maximum prominence.
[2]Head doming 0% = flat head shape; 100% = very domed head (triangular cross section).
[3]Disease susceptibility in the field according to Robertson *et al.* (1993);
S = susceptible, R = resistant, N = not determined
t =Data transformed with arc sine (Schefler 1969)
Text in bold = highest value obtained
Text in red = value significantly lower than the highest value (shown in bold) obtained for that character

Table 3.4 illustrates that significant differences were found between the cultivars for all characters studied.

Bud prominence: Headline had significantly more prominent buds than all of the other cultivars tested. The buds of Marathon, Premium Crop and Shogun showed the least prominence, with a score significantly smaller than the other cultivars tested. No relationship was observed between bud prominence and disease susceptibility.

Head diameter: Susceptible cultivar Premium Crop had the largest head size and resistant Shogun the smallest. Interestingly there was a statistically significant divide between the three susceptible cultivars (which all had significantly larger heads) and the resistant and unclassified cultivars.

Bud number: Both Shogun and Marathon had a significantly higher bud number (both centrally and at the side of the head) than the majority of the other cultivars tested. Susceptible Premium Crop had the lowest bud number and this was significantly lower than all of the other cultivars tested.

Head doming: The most domed cultivar was found to be resistant Shogun, whilst the least domed was susceptible Premium Crop. Shogun was significantly more domed than the three susceptible cultivars and the resistant cultivar (Samurai) tested, indicating that there may be a relationship between doming and susceptibility.

No naturally occurring disease symptoms were observed during the field trial, but some heads were watersoaked i.e. showed the early stages of head rot. However, there was no significant difference between the doming values of watersoaked heads compared with those of non-watersoaked heads of the same variety (results not shown).

To determine whether there was a relationship between the various head morphological traits and disease susceptibility, correlation coefficients were calculated using the field susceptibility values of Robertson *et al.* (1993) (Table 3.5).

Table 3.5: Correlation coefficients obtained for the five head morphology traits, with cultivar disease susceptibility ratings obtained in the field (Robertson *et al.*, 1993) or in the laboratory (Chapter 2, Section 2.3.3)

	Head character	Correlation coefficient ($P<0.05$)		
		laboratory susceptibility[1]	laboratory susceptibility[2]	field susceptibility[3]
1	Head doming	-0.52	-0.56	**-0.71**
2	Prominence	0.4	0.064	0.02
3	Head size	**0.65**	**0.66**	**0.76**
4	Bud number (central)	-0.1	-0.031	-0.61
5	Bud number (side)	-0.4	-0.16	-0.58

Significance = **0.632(8df); 0.71 (6df)**
Values in bold = statistically significant correlation
[1]Laboratory susceptibility without Trixie and Headline i.e. 6df (because these cultivars were not present in the field data of Robertson *et al.*, 1993)
[2]Laboratory susceptibility with all ten cultivars i.e. 8df
[3]Field susceptibility = from Robertson *et al.* (1993) i.e. 6df

As highlighted in Table 3.5, the only two characters which correlated significantly with disease susceptibility were head doming (r = -0.71 @ $P<0.05$ with field susceptibility) (Figure 3.4), and head size (r = 0.76 @ $P<0.05$ for field susceptibility, and 0.66 and 0.65 @ $P<0.05$ for laboratory susceptibility) (Figure 3.5).

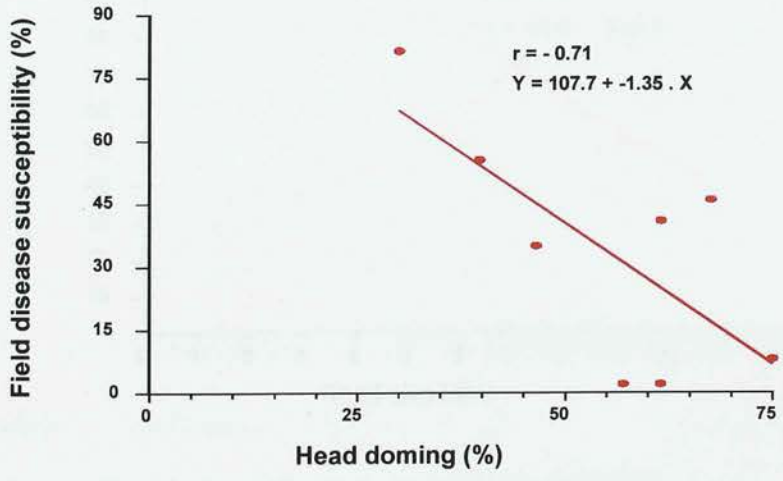


Figure 3.4: Correlation of head doming against disease susceptibility in the field (field data determined by Robertson *et al.* (1993)

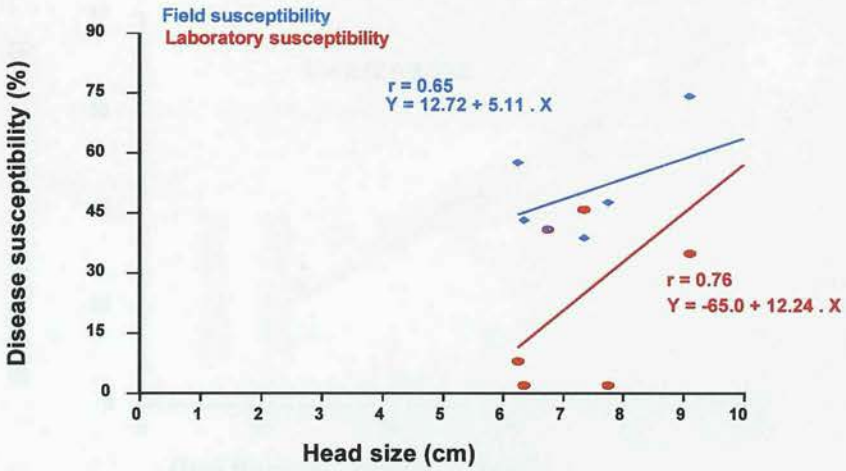


Figure 3.5: Correlation of head size against disease susceptibility (in the field as determined by Robertson *et al.*, 1993, and in the laboratory - Section 2.3.3, Chapter 2)

In addition to the results presented in Table 3.5, head size correlated significantly with head doming ($r = -0.87$, $p<0.05$) (Figure 3.6), and bud number at the side of the head correlated with central bud number ($r= 0.98$, $p<0.05$) (Figure 3.7).

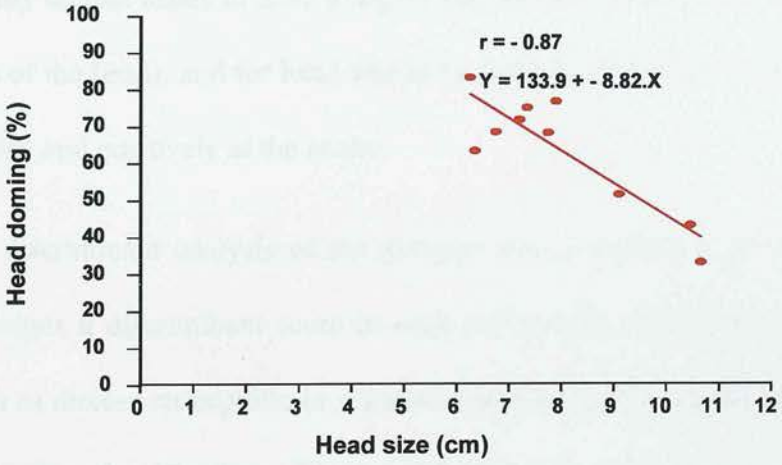


Figure 3.6: Correlation of head size against head doming

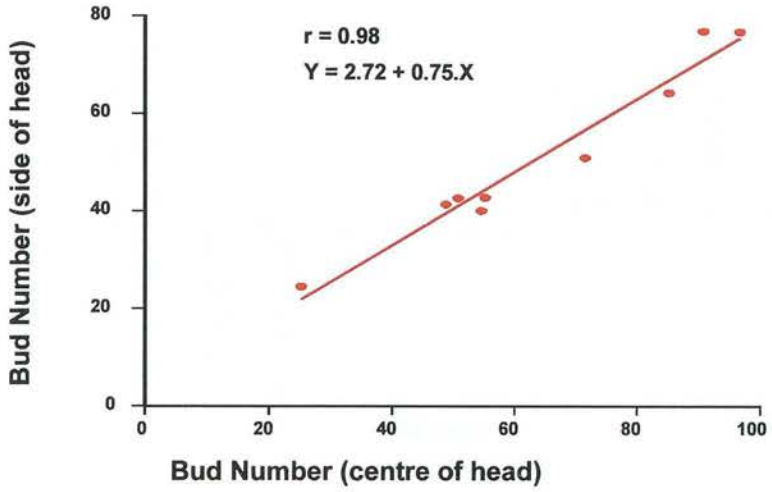
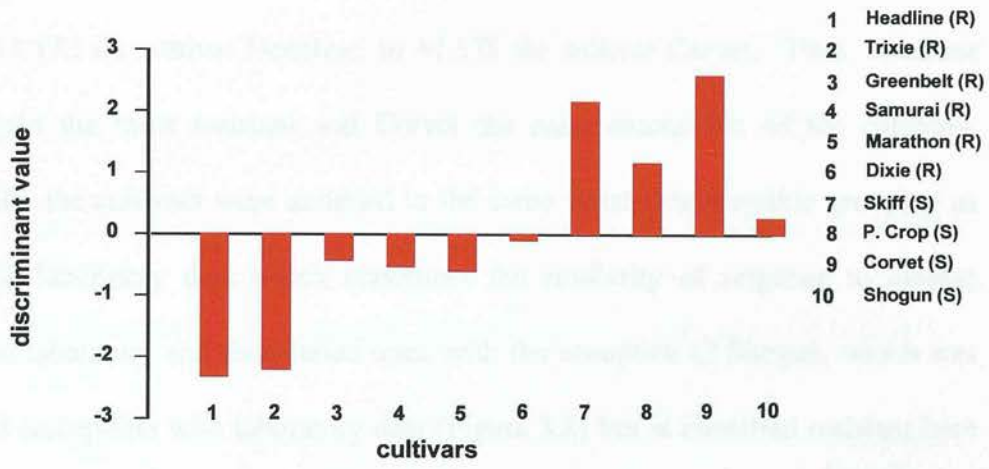


Figure 3.7: Correlation of bud number at the side of the head against bud number located centrally.

Although not statistically significant, head doming and laboratory susceptibility ($r = -0.56$, $p<0.05$), and bud number and field susceptibility ($r = -0.61$ @ $p<0.05$ centrally on the head, and $r = -0.58$ @ $p<0.05$ at the side of the head), suggested there may be a weak relationship between these factors. In addition, there is a trend for highly domed heads to have a higher bud number (both at the sides and central regions of the head), and for head size to be influenced by bud number - negatively at the side and positively at the centre.

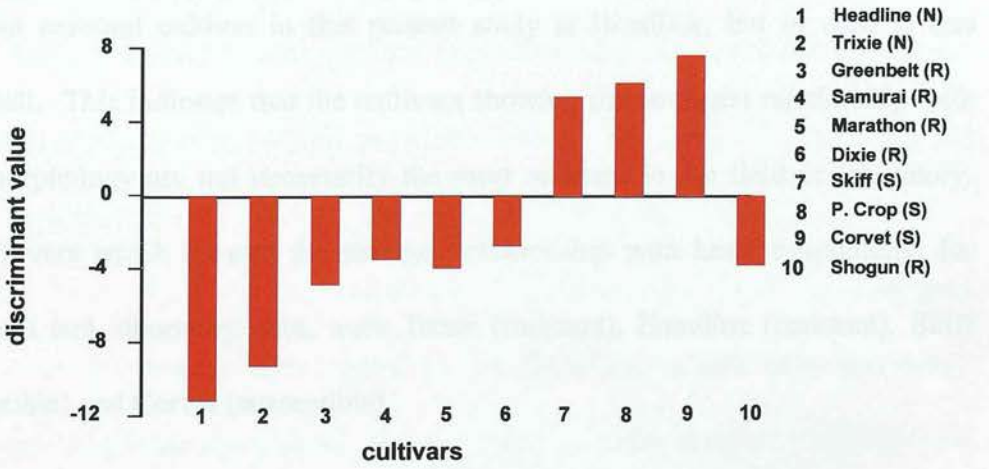
Linear discriminant analysis of the grouped morphological data was carried out: This assigns a discriminant score to each cultivar for its morphological traits, to group it as disease susceptible or resistant based on the field and laboratory disease susceptibility classifications (Figures 3.8 and 3.9). Increasing negative scores indicate increasing resistance and increasing positive scores indicate increasing susceptibility to disease.



S = Laboratory susceptible, as determined in the laboratory test described in Chapter 2
R = Laboratory resistant, as determined in the laboratory test described in Chapter 2

Figure 3.8: Linear discriminant scores of head morphology traits and laboratory cultivar susceptibility values (Section 2.3.3, Chapter 2).

As shown in Figure 3.8, the cultivars' discriminant scores for the laboratory data ranged from -2.315 for Headline to +2.596 for Corvet. Therefore from this analysis Headline is the most resistant cultivar, while Corvet in the most susceptible.



S = Field susceptible (Robertson *et al.* (1993), as determined in the field trial of Robertson *et al.*, 1993.
R = Field resistant (Robertson *et al.* (1993), as determined in the field trial of Robertson *et al.*, 1993.

Figure 3.9: Linear discriminant scores of head morphology traits and field cultivar susceptibility values (Robertson *et al.*, 1993).

From Figure 3.9 it can be seen that the discriminant scores for field data ranged from -11.172 for cultivar Headline, to +7.575 for cultivar Corvet. Thus, Headline was again the most resistant and Corvet the most susceptible of the cultivars. Generally the cultivars were assigned to the same resistant/susceptible grouping as with the laboratory data which underlines the similarity of response to disease between laboratory and field-based tests, with the exception of Shogun, which was deemed susceptible with laboratory data (Figure 3.8) but is classified resistant here (Figure 3.9). The field discriminant scores are higher (for the susceptible cultivars) and lower (for the resistant cultivars), than those obtained with the laboratory based scores, indicating that the morphological characters studied have a stronger relationship with the field data.

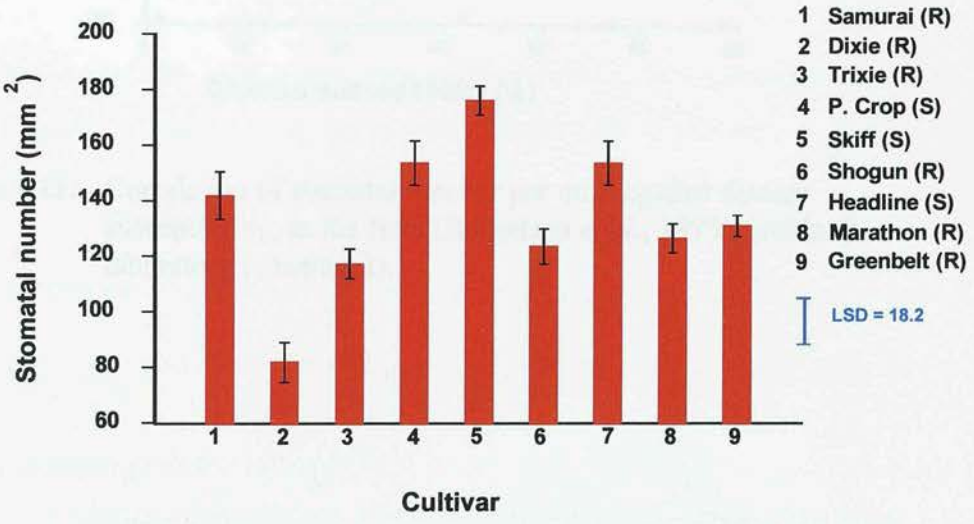
The degree of resistance/susceptibility assigned to the cultivars in the linear discriminant analyses was not necessarily equivalent to that found in the original susceptibility experiments (Robertson *et al.*, (1993); Section 2.3.3, Chapter 2) i.e. the most resistant cultivar in this present study is Headline, but *in vitro* it was Greenbelt. This indicates that the cultivars showing the strongest relationship with head morphology are not necessarily the most resistant in the field or laboratory. The cultivars which showed the strongest relationship with head morphology, for both field and laboratory data, were Trixie (resistant), Headline (resistant), Skiff (susceptible) and Corvet (susceptible).

Therefore, to summarise the results, head diameter and degree of doming appear to contribute most to head rot resistance: Larger heads were more susceptible to

disease, and highly domed heads were more resistant to disease than flat heads. In addition, the cultivars Headline, Corvet, Skiff and Trixie show the strongest relationships between disease susceptibility and head morphology.

3.3.6 Determining whether cultivar stomatal number influences susceptibility to disease

Figure 3.10 shows the stomatal number per mm^2 found for nine broccoli cultivars.



R = Resistant, as determined in the field by Robertson *et al.*, 1993
S = Susceptible, as determined in the field by Robertson *et al.*, 1993

Figure 3.10 : Stomatal number per mm^2 in nine broccoli cultivars (with SEM bars)

Significant differences in cultivar stomatal number were found (Figure 3.10), but they did not correlate significantly with *in vitro* ($r = 0.51$ @ $P< 0.05$), or field ($r = 0.50$ @ $p<0.05$) (Figure 3.11) disease susceptibility ratings. However, the three highest stomatal counts were found in the three susceptible cultivars tested. Susceptible Skiff had the highest stomatal number, while resistant Dixie had the lowest.

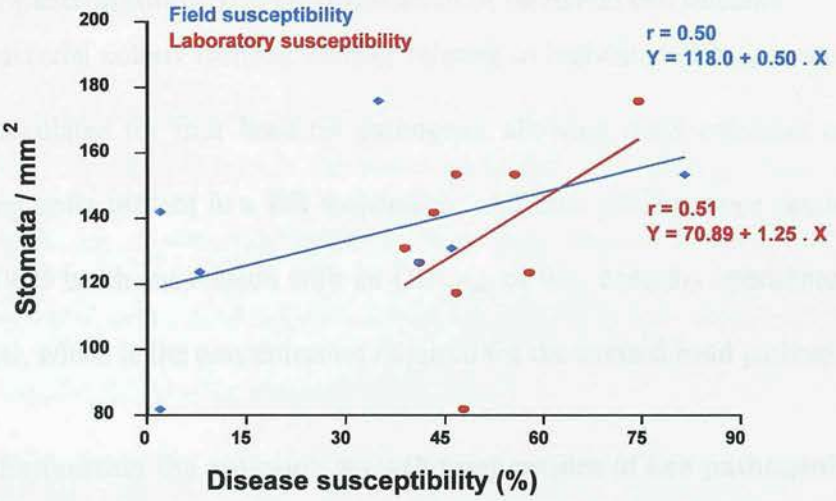


Figure 3.11: Correlation of stomatal number per mm^2 against disease susceptibility, in the field (Robertson *et al.*, 1993), and in the laboratory (Chapter 2).

3.4 Discussion

3.4.1 Spectrophotometric quantification of bacterial cell number

The bacterial colony forming number relating to individual OD_{500nm} values over time was calculated for four head rot pathogens, allowing rapid estimates of the colony forming units present in a KB suspension. All four profiles were similar, indicating that a KB broth suspension with an OD_{500nm} of 0.1, contains approximately 1×10^8 cells/ml, which is the concentration required for the excised head pathogenicity test.

3.4.2 Estimating the optimum growth temperature of two pathogenic bacteria

Both *Erwinia carotovora* ssp. *atroseptica* P5067 and *P. fluorescens* P5038 were found to multiply optimally at 25°C (±2°C). This corresponds to the published data for these species which states that *Erwinia carotovora* grows optimally between 27-30°C, whilst *P. fluorescens* maximal growth is reported at 25-27°C (Bergey, 1974).

3.4.3 Bacterial growth rate at 20°C

All three pathogenic isolates (two *P. fluorescens* and one *E. carotovora*) grew well at 20°C despite the fact that this incubation temperature is 5°C below the optimal growth temperature for isolates P5067 and P5038. The isolates all showed similar growth curves, reaching stationary growth by 25h with a concentration of 2.0×10^9 cells/ml. Therefore it would appear that these isolates are equally successful at multiplying at the 20°C temperature employed in the excised head pathogenicity test, and thus no one isolate has a growth advantage over the others. As the *Erwinia* strain grew equally to the two Pseudomonads, this contradicts the observation in Section 3.3.2 that the *Erwinia* strain produced a significantly lower OD_{500nm} increase over a 4h period than *P. fluorescens* P5038. Possibly, the *E. carotovora* strain

received a lower bacterial inoculum concentration than predicted in the experiment described in Section 3.3.2.

3.4.4 Bacterial growth *in planta*

To recap, an initial screen of the antibiotic-resistant mutants produced showed that they were not significantly different from their respective wild-types in terms of pathogenicity on excised broccoli heads, or growth in KB broth, and so they were considered to be suitable mutants for this study.

An attempt was also made to obtain spontaneous spectinomycin mutants from pathogenic *Erwinia carotovora* ssp. *atroseptica* isolate P5067, but when the opaque suspension was plated on to NA, milky growth was obtained, and as a result, discrete colonies could not be located. It is likely that this was due to the mutants having been altered in extracellular polysaccharide (EPS) production - mucilaginous secretions that coat the bacterium's outer surface to form an interface with its environment - making the culture very runny, and due to these difficulties the *Erwinia*-spectinomycin mutants were not used further.

For the mutant strains tested, colonies were successfully isolated from broccoli tissue, with significantly higher numbers being isolated over time to indicate that bacterial growth had occurred *in planta*. No significant difference in colony number was obtained from the inoculation of pathogenic against non-pathogenic bacteria; the inoculation of bacteria on to susceptible against resistant tissue, or from the two mutant strains following inoculation together. Therefore this may demonstrate that the pathogenic strain does <u>not</u> induce disease simply by: (1) multiplying more rapidly

than the non pathogenic strain, (2) multiplying more successfully on susceptible cultivars compared with resistant ones, or (3) out-competing the non pathogenic strain tested. However, the results were extremely variable and so no firm conclusions can be drawn. In addition, it would have been expected that significantly more colonies would have been isolated when the mutant strains were inoculated alone (1×10^8 cells) as opposed to together (4.9×10^7 cells of each), but this was not the case, as the colony number isolated was similar following both treatments. This may indicate that either (1) inoculation of a lower inoculum concentration has allowed the mutant strains to establish and multiply more; (2) the heads inoculated with 1×10^8 cells, may have been subject to competition from a residual bacterium which inhibited its growth, or (3) the isolation protocol has failed to detect differences in the number of bacteria present.

Although considerably more bacterial colonies were isolated from the heads incubated for 6 days compared with those tested by Robertson and Brokenshire (1992) (which showed soft rot symptoms - 1.5×10^8 pectolytic and 3.6×10^7 fluorescent *Pseudomonas* bacteria), the difference is likely to be due to the fact that here, the heads were inoculated with a high inoculum concentration, whilst Robertson and Brokenshire (1992) worked with non-inoculated field material. However, it would have been expected that with the high colony number isolated from the heads in this study, the pathogenic mutant inoculated would have induced more significant disease symptoms. Possibly, after the phenotypic comparison was carried out, a second spontaneous mutation occurred affecting pathogenicity.

As expected, considerably more bacteria were isolated on the NA plates compared to those containing antibiotics, demonstrating that residual bacteria were present during testing. These bacteria may have strongly influenced the results obtained by competing with the inoculated strains, reducing their ability to colonise the broccoli tissue. This, in turn, may have led to the variable results which were obtained between replicates. Indeed, the poor disease symptoms induced by the pathogenic strain, even after 6 days, may have been due to interference from residual bacteria.

Loss of antibiotic resistance, the isolation protocol, or inherent variability in the plant material (and its resident saprophytic bacterial population), may have been responsible for the variable results obtained. As some replicates produced an extremely low bacterial number it is particularly likely that there may have been a problem with the isolation procedure. Altering the protocol may lead to more accurate results. Moreover, the mutants should now be assessed more thoroughly to determine whether any other changes in phenotype could have occurred, because the mutations may have affected their ability to colonise or grow *in planta*, resulting in variability between replicates.

The variability found introduces doubt over the reliability of the results obtained rendering the significant differences inconclusive. After locating the factor(s) responsible for the variability, the study should be repeated with a larger number of replicates to improve accuracy.

3.4.5 Determining whether broccoli head morphology influences cultivar susceptibility to disease

Head doming and head size correlated significantly with field susceptibility data and so it can be inferred that these two characters are influential in cultivar susceptibility to disease in the field (head size also correlated significantly with laboratory disease susceptibility data). Head doming produced a high, but non-significant, correlation coefficient with laboratory data. Head size also correlated significantly with head doming, indicating that the larger heads were, the flatter they became. Therefore the flatter and larger heads are, the more susceptible they are to infection. This supports the work of both Canaday (1989), and Robertson and Brokenshire (1992), who also found a relationship between head doming and disease resistance (but in a different range of cultivars). Canaday (1989) found a regression correlation of 0.935 ($P<0.05$) for head tightness and doming with disease incidence.

While head doming and size correlate significantly with disease susceptibility, they will only be partially responsible for disease resistance. Other biochemical and structural defence products are produced actively against pathogen attack, and there may even be an additive effect between head morphology and active defence products. Previous research has already indicated that gluosinolates (Conn *et al.*, 1988), phytoalexins and callose (Simpson, 1994) production may be involved in protection against bacterial disease. However, the role of morphology in disease resistance or susceptibility appears to be more important in certain cultivars, such as Headline, Corvet, Trixie and Skiff which showed the most profound linear discriminant scores (Figures 3.8 and 3.9).

Whilst the other head characters tested (bud prominence and bud number at the side and centre of each head) did not correlate significantly with field disease susceptibility, central and side bud number correlated highly. Moreover, highly domed heads tended to have a higher bud number both centrally and at the sides of the head (although again this is not a significant correlation). This may be a structural requirement in the formation of such highly domed heads.

The linear discriminant analyses highlighted the difference between the laboratory and field susceptibility data, with the field data produced much higher scores, indicating that there was more of a relationship between the morphological traits and cultivar susceptibility. However, the same cultivar susceptibility trend being found for both (with one exception, Shogun).

However, for all comparisons between laboratory and field susceptibility data, it should be noted that the data do not follow the same scoring scale, and therefore there are quantitative differences between them. Consequently, it may be advisable to repeat this work using the same scoring scale for each, as there is considerable variation between the two scoring scales used.

Significant differences in cultivar stomatal number were found, with the three susceptible cultivars tested recording the highest numbers. This indicates that a high stomatal number may predispose cultivars to disease, although no significant correlation was found between stomatal number and disease susceptibility. Possibly a high stomatal count predisposes cultivars to disease, but those cultivars with an

efficient resistance response to pathogen attack are able to defend themselves sufficiently.

3.4.6 Conclusions

The research conducted in this study has provided knowledge about the growth characteristics of the pathogenic bacteria responsible for head rot disease: The colony forming units present in a King's B broth suspension, can now be extrapolated, simply from measuring the OD_{500nm} value; the optimum *in vitro* growth temperature for two pathogenic isolates has been identified between 23 - 27°C, and the growth curves of three pathogenic bacteria have been constructed.

In addition, the growth of one pathogenic and one non pathogenic strain was monitored *in planta* on both resistant and susceptible broccoli tissue. Although results were variable, the two bacterial strains multiplied at an equal rate on resistant and susceptible tissue.

Head doming and size have been implicated as important factors in determining broccoli cultivar resistance to disease, and finally, there may have been a relationship with stomatal number and susceptibility.

Chapter 4: Bacterial biosurfactant production - literature review

4.1 Introduction to biosurfactants

Biosurfactants are metabolites, secreted by micro-organisms, which are composed of both a hydrophilic and a hydrophobic region, enabling them to affect the surface tension of fluid interfaces. The mechanics of biosurfactant action will be addressed shortly. The majority of broccoli head rot pathogens produce biosurfactants and they are considered to be a major virulence determinant of the disease (Hildebrand, 1989). By reducing the surface tension of rain water or dew droplets present on the broccoli head, biosurfactants allow water to spread over the waxy tissue leading to the development of watersoaking, thereby enhancing bacterial colonisation and aiding disease progression (Hildebrand, 1989). In addition, the spreading water may redistribute micro-organisms on the plant surface, and biosurfactants have been shown by Hildebrand *et al.* (1990) to alter membrane permeability, resulting in nutrient leakage from epidermal cells which can be utilised by the bacteria.

Hildebrand (1989) claimed that both biosurfactants and pectolytic enzymes were essential for disease development, because strains lacking either factor could only induce disease in wounded tissue. It was suggested that both factors work in combination to provide a suitable environment for pathogenesis. However, the exact role of biosurfactants has not yet been established *in planta*, because there are non-biosurfactant producers which are fully pathogenic on unwounded tissue (Robertson *et al.*, 1993). Therefore, in order to expand our knowledge of the role of biosurfactants in head rot, Tn5 mutagenesis was employed to produce biosurfactant-minus mutants of a pathogenic *P. fluorescens* strain, and then the effect of

biosurfactant-loss on pathogenicity was analysed on excised broccoli heads (Chapter 6). Prior to this work, it was necessary to develop a sensitive biosurfactant detection test and to study biosurfactant production in several head rot pathogens - the results of this research are reported in Chapter 5. Thus, Chapter 4 introduces and summarises the current knowledge available about biosurfactants.

4.2 Chemistry of biosurfactants and industrial synthetic surfactants

As mentioned previously, biosurfactants are amphipathic, containing both a hydrophilic region and a hydrophobic region. The hydrophobic moiety of a surfactant molecule always consists of the hydrocarbon chain of a fatty acid, whilst the hydrophilic moiety affects their classification and may consist of either:

(1) the ester or alcohol function of neutral lipids (neutral charge);

(2) the carboxylate group of fatty acids or amino acids or the phosphate-containing portion of phospholipids (positive charge);

(3) the carbohydrate moiety of glycolipids (negative charge) (Fiechter, 1992).

As noted above, this results in biosurfactants having either a positive, negative or neutral charge. Due to their amphipathic properties, surface-active molecules concentrate at the interface of a solution with their hydrophobic regions aligned away from the liquid and the hydrophilic ones immersed (Fiechter, 1992). This then alters the physical forces governing the arrangement of liquid molecules, by influencing the formation of hydrogen-bonds, and results in a reduction in surface tension - the force required to keep a water droplet spherical (Denny, 1993). Consequently, their structure infers detergency, emulsifying, foaming and dispersing properties (Desai *et al.*, 1994). As the biosurfactant concentration increases, individual molecules

aggregate in structures known as micelles where the hydrophilic regions cluster to shield the hydrophobic regions from the aqueous solution (Desai *et al.*, 1994). This is illustrated in Figure 4.1. This aggregation occurs at the Critical Micelle Concentration (CMC) and is used as a measure of the biosurfactant's efficiency. The efficiency can range from 0.02 to 150μg/ml (Desai *et al.*, 1994) and it increases with an increase in the hydrophobe length, and decreases with increased unsaturation or branching (Zajic and Seffens, 1984). The effectiveness of a biosurfactant is measured by the minimum surface tension value reached. While efficiency depends on the concentration of a biosurfactant at an interface, effectiveness depends on the cohesiveness of its hydrophobic groups (Zajic and Seffens, 1984).

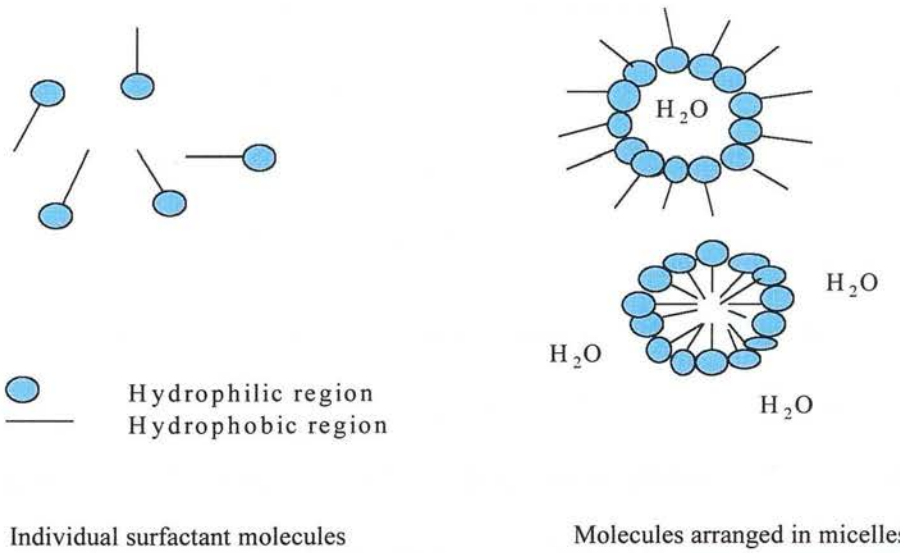


Figure 4.1: Micelle formation by surfactants (Reproduced from Desai *et al.*, 1994)

Five classes of biosurfactant can be distinguished, based on their chemical structure, namely: (i) glycolipids, which are the most common group of biosurfactants, and are composed of carbohydrates with long-chain aliphatic acids or hydroxy aliphatic

acids. Examples, include the extensively studied rhamnolipids, produced by *Pseudomonas aeruginosa* and other *Pseudomonas* species (Guerra-Santos *et al.*, 1986; Koch *et al.*, 1991), and the trehalose lipids from *Rhodococcus erythropolis*; (ii) phospholipids which are found in every micro-organism and are composed of a glycerol unit esterified to two fatty acids and one phosphate group (e.g. the fatty acids produced by *Corynebacterium lepus*, and the fatty acid and neutral lipids produced by *Nocardia erythropolis*); (iii) lipopeptide/lipoproteins, which include surfactin produced by *Bacillus subtilis*, which has been researched as a substitute for synthetic surfactants (Nakano *et al.*, 1988), the antibiotic gramicidin, and viscosin, produced by the head rot pathogen *Pseudomonas fluorescens* (Laycock *et al.*, 1991); (iv) polymeric surfactants which have a high molecular weight and includes the mannan-lipid from the yeast *Candida tropicalis* and other polysaccharide-protein complexes, and (v) particulate surfactants such as the extracellular membrane vesicles in *Acinetobacter* species, composed of protein, phospholipid and lipopolysaccharide vesicles and whole cells (Desai *et al.*, 1994).

Of particular interest to head rot research is the lipopeptide biosurfactant viscosin produced by *Pseudomonas fluorescens* (Laycock *et al.*, 1991). Viscosin was first isolated from *Pseudomonas viscosa* by Kochi (cited in Groupe *et al.*, 1951) and was found to be active against other mycobacteria. It is a cyclic depsipeptide (containing a peptide lactone) with alternating D and L amino acids and an N-terminal D-3-hydroxydecanoyl group (Burke *et al.* 1989). Purified extracellular product containing viscosin was found to reduce the surface tension of water to 27mN/m, and approximately 4-9mg/l is required to reach the CMC (Laycock *et al.*, 1991).

4.3 The role of biosurfactants

The main physiological role of a biosurfactant is to permit growth of micro-organisms on water-immiscible substrates, such as hydrocarbons, by reducing the surface tension at the phase boundary, thus influencing microbial adhesion to the substrate and making it more readily available for uptake and metabolism (Fiechter, 1992).

However, biosurfactant properties have been widely exploited by humans, with the development of chemically synthesised surfactants for emulsification, phase separation, wetting, foaming, solubilisation, de-emulsification, corrosion-inhibition and viscosity reduction in the pharmaceutical, cosmetic, petroleum and food industries (Fiechter, 1992). In agriculture, surface active compounds are used to obtain good wettability of soils necessary for equal distribution of fertilisers (Fiechter, 1992) and to improve the stability, emulsification, and dispersal properties of pesticide sprays (Knoche *et al.*, 1992). Interestingly, biosurfactants are now considered more desirable than industrial surfactants due to the advantageous properties they possess of biodegradability; high specificity; low toxicity; effectivity at extreme conditions of pH, temperature and salinity and ease of production on cheaper, renewable-resource substrates (Desai *et al.*, 1994). Furthermore, it is expected that the demand for biosurfactants will increase by 35% towards the end of the century (Desai *et al.*, 1994), but the main problem is that they are presently economically uncompetitive with their synthetic counterparts (Fiechter, 1992).

4.4 Bacterial surfactant production

Generally, the biosynthetic steps for secondary metabolites, such as biosurfactants, are carried out by enzymes specific for each metabolite, but some have a broad substrate specificity giving rise to a family of related compounds (Martin and Liras, 1989). A picture of the genes involved in synthesis, being organised in clusters on the bacterial chromosome is emerging, and subclustering of genes may control the timing with which enzymes appear in the pathway to ensure that intermediates appear in the proper sequence of biochemical reactions (Martin and Liras, 1989). Genes involved in the biosynthesis of secondary metabolites are not usually expressed at high growth rates, because several synthetases of secondary metabolites are either inhibited or repressed by easily utilisable phosphate, carbon or nitrogen sources during the growth phase in liquid culture. Indeed, many genes are regulated by the phosphate concentration in culture broth and glucose may also have a regulatory role by inhibiting secondary metabolite synthesis (Martin and Liras, 1989). This type of regulation has been observed in several situations. For example, extracellular production of rhamnolipids, occurs with the onset of nitrogen limitation during the stationary phase (Oschner *et al.*, 1994a), and biosurfactant production in *P. fluorescens* requires low oxygen and nitrogen concentrations (Persson *et al.*, 1988). Production ceases when environmental conditions become unfavourable and this allows departitioning of the cell from the interface (Rosenberg, 1986). In the case of the synthesis of antibiotic products (which many biosurfactants are), antibiotic-resistance genes are linked to the biosynthetic genes and are expressed in a co-

ordinated way to avoid the suicide of the producing organism (Martin and Liras, 1989).

Lipopeptides are synthesised non-ribosomally by large multifunctional enzyme complexes situated near to the bacterial membrane, in a process known as the thiotemplate mechanism (Georgiou *et al.*, 1992). This multienzyme thiotemplate system has been described in detail for the decapeptide antibiotic gramicidin S (which has surfactant activity) produced by *Bacillus brevis* by Lipmann (1980). The first step in the formation of gramicidin S is the activation of the constituent amino acids in the form of amino acyl adenylates via adenylation by ATP. The activated intermediates are then covalently attached to specific sites on the gramicidin S synthetase complex of the multienzyme complex by thioester linkages. The amino acid intermediates are arranged on the enzyme in a linear fashion corresponding to the sequence with which they will be incorporated into the growing peptide (Lipmann, 1980). Peptide bond formation and the movement of the growing peptide chain from one position of the thiotemplate to the next is catalysed with the aid of a 4' phosphopantetheine cofactor. The cofactor functions as an internal swinging arm to mediate the transport of the growing peptide between sites of attachment of the amino acids. Often the pathway concludes with a cyclization that result in the formation of a cyclic polypeptide (Nakano *et al.*, 1992).

The lipopeptide biosurfactant, surfactin, produced by *Bacillus subtilis* is one of the most studied biosurfactants and it is known to be synthesised by a multienzyme thiotemplate mechanism in the stationary phase of growth (Kluge *et al.*, 1988).

Surfactin has been shown to reduce the surface tension from 72 to 27mN/m (Nakano *et al.*, 1988). Three loci are involved in surfactin synthesis; *srfA*, which encodes specific enzymes, *srfB* which contains *comP* and *comA* genes involved in a signal transduction system (Nakano *et al.*, 1992), and *sfp* which is responsible for the activation of the synthetase (Lambalot *et al.*, 1996). *In vitro* synthesis of surfactin by a cell free system requires ATP, Mg^{+2}, precursors and sucrose (Georgiou *et al.*, 1992). Both the lipid and peptide domains are directly synthesised from carbohydrates. Addition of amino acids or fatty acids in the growth medium can affect yield, but not the structure of the product (Georgiou *et al.*, 1992). Syringotoxin, from *P. syringae* pv. *syringae*, is also produced by a thiotemplate mechanism, with the toxin being ejected from the cell before it can damage any cell component (Zhang *et al.*, 1995).

Rhamnolipid synthesis, by *P. aeruginosa*, is known to proceed by sequential glycosyl transfer reactions. This involves two genes *rhl*A and *rhl*B that encode a rhamnosyltransferase responsible for catalysing the transfer of rhamnose from TDP-rhamnose to β hydroxydecanol-β-hydroxydecanoate (Oschner *et al.*, 1994a). A regulatory gene *rhl*R encodes for a transcriptional activator protein RhlR that positively regulates rhamnolipid synthesis (Oschner *et al.*, 1994b). A *rhl*I gene had been identified that encodes for an autoinducer that binds and activates the RhlR protein (Oschner and Reiser, 1995). Guerra-Santos *et al.* (1986) found better yields of rhamnolipids were obtained when the concentration of magnesium, calcium, potassium, sodium and the trace elements were kept to a minimum, and glucose was increased to 73g/l. The optimum pH for production was 6.25 and the optimum

temperature was between 31°C and 34°C (Guerra-Santos *et al.*, 1986). Production of extracellular rhamnolipids is strictly regulated with maximal production being found during the stationary phase of growth (Oschner *et al.*, 1994b).

Unfortunately, little is known about the factors governing viscosin synthesis, although it is likely that it is regulated by a thiotemplate mechanism especially because it is a cyclic depsipeptide containing D and L form amino acids, as well as some non-protein amino acids, which cannot be synthesised ribosomally. Notably, surfactant production can be unstable in liquid culture, as viscosin was found to be metabolised after about 48h and then only low levels remain (Hildebrand, per. communication), so this must be taken into account when carrying out biosurfactant studies. However, head rot pathogens may produce biosurfactants other than viscosin. For example, *Pseudomonas fluorescens* was found to produce a rhamnolipid when grown on virgin olive oil (Healy *et al.*, 1996) and Burd and Ward (1996) describe a biosurfactant known as PM Factor produced by *P. marginalis,* containing lipopolysaccharides, protein and phospholipids. In addition, Hildebrand (1989) has described head rot biosurfactants which are not haemolytic like viscosin (Ullrich *et al.*, 1991).

Carbon source plays an important role in yield and structure of microbial surfactants. Some micro-organisms only produce them when grown on hydrocarbons and others require simple, water soluble substrates such as carbohydrates and amino acids (Georgiou *et al.*, 1992). Viscosin production by *P. fluorescens* is optimum *in vitro* on a medium containing glycerol, as the carbon source for its synthesis (Georgiou *et*

al., 1992). Desai *et al.* (1988) noted that for *P. fluorescens,* maximum biosurfactant yield on hydrocarbons was obtained with gasoline as a substrate, and the biosurfactant produced on different hydrocarbons sources differed quantitatively in carbohydrate and protein content, indicating various types of biosurfactants may be produced by a bacterial species depending on the carbon source available (Desai *et al.*, 1988). Moreover, Persson *et al.* (1988) noted that *P. fluorescens* strain 378 produced biosurfactant optimally at a pH of 8, with the yield varying considerably depending on pH.

Another factor which may affect biosurfactant production by plant pathogenic bacteria is the plant environment. Syringomycin, is known to be stimulated by plant metabolites because the *syr*B gene, involved in its production, is strongly induced by the plant phenolic compounds arbutin, phenyl-β-D-glucopyranoside and salicin, while esculin and helicin are moderate inducers (Mo and Gross, 1991). Furthermore, certain mutants of *Pseudomonas syringae* pv. *syringae*, which no longer produced syringomycin *in vitro*, were found to produce it *in planta* (Quigley and Gross, 1994) possibly due to the activation of its genes by a specific plant metabolite. Other examples of genes being induced by plant metabolites can be found. Virulence genes of *Agrobacterium tumefaciens*, such as the gene for acetosyringone, are activated by phenolic compounds and sugar molecules, and in *Rhizobium*, flavanoids are required for induction of nodulation (Sigee, 1993). Plant induction of genes is also involved in the hypersensitive response elicited by *P. syringae* pv. *vesicatoria* (Rahme *et al.* 1992, cited in Sigee, 1993). Control of virulence factors by plant signal molecules

ensures that the bacterium is attuned to a dynamic plant environment governing disease development (Mo and Gross, 1991).

4.5 Biosurfactant activity on the plant surface

Little is known of the role biosurfactants play in head rot disease aetiology, but research is expanding into biosurfactant-plant interactions in general.

Bunster *et al.* (1989) found that strains of *P. fluorescens* and *Pseudomonas putida* with surface-active properties, significantly increased leaf wettability when inoculated on to wheat leaves. They suggested biosurfactants may increase the duration of leaf wetness, and affect the abundance, distribution and attachment of micro-organisms present on plant tissue, thus having implications for bacterial and fungal disease development (Bunster *et al.*, 1989). Netting and von Wettstein-Knowles (1973, cited in Bunster *et al.*, 1989) claimed that wettability was determined by the chemical nature of the surface wax, the shape of the wax crystals, and the roughness of the leaf surface - possibly broccoli cultivars differ in their wax structure, predisposing certain varieties to infection, assisted by surfactant-induced wetting? However, Rentschler (1971) described that a comparison of wettability and the different structures of wax showed no true distinctions. Environmental factors which influence leaf wettability include weathering, differences in turgidity of the whole plant, and the presence of surfactants in agrochemicals (Bunster *et al.*, 1989). Moreover, Hildebrand (1989) suggests that increased leaf wettability enhances bacterial colonisation of broccoli tissue and stated that biosurfactants may induce watersoaking, thereby aiding head rot disease progression (Hildebrand, 1989). In addition, he found biosurfactant-producing strains of *P. fluorescens* caused solute leakage and black lesions to

develop around the guard cells of stomata, which were only induced by surfactant-producing strains, suggesting that a surfactant-mediated mechanism of stomatal entry may occur (Hildebrand, 1989). The biosurfactant responsible was found to be the lipopeptide viscosin (Hildebrand *et al.*, 1990), and therefore it was claimed that viscosin enables pectolytic enzymes to attack the otherwise hydrophobic waxy tissues of broccoli, to induce stomatal lesions (Hildebrand, 1989; Hildebrand *et al*, 1990). However, epicuticular wax was unaltered on plants exposed to viscosin (Hildebrand *et al*, 1990). Interestingly, black lesions were also found by Gladstone (1997) after inoculating broccoli with either a biosurfactant-producing strains of *P. fluorescens* or purified viscosin, but in contrast the wax crystals on the plant's surfaces were also found to be damaged. The exact mechanism of these biosurfactant interactions are unknown. Although both researchers studied head rot of broccoli with purified viscosin, the cultivars used may have varied with certain cultivars having wax which provides less defence against the toxicity of surfactants. In support of Gladstone (1997), Bussotti *et al.* (1997) noted that surfactant molecules, absorbed through the stomata of pine needles, caused a deterioration in the epistomatal wax structures, and Schreiber *et al.* (1995) demonstrated that the surfactant Brij 30 had a reversible plasticizing effect on the cuticle wax permeability of *Prunus laurocerasus*, which they suggested may be due to a physical alteration in the wax structure or solubilisation of wax molecules in the micelles of the surfactant. Possibly this reversibility is a common feature of all surfactants and could explain why Hildebrand *et al.*, (1990) found no alterations in the surface wax of plants. Support for Hildebrand *et al.* (1990), comes from Bunster *et al.* (1989) who described wettability as reflective of

the chemical nature of the wax, indicating that certain forms of wax are more resistant to the harmful effects of biosurfactants than others. In addition, there was no evidence that this altered the wax structure. Furthermore, Knoche *et al.* (1992) found disruption or removal of epicuticular wax significantly changed the ethylene oxide-dependent toxicity pattern, indicating that epicuticular wax plays an important part in the toxicity pattern of *Brassica* species.

Viscosin is structurally similar to the lipopeptide biosurfactants, syringomycin and syringopeptin, which are toxins produced by *Pseudomonas syringae* pv. *syringae*, and are known to be virulence factors causing lesions in plant tissue (Hutchison *et al.*, 1995). The toxin, tolaasin, produced by *Pseudomonas tolaasii* also has biosurfactant activity. Toxins are non-enzymatic, metabolic, virulence factors which are released by the pathogen to chemically injure the host. They have a wide range of physiological and biochemical effects on the host causing symptoms such as chlorosis, water-soaking, necrosis, growth abnormalities and wilting (Sigee, 1993). Furthermore, syringomycin causes an influx of H^+ and Ca^{2+} ions and an efflux of K^+ ions, leading to a lowering of cytoplasmic pH and stomatal closure, which results in cell death and provides nutrients for the bacteria. Although the majority of pathogenic effects occur below the CMC, indicating that biosurfactant-activity alone is not crucial to pathogenesis, Hutchison *et al.* (1995) suggest that the evaporation of water film on plant surfaces may concentrate the toxin and thereby enhance the surfactant-activity. It is thought that the biosurfactant-activity also facilitates the spread of bacteria on the plant surface by reducing the surface tension of water (Hutchison and Gross, 1997). This is the mode of action for tolaasin which causes

water to spread over the surface of mushroom tissue, due to its surfactant properties (Hutchison and Johnstone, 1993). Although, as the water evaporates and concentrates the biosurfactant, conditions may become less favourable for bacterial growth, there may be a trade off between the increased ability of bacteria to spread over the plant surface and reducing the time available for infection.

In a model proposed for the phytotoxic effects of syringomycin's biosurfactant activity, Hutchison *et al.*, (1995) suggested that at low syringomycin concentrations (below the CMC), individual monomers of the toxin may aggregate in the lipid bilayer to form functional pores (individual monomers are unable to form pores) (Hutchison *et al.*, 1995). Moreover, they claim that if the toxin level increases above the CMC, micelles may fuse with the plasma membrane leading to non-specific membrane solubilisation, giving the toxin's biosurfactant activity a dominant role in the membrane attack. However, as syringomycin has a CMC of 1.25mg/ml, it seems unlikely that this concentration would be exceeded often, so the role of syringomycin's biosurfactant activity *in planta* requires further analysis.

4.6 Effect of industrial surfactants on plant tissue

Industrial surfactants have been found to be phytotoxic, inducing lesions on plants which allow bacteria to enter (Knoche *et al.*, 1992). This broad mode of action may be shared with some of the bacterial biosurfactants. For example, Bussotti *et al.* (1997) studied the effect of the anionic surfactant, sodium alkylbenzene sulphonate (SABS), on the anatomy and ultrastructure of *Pinus pinea* L. needles after spraying the trees with an aqueous solution containing 1kg/l. Within a few days, needles had turned yellow and it was found that the surfactant had been absorbed through the

stomata, inducing alterations in the stomatal guard cell walls. This reflects the results of Hildebrand (1989) which indicated that bacterial biosurfactants induced stomatal guard cell damage. The SABS also caused degeneration of the protoplasm in the mesophyll cells, beginning with cells closest to the stomatal opening and consequently the efficiency of both photosynthesis and gaseous exchange may have been affected (Bussotti *et al.*, 1997). Further evidence for the phytotoxic effects of surfactants was demonstrated by Johnson *et al.* (1996) who found the addition of a synthetic surfactant to a suspension of the phytopathogen *Pseudomonas syringae* pv. *putagetis,* enabled the bacterium to induce disease on unwounded weed tissue, where previously it had been unable to penetrate. Interestingly, however, Falk *et al.* (1994) treated the leaves of three species of weed, with nine different surfactants, at a 0.1% w/v concentration, and found tissue damage occurred after 24h, but the effects of a particular surfactant were not uniform across the three weed species, indicating that tissue composition strongly determines surfactant phytotoxicity.

Solubilisation of membranes and interactions with proteins were found to be the primary factors causing necrosis and tissue damage after surfactant application (Helenius and Simons, 1975). Industrial surfactants with medium length oxyethylene chains were found to be the most phytotoxic towards *Brassica oleracea* var. *gemmifera,* which was hypothesised to be due to their ability to penetrate its tissue rather than actual cellular phytotoxicity (Knoche *et al.*, 1992). Coupland *et al.* (1989) reported that surfactants, causing growth inhibition and decreased CO_2 fixation, could be ranked in order of toxicity depending on their chemical charge,

with the order being cationic > anionic > non-ionic, and toxicity was also found to decrease with an increase in hydrophobe size.

As mentioned previously, as hydrophobe length increases, the efficiency increases (concentration required to form micelles) (Desai *et al.*, 1994) and so the toxicity decreases (according to Coupland *et al.*, 1989). This suggests that surfactants are most phytotoxic when they are less efficient, i.e. require large quantities to form micelles, and is based on the idea that toxicity results from the direct insertion of surfactant molecules into the membrane. In addition to the work of Coupland *et al.* (1989) and Knoche *et al.* (1992), this theory is supported by Silcox and Holloway (1986) who evaluated surfactant toxicity towards plants and found that the degree of toxicity was found to be inversely proportional to the ethylene oxide content, so the shortest chain length was the most phytotoxic.

Possibly hydrophobe length is the critical factor in determining biosurfactant phytotoxicity, with shorter hydrophobe surfactants being able to insert into, and disrupt, the plasma membrane more successfully. However, Hutchison *et al.* (1995) proposed that micelles insert directly into the plasma membrane causing non-specific solubilisation, and as more efficient surfactants form micelles at lower concentrations they would therefore be more phytotoxic. Therefore this suggests that more efficient biosurfactants are more phytotoxic, than less efficient ones, which contradicts the authors mentioned above (i.e. Coupland *et al.*, 1989; Knoche *et al.*, 1992; Silcox and Holloway, 1986).

Although, there is considerable evidence demonstrating the phytotoxic effects of surfactant molecules, they can also positively influence plant growth depending on the type and concentration of a given surfactant (Lang and Wagner, 1993). For example, microporous membranes treated with an unspecified surfactant are commercially available to stimulate the growth of cultured plant cells by facilitating nutrient supply (Sigma Plant Cell Culture Catalogue, 1990). Furthermore, Kumar *et al.* (1992) found that a 0.01% (w/v) concentration of the non-ionic surfactant, Pluronic F68, resulted in an increase in the fresh weight of the roots of *Solanum dulcamora* after 5 days incubation. The growth of callus was also stimulated and it was found that both protein and carbohydrate levels increased. Furthermore, malate dehydrogenase (MDH) and acid phosphatase (APase) activity were raised, indicating an altered biochemical status of the exposed tissues. Malate dehydrogenase is a key enzyme involved in the oxidation of carbohydrates via tricarboxylic acid cycle, enhancing substrate utilisation and therefore growth, while APase increases cellular phosphate utilisation and metabolism. However, at lower or higher surfactant concentrations growth was inhibited, indicating that surfactants can only be beneficial to plant growth at specific concentrations. These results must be treated carefully since they may not relate to the effects observed on whole plants.

4.7 The antibiotic effects of biosurfactants

Certain biosurfactants are also known for their antibiotic properties, having inhibitory effects on bacteria, filamentous fungi and yeasts, due to their ability to bind hydrophobic molecules, and to partition within membranes (Georgiou *et al.*, 1992). If a pathogen possesses antibiotic properties, competing microbes may be eliminated

allowing the pathogen to flourish and initiate disease, while a saprophytic strain with such activity may prevent pathogenic strains from causing disease and so would be a potential biocontrol agent. For example, the rhamnolipid biosurfactant of *P. aeruginosa* solubilises the cell envelope components of competing microbes (Georgiou *et al.*, 1992; Stanghellini and Miller, 1997). Haferburg *et al.* (1987) investigated the application of rhamnolipids with regard to antiphytoviral activity. The use of a 1% emulsion of rhamnolipid to treat leaves *of Nicotiana glutinosa* infected with Tobacco Mosaic Virus (TMV) led to a 90% reduction in the number of lesions, and in another experiment, 1% rhamnolipid decreased the Potato Virus X content of systemically infected *Nicotiana tabacum* by 46% and secondary leaves by 43% (Haferburg *et al.*, 1987). Therefore this demonstrates that the surfactant is either stimulating the plant's resistance mechanisms or directly affecting the virus. Stanghellini and Miller (1997) tested rhamnolipids from *P. aeruginosa*, ranging in concentration from 5 to 30μg/ml, against three genera of zoosporic plant pathogens: *Pythium aphanidermatum*, *Phytophthora capsici* and *Plasmopara lactucae-radicis*. All were immobilised and lysis of the entire zoospore population occurred in less than one minute (Stanghellini and Miller, 1997). It was suggested that lysis occurred due to the rhamnolipid intercalating and disrupting the plasma membrane. Moreover, surfactin from *Bacillus subtilis* has antifungal and anti-tumour activity which represses the growth of micro-organisms belonging to the genus *Mycobacterium* (Lang and Wagner, 1993), and Asaka and Shoda (1996) found that surfactin and iturin A (another lipopeptide produced by *B. subtilis*), suppress damping-off of tomato seedlings caused by *Rhizoctonia solani*.

Viscosin, produced by *P. viscosa* and *P. fluorescens*, was found to be active against various pathogenic and saprophytic mycobacteria and bronchitis virus of chickens, but inactive against representative species of other groups of bacteria (Groupe *et al.* 1951). Biosurfactants produced by the broccoli head rot pathogen *P. fluorescens*, (which includes viscosin) have recently been found to lyse fungal zoospores (J. Ligon, Pers. communication), and Gladstone (1997) found viscosin decreased spore germination of *Fusarium poae* and *Botrytis cinerea* maximally at a concentration of 150μg/ml and significantly reduced germ-tube length. The biosurfactant was found to have no effect on the growth of white yeasts, but caused a slight reduction in the growth of *Flavobacter* (Gladstone, 1997). It is possible that biosurfactant production by the head rot pathogen reduces competition from other microbes, enabling them to induce disease.

Industrial surfactants may also alter the niche of epiphytic micro-organisms present on leaf surfaces, or the phyllosphere environment in a similar manner proposed for biosurfactants by Bunster *et al.* (1989). For example, as Young and Kauss (1984) found the addition of the surfactant Tween 20 interfered with the hydrophobic attachments of the pathogenic fungi, *Colletotrichum lindemuthianum*, to bean (*Phaseolus vulgaris* L.), and so possibly biosurfactant producing bacteria have a competitive edge over non-producers. In general, anionic and non-ionic surfactants are less toxic and more active against gram positive bacteria, than gram negative bacteria because the protein and lipopolysaccharide moieties of the gram negative

bacteria's cell wall protects their cell membranes from surfactant attack (Lang and Wagner, 1993).

4.8 Biosurfactants and head rot

Although Hildebrand (1989) found that co-inoculating biosurfactant-producing strains with pectolytic strains led to disease development in unwounded heads, pathogenic strains of head rot bacteria produce varying amounts of biosurfactants (unpublished observation), and some strains (pathogenic to unwounded heads) produce no biosurfactant in culture (Harling *et al.*, 1994). It is possible that residual bacteria on the excised heads used to determine pathogenicity fulfilled this purpose (as head sterilisation is not possible), but this seems unlikely given the experimental controls used. This suggests that biosurfactants are not essential pathogenicity factors in head rot, but they may still be involved in virulence, enhancing disease severity i.e. pathogenicity factors such as pectolytic enzymes may induce disease symptoms, but the presence of biosurfactants may enhance the disease severity. Therefore, the role of biosurfactants in head rot requires clarification, and so research into biosurfactant production and activity will be conducted in Chapters 5 and 6.

Chapter 5: Development of a sensitive test to detect biosurfactants from head rot pathogens

5.1 Introduction

This chapter presents results on the production of biosurfactant in pathogenic *P. fluorescens* strains, and assesses the sensitivity of various biosurfactant detection tests.

5.1.1 Biosurfactant production

The *P. fluorescens* isolates used in this study produce the biosurfactant viscosin (Harling and Dubickas, 1998 unpublished), as do the broccoli isolates of Laycock *et al.* (1991). There is evidence that our isolates produce at least one other biosurfactant type (Harling and Dubickas, 1998 unpublished). This reflects the findings of Hildebrand (pers. comm.) and Healy *et al.* (1996) who found that *P. fluorescens* produced a non-haemolytic and a rhamnolipid biosurfactant, respectively. Therefore there may be more than one biosurfactant type involved in head rot.

To summarise the important aspects of biosurfactant production (which were discussed fully in Chapter 4), each biosurfactant produced by the head rot pathogens may be synthesised via a different metabolic pathway, but in the case of viscosin, it is likely that its synthesis is via a thiotemplate mechanism due to the constraints of its cyclic depsipeptide structure (Chapter 4). Culture conditions considerably affect biosurfactant production *in vitro*. In *P. fluorescens*, production generally requires low oxygen and nitrogen concentrations in liquid culture (Persson *et al.*, 1988) presumably due to the secondary metabolite synthetases being repressed by phosphate, carbon or nitrogen sources during logarithmic growth (Martin and Liras, 1989). Carbon source is equally important, affecting the biosurfactant type

produced, as Desai *et al.* (1988) noted that in *P. fluorescens* the biosurfactants produced differed quantitatively in carbohydrate and protein content, depending on the insoluble carbon source available. Viscosin production is optimal *in vitro* with a glycerol carbon source (Georgiou *et al.*, 1992). Other components of liquid medium, such as ATP, Mg^{+2}, amino acids, fatty acids and sucrose have been shown to affect the yield, but not the structure, of the biosurfactant surfactin (Georgiou *et al.*, 1992) but no information is available for head rot biosurfactants. Guerra-Santos *et al.* (1986) found rhamnolipid yield improved when the concentration of magnesium, calcium, potassium, sodium and the trace elements were kept to a minimum, and glucose was increased to 73g/l. pH also considerably affects yield, with one strain of *P. fluorescens* producing optimally at pH8 (Persson *et al.*, 1988), while *P. aeruginosa* favoured pH6.25 (Guerra-Santos *et al.*, 1986). However, the plant environment may also influence biosurfactant production: Syringomycin is known to be stimulated by the plant metabolites arbutin, phenyl-β-D-glucopyranoside and salicin, while esculin and helicin are moderate inducers (Mo and Gross, 1991). Therefore it is important to consider the effect of environmental conditions when studying biosurfactant production.

5.1.2 Biosurfactant assays

To study biosurfactant production requires an accurate, rapid and sensitive test for their detection. Various tests have been utilised for detecting and/or quantifying the biosurfactants produced by different bacteria, with the most popular methods briefly described below:-

(a) Water droplet test

Hildebrand (1989) placed a loopful of bacteria from an agar colony into a water droplet and noted the reduction in surface tension brought about by biosurfactants. This water droplet method is rapid, requiring no specialised equipment, but sensitivity may be a problem if low biosurfactant levels are present, and it is not quantitative.

(b) Haemolysis of blood agar

As many biosurfactants lyse erythrocytes, by forming plasma membrane pores, a method has been developed where bacteria are inoculated on to agar plates containing blood, and then biosurfactant production is detected by zones of haemolysis which develop around the colonies (Mulligan *et al.*, 1994; Koch *et al.* 1991). Viscosin is haemolytic (Hildebrand, pers. comm.) so this method may be suitable for testing biosurfactant production by head rot pathogens. It is simple to implement and is not labour intensive, but as it requires specific, complex media, productivity cannot be tested under different culture conditions (Siegmund and Wagner, 1991). In addition, the type of biosurfactant produced cannot be determined and lytic enzymes, which may also be produced, induce the same reaction. Pseudomonads, in particular, contain β hemolysins that lyse blood cells and may produce inaccurate results (Siegmund and Wagner, 1991). Non-haemolytic biosurfactants cannot be detected.

(c) Siegmund and Wagner agar

Glycolipid biosurfactants, such as rhamnolipids excreted by *Pseudomonas* species, can be detected by simply plating them on to a medium developed by Siegmund and Wagner (1991). The anionic nature of glycolipids means that they form an insoluble

ion pair with the cationic chemical cetyl-trimethylammonium bromide (CTAB), and the basic dye, methylene blue, which is present in the agar, leading to the development of dark blue halos around producing colonies (Siegmund and Wagner, 1991). This method is semi-quantitative as the diameter of the coloured halo increases with surfactant concentration, but only glycolipids can be detected. This test could be used to determine whether the head rot strains of *Pseudomonas fluorescens* produce any such biosurfactants, as reported by Healy *et al.* (1996).

(d) Hydrocarbon utilisation

Most biosurfactants solubilise water immiscible hydrocarbons, to sustain growth, and this ability has been exploited to determine the presence of biosurfactant producers. Koch *et al.* (1991) screened for rhamnolipid-minus mutants of *Pseudomonas aeruginosa* PG201 in this way and found that growth was only obtained if purified rhamnolipids were supplemented. *Pseudomonas fluorescens* has been described as a potent hydrocarbon degrader (Persson *et al.*, 1988) so this method may be successfully adapted for head rot research.

(e) Measurement of contact angles

Bunster *et al.* (1989) studied the surface-activity of *P. fluorescens* and *Pseudomonas putida* strains, by measuring the contact angles obtained after adding the bacteria to a water droplet. The contact angle is located at the interface between the droplet and the solid surface. In the absence of surfactant, water molecules adhere strongly to each other and so the water droplet retains a round appearance with a contact angle of $>90^{\circ}$, while in the presence of biosurfactants the adherance forces reduce causing the droplet to spread out flat creating a contact angle of $<90^{\circ}$. Bunster *et al.* (1989)

used a 5µl aliquot from a broth suspension to form a droplet and measured the contact angle obtained. This method has the advantage of being quantitative and will detect all types of biosurfactant produced, although it may lack sensitivity towards low biosurfactant levels.

(f) Thin layer chromatography

Matsuyama *et al.* (1987) identified *Serratia macrescens* biosurfactants by thin layer chromatography (TLC) of a single colony. Bacteria were applied directly to the TLC plate, avoiding the need for sample preparation. This method may be applicable for the biosurfactants produced by *P. fluorescens*, but would be relatively time-consuming compared to the other methods described.

(g) Surface tensiometry

Testing a solution's surface tension with a surface tensiometer (Du Nuoy) measures the force required to detach a wire loop from the liquid's surface, leading to an accurate quantification of the biosurfactant present. However, the biosurfactant to be studied requires initial purification to obtain experimental standards for comparison and therefore it is time consuming, and furthermore, it requires technical expertise. In addition, the instrument is expensive to purchase.

Problems which may arise with many of the biosurfactants detection methods described include: The requirement for prior isolation and characterisation of the biosurfactant; an insufficient quantity of biosurfactant present for successful detection (Koch *et al.*, 1991), and ingredients in the media and bacterial membranes interfering with the results because they too have surface active properties.

5.1.3 Aims

Biosurfactant production, in three *P. fluorescens* strains, was studied over the course of bacterial growth to determine: When production starts; how long it continues for, and the similarity between different head rot strains. Since different biosurfactants may be produced simultaneously by these strains, detection of general surface activity is required. Subsequently, the following five biosurfactant assays were evaluated for sensitivity: 1) a water droplet test; 2) a blood agar test; 3) Siegmund and Wagner agar test; 4) a hydrocarbon utilisation test, and 5) a contact angle measurement test. The results obtained allowed for more detailed research into head rot biosurfactants to be undertaken in Chapter 6.

5.2 Materials and methods

5.2.1 Bacterial strains studied

Bacterial strains used included the pathogenic biosurfactant-producers (previously determined by the water droplet test): *P. fluorescens* Group IVb (Lelliot and Stead, 1987) isolates P5038, P5049 and P5064; the non-pathogenic biosurfactant producer *P. fluorescens* Group Vb isolate N5024, and the non-producing pathogen *Erwinia carotovora* ssp. *atroseptica* P5067. All were previously isolated from diseased broccoli heads and re-inoculated on to healthy heads to test pathogenicity (Robertson *et al.*, 1993; Campbell *et al.*, 1995). Strains were maintained on KB agar prior to testing to ensure abundant biosurfactant production. Table 5.1 shows which strains were used in each experiment.

Table 5.1: Bacterial isolates used in each experiment carried out in Chapter 5

Bacterial Isolate	Pathogenic	Biosurfactant producer	Experiment					
			Dynamics of biosurfactant production	Biosurfactant tests				
				Water droplet	Blood Agar	SW agar	Hydro-carbon Test	Contact Angle
P5038	✓	✓	✓	✓	✓	✓	-	-
P5049	✓	✓	✓	✓	✓	✓	✓	✓
P5064	✓	✓	✓	-	-	-	-	-
P5067	✓	-	-	✓	✓	✓	-	✓

N.B. The negative control in all tests, except for the hydrocarbon utilisation test, was to test a non-biosurfactant producer. In the hydrocarbon utilisation test no bacterial inoculum was used.

5.2.2 Bacterial growth and biosurfactant production

Production was studied over time in three *Pseudomonas fluorescens* isolates (Table 5.1). A 0.1ml (3.5×10^8 cells) aliquot from an overnight suspension was inoculated into 100 ml KB broth, in a 250ml flask, and incubated at 25°C (±2°C) with orbital shaking at 200 rpm. Three replicate flasks were used. The OD_{500nm} was recorded

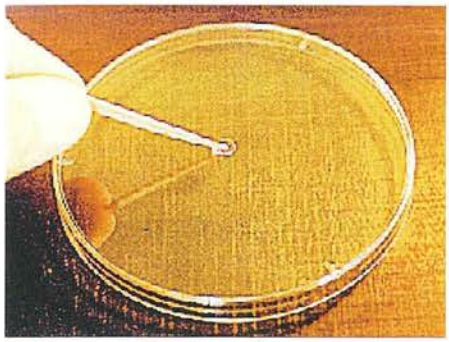
immediately on inoculation and then every 4h until late stationary phase and at every reading a 20µl droplet of suspension was tested for the presence of surfactant using the water droplet method described below (Section 5.2.3i).

5.2.3 Comparison of five test methods for biosurfactant detection in *P. fluorescens*

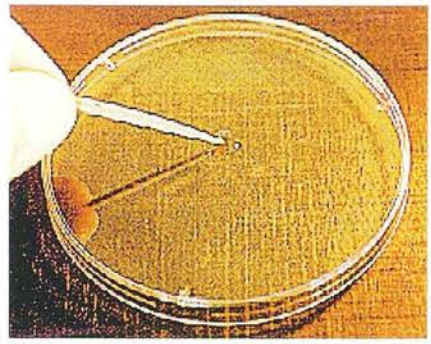
Two biosurfactant producers and one non-producing strain were used, unless specified (Table 5.1). The five methods evaluated for biosurfactant detection are described below:

5.2.3 (i) Water droplet test

This was based on the method of Hildebrand (1989). After 48h growth on KBA, bacteria were transferred using a toothpick from a bacterial colony to a 50µl water droplet situated on a plastic petri dish. Bacteria from 48h liquid cultures were also tested by pipetting 20µl (7.0×10^7 cells) of suspension on to the 50µl droplet. Twenty replicates were carried out for each treatment. If biosurfactants are produced the surface tension of the droplet reduces rapidly (Plate 5.1), although the rate of reduction depends on the biosurfactant's effectivity (cohesiveness of it's hydrophobic groups) and the quantity produced.



a) No biosurfactant present



b) Biosurfactant present

Plate 5.1: Effect of adding a biosurfactant-producing strain of bacteria to a water droplet
a) No biosurfactant present, and b) biosurfactant present

5.2.3 (ii) Blood agar plates

This was based on the method used by Koch *et al.* (1991). Bacteria (48h old) from both plate and liquid cultures were inoculated on to KBA containing 5% horse blood (Oxoid) to test for haemolytic activity, which results in zones of clearance around biosurfactant-producing colonies. Luria-Bertani (LB; Bertani G, 1952) agar plus 5% blood was tested as a control because glycerol is absent from this medium and so theoretically viscosin production should be prevented. Consequently, the presence of any other biosurfactants or lytic substances produced should be determined. Bacteria were transferred from KB plate cultures, using sterile toothpicks, and placed directly on to the agar and for the liquid suspensions, either 30µl (1.05×10^8 cfu) or 60µl (2.1×10^8 cfu) of suspension was pipetted on to sterile

1.5cm^2 pieces of 0.45μm nitrocellulose membrane (Whatman) which were then placed on to the agar. This was to maintain the bacteria within localised regions which would allow a comparison between the agar exposed to bacteria and that which was not. Bacteria grew on to the agar from the filters. Two plates were used for each treatment, with 10 replicates on each plate. Plates were incubated at 25°C (±2°C) and monitored for zones of clearance every 24h for 14 days.

5.2.3 (iii) Siegmund and Wagner agar

SW agar was made according to Siegmund and Wagner (1991). Both liquid and plate bacterial cultures (48h old) were inoculated as described for the blood agar plates (Section 5.2.3ii). Two plates were used for each treatment, with 10 replicates on each plate. Plates were incubated at 25°C (±2°C) and monitored every 24h for 14 days, for the development of colour halos to indicate biosurfactant activity.

5.2.3 (iv) Hydrocarbon utilisation method

Two hydrocarbon methods were tested on *P. fluorescens* isolate P5049: (a) an agar plate method, and (b) a flask method. The insoluble carbon sources tested included toluene, petroleum ether, paraffin and a mixture of toluene and paraffin. Controls were carried out with either no carbon source present or no bacterial inoculum.

(a) Hydrocarbon - agar method

Agar plates containing 25ml Ayer's minimal medium (MM) (Ayers *et al.*, 1919) (which contains no organic nutrients and is used for carbohydrate utilisation tests) were supplemented with 2% of insoluble carbon source and inoculated with 100μl of a 48h KB suspension (3.5 x 10^8 cells). The carbon source, including toluene; liquid paraffin; petroleum ether and hexadecane, was added after autoclaving the agar and

allowing it to cool to 55°C. Plates were incubated at 25°C (±2°C) and monitored every 24h, for 7 days, for bacterial growth. Three replicate plates were carried out for each hydrocarbon.

(b) Hydrocarbon - flask method

Based on the method of Koch *et al.* (1991), bacteria were cultured in either minimal medium (MM) or KB broth (excluding the glycerol carbon source), with the addition of a hexadecane carbon source. Two MM were tested: Guerra-Santos MM (Guerra-Santos *et al.*, 1986) which is specifically designed for high biosurfactant production in *P. aeruginosa,* and Ayer's MM.

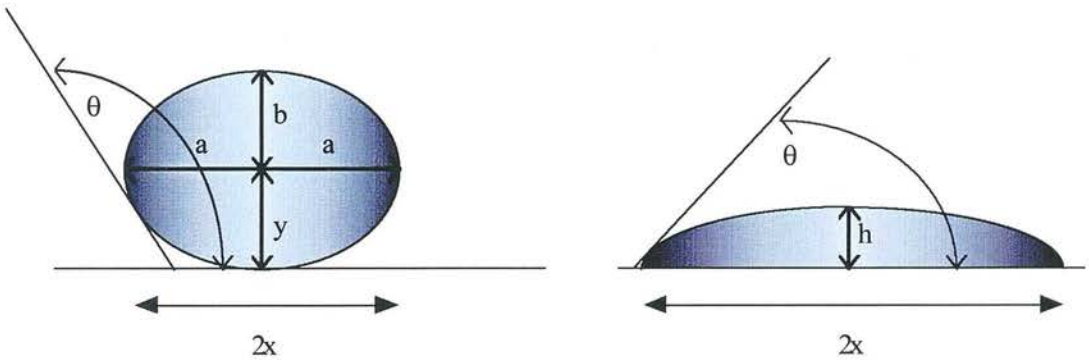
Flasks (250 ml) containing 100ml of broth had either 2%, or 4%, hexadecane added by filter sterilising it through a 0.2µm syringe filter. Either 500µl (1.75×10^9 cells) or 1000µl (3.5×10^9 cells) of bacterial suspension was added from a 48h KB broth culture. Cultures were either shaken at 200rpm or left static (three replicates each) and incubated at 25°C (±2°C). Growth was recorded by measuring OD_{500nm} immediately and then after 24, 48 and 72h.

5.2.3 (v) Contact angle measurement method

Based on a modified version of Bunster *et al.* (1989), a 50µl droplet of sterile water was placed on a $2cm^2$ piece of plastic derived from a disposable weighing boat. Twenty microlitres of bacterial suspension (7.0×10^7 cells) from a 48h KB broth culture, of either *P. fluorescens* P5049 or *Erwinia carotovora* P5067, was pipetted on to the droplet and the suspension was allowed to rest for two minutes. The droplet's height and width was recorded before and after the addition of bacterial suspension using an ocular micrometer in the eyepiece tube of a stereomicroscope.

The microscope was positioned on its side to view the droplet's height. Contact angles were measured every 4h from inoculation through to 80h growth.

If surfactants are present the droplet will collapse altering the shape of the droplet and its contact angle (Figure 5.1).



θ >90° (before biosurfactants) θ <90° (after biosurfactants)

Contact angle equations: $\theta = 180^{\circ} - \text{arc tg } (x/y.b2/a2)$ $(\theta > 90^{\circ})$

$\theta = 2\text{arc tg } (h/x)$ $(\theta < 90^{\circ})$

where: arc tg = tangent of the angle

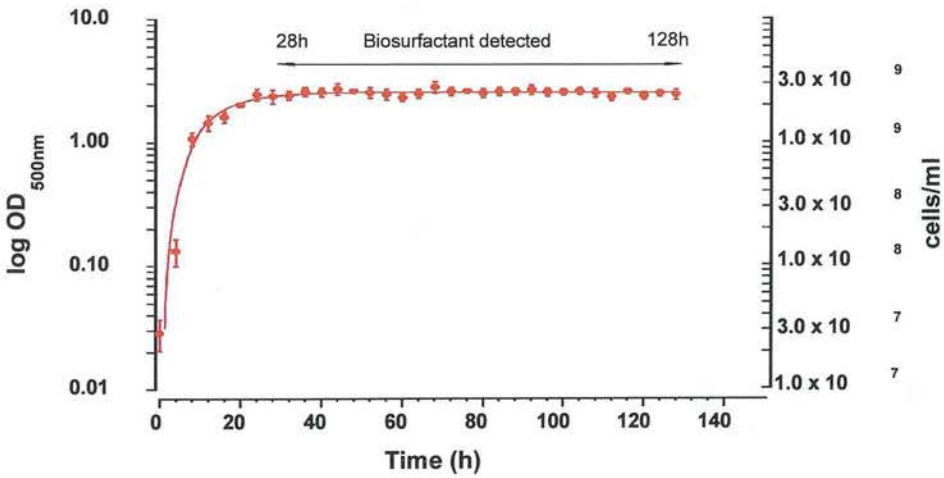
Figure 5.1: Effect of biosurfactants on the shape of a water droplet (Source: Rentschler, 1971)

Three replicates were carried out for both isolates at each reading. The contact angles were then calculated from the measurements recorded.

5.3 Results

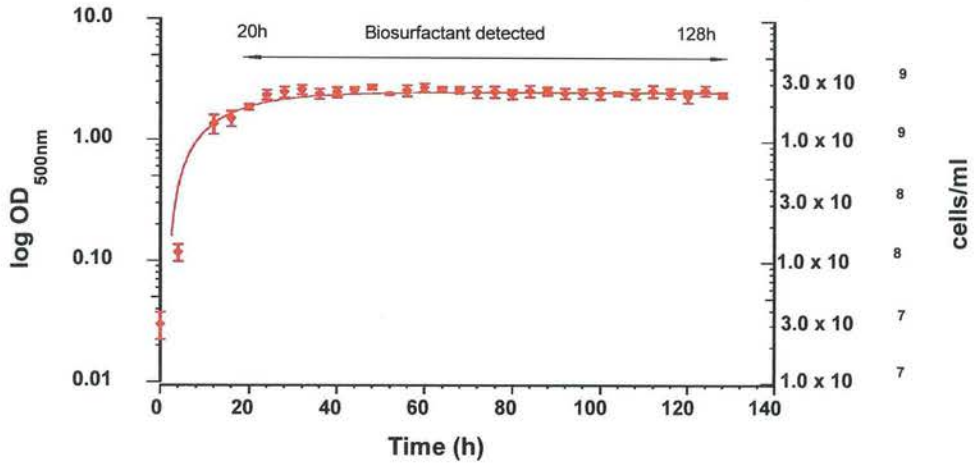
5.3.1 Growth and biosurfactant production

Biosurfactant was initially detected after 20h in *P. fluorescens* isolate P5049, 24h in *E. carotovora* P5064 and 28h in *P. fluorescens* isolate P5038 (Figures 5.2, 5.3 and 5.4). In all cases these results coincide with the onset of the stationary growth. Biosurfactant was then continually detected to the end of the time course (128h). Isolates P5049 and P5064 showed much stronger biosurfactant activity with the droplet test than isolate P5038, because the droplet spread further and more quickly.



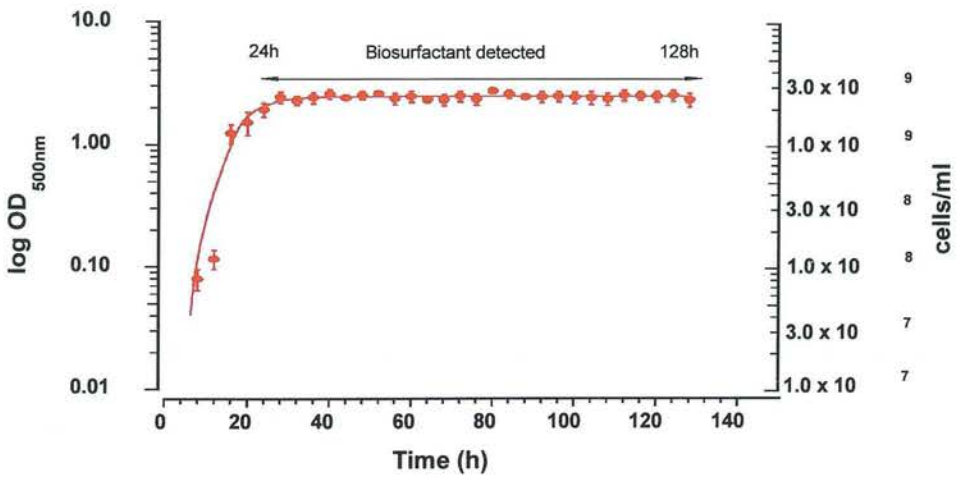
Log cells/ml was obtained from Figure 3.2 in Chapter 3

Figure 5.2: Growth (OD_{500nm}) and biosurfactant detection in *Pseudomonas fluorescens* P5038 (with SEM bars)



Log cells/ml was obtained from Figure 3.2 in Chapter 3

Figure 5.3: Growth (OD_{500}) and biosurfactant detection in *Pseudomonas fluorescens* P5049 (with SEM bars)



Log cells/ml was estimated from Figure 3.2 in Chapter 3

Figure 5.4: Growth (OD_{500}) and biosurfactant detection in *Pseudomonas fluorescens* P5064 (with SEM bars)

5.3.2 Comparison of five test methods for biosurfactant detection in *P. fluorescens*

5.3.2 (i) Water droplet test

Table 5.2: Biosurfactant detection in three bacterial isolates using the water droplet test

		Bacterial Isolates*			
		P5038[1]	P5049[1]	P5067[2]	
Growth Medium	**Inoculum**	**Positive detection of biosurfactant in 20 replicates**			**Mean % detection in 20 replicates for P5038 and P5049**
KB broth	Liquid[3]	16/20	18/20	0/20	85%
KB agar	Colony on agar[4]	20/20	20/20	0/20	100%

* = Bacterial isolates all 48h old when tested

1= *P. fluorescens*; 2 = *Erwinia carotovora* - the control isolate, i.e. no biosurfactants produced

[3]Liquid inoculum: 20µl (7.0×10^7 cells) transferred from liquid culture

[4]Bacterial colony transferred from agar culture via toothpick, cell no. not quantified

This was the most sensitive and rapid assay for detecting *P. fluorescens* biosurfactants with 100% detection (Table 5.2). For isolate P5049, the water droplets collapsed immediately when cells from an agar plate colony were used, although P5038 took longer to spread the droplet (ca. 2 seconds). For liquid cultures the droplet spread slowly and less extensively than the plate cultures, resulting in several replicates being classed as non-producers. No change in droplet shape was observed with *Erwinia carotovora* P5067.

5.3.2 (ii) Blood agar plates

Table 5.3: Biosurfactant detection in three bacterial isolates using the blood agar test

Test Medium containing 5% blood cells	Inoculum	Bacterial Isolates* P5038[1]	P5049[1]	P5067[2]	Mean % detection in 20 replicates for P5038 and P5049
		Positive detection of biosurfactant in 20 replicates			
KB agar	Liquid 30µl[3]	0/20	0/20	0/20	0%
KB agar	Liquid 60µl[3]	0/20	0/20	0/20	0%
KB agar	Colony on agar[4]	0/20	0/20	0/20	0%
LB agar	Liquid 30µl[3]	0/20	0/20	0/20	0%
LB agar	Liquid 60µl[3]	0/20	0/20	0/20	0%
LB agar	Colony on agar[4]	0/20	0/20	0/20	0%

* = Bacterial isolates all 48h old when tested
1 = *P. fluorescens*; 2 = *Erwinia carotovora* - the control isolate, i.e. no biosurfactants produced
[3]Liquid inoculum: 30µl (1.05×10^8 cells); 60µl (2.1×10^8 cells) transferred from liquid culture
[4]Bacterial colony transferred from agar culture via toothpick, cell no. not quantified

No haemolysis was detected in either *P. fluorescens* strain regardless of the medium used or inoculation method (Table 5.3). *Erwinia carotovora* P5067, which produces no biosurfactants, showed no difference to the *P. fluorescens* isolates. The plates were monitored for haemolysis over 14 days and substantial growth did occur for both the plate and broth inoculations, with the growth being observed on the agar surrounding the filter following the broth inoculation.

5.3.2 (iii) Siegmund and Wagner agar

Table 5.4: Biosurfactant detection in three isolates using the Siegmund and Wagner agar test

Growth medium prior to inoculation	Inoculum	Bacterial Isolates* P5038[1]	P5049[1]	P5067[2]	Mean % detection in 20 replicates for P5038 and P5049
		Positive detection of biosurfactant in 20 replicates			
KB broth	Liquid 30µl	0/20	0/20	0/20	0%
KB broth	Liquid 60µl	0/20	0/20	0/20	0%
KB agar	Colony on agar[4]	0/20	0/20	0/20	0%

* = Bacterial isolates all 48h old when tested
1 = *P. fluorescens*; 2 = *Erwinia carotovora* - the control isolate, i.e. no biosurfactants produced
[3]Liquid inoculum: 30µl (1.05×10^8 cells); 60µl (2.1×10^8 cells) transferred from liquid culture
[4]Bacterial colony transferred from agar culture via toothpick, cell no. not quantified

All three isolates P5038, P5049 and P5067 grew on the plates, but no colour reaction was seen, indicating that either no glycolipid surfactants were produced by these strains or the test had failed to detect them (Table 5.2). Growth was less vigorous than on the blood agar plates.

5.3.2 (iv) Hydrocarbon utilisation method

(a) Hydrocarbon-agar method

Cultures grew poorly, after 5 days, when using either hexadecane, petroleum ether or paraffin as a carbon source. In addition, the insoluble carbon sources formed a greasy layer on the agar surface, resulting in bacterial growth being 'milky', presumably due to bacterial attachment being affected, so colony counts could not be made. Toluene dissolved the plastic Petri-dish.

(b) Hydrocarbon-flask method

The data obtained (Appendix 1, Chapter 9) were analysed with a 5 factor ANOVA (Genstat 5, Rothamstead Experimental Station, 1995) and the results are shown in Table 5.5.

Table 5.5: ANOVA results for growth of *Pseudomonas fluorescens* isolate P5049 in three media containing hexadecane

Factor	variance ratio	significance value	degrees of freedom	Mean growth as OD_{500nm}				LSD value p =0.001
				Ayers	**GS**	**KB**		
medium tested	809.67	3.63	2	0.23	0.23	1.08	-	0.05
				yes	**no**			
culture shaken?	48.55	4.49	1	0.44	0.58	-	-	0.04
				0.5ml	**1ml**			
inoculum volume	191.66	4.49	1	0.37	0.65	-	-	0.04
				0%	**2%**	**4%**		
hexadecane concentration	12.21	3.63	2	0.45	0.50	0.57	-	0.02
				0	**24**	**48**	**72**	
time (h)	60.96	2.68	3	0.21	0.54	0.63	0.66	0.04

Significant differences were found between all factors: with the most influential factors (based on variance ratio) being medium; inoculum; time; shaking; and finally hexadecane.

Medium: The mean OD_{500nm} obtained in KB broth was significantly higher than those from Ayer's and Guerra-Santos MM. However, it was subsequently found that bacteria could still grow in KB broth without the addition of hexadecane (results not shown), so this medium was eliminated from the trial because there must be another carbon source present, possibly starch. Therefore another 5 factor ANOVA was conducted on the data, excluding KB broth as shown in Table 5.6.

Table 5.6: ANOVA results for growth of *P. fluorescens* isolate P5049 in Ayer's and Guerras-Santos media containing hexadecane

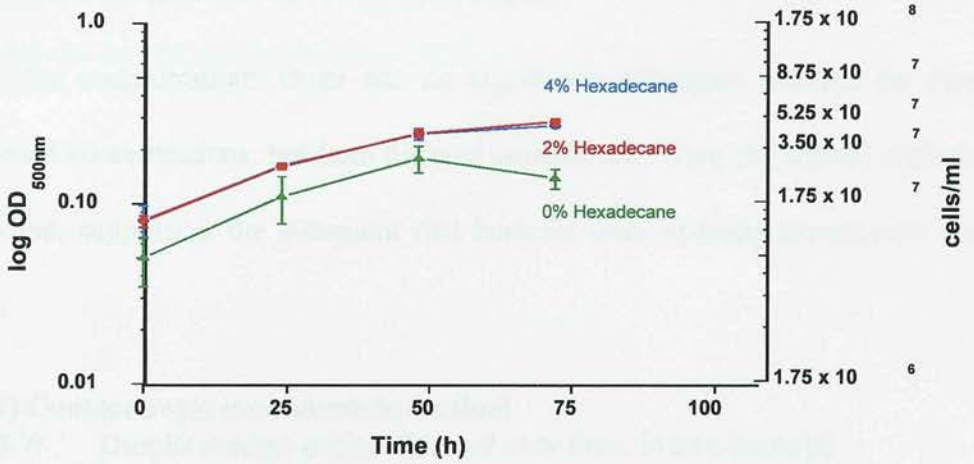
Factor	variance ratio	significance value	degrees of freedom	Mean growth as OD_{500nm}				LSD value
				Ayers	GS			
medium tested	0.02	3.63	1	0.23	0.23	-	-	0.0176 ($p < 0.9$)
				yes	no			
culture shaken?	37.81	4.49	1	0.20	0.25	-	-	0.0176 ($p < 0.001$)
				0.5ml	1ml			
inoculum volume	346.49	4.49	1	0.14	0.31	-	-	0.0176 ($p < 0.001$)
				0%	2%	4%		
hexadecane concentration	53.58	3.63	2	0.16	0.25	0.26	-	0.0215 ($p < 0.001$)
				0	24	48	72	
time (h)	106.51	2.68	3	0.14	0.20	0.27	0.30	0.0215 ($p < 0.001$)

No significant growth differences were found between Ayer's and Guerra-Santos media, but all other factors showed significant differences with the greatest influence being generated by inoculum; time; hexadecane; then shaking (as shown by the variance ratios).

Time: Over the time course of the experiment there was generally an increase in OD_{500nm} value in all treatments, with the most growth occurring before 48h. The no hexadecane controls did not show an increase in OD_{500nm} over time (Appendix 1, Chapter 9), demonstrating that the hexadecane was responsible for the OD_{500nm} increase observed.

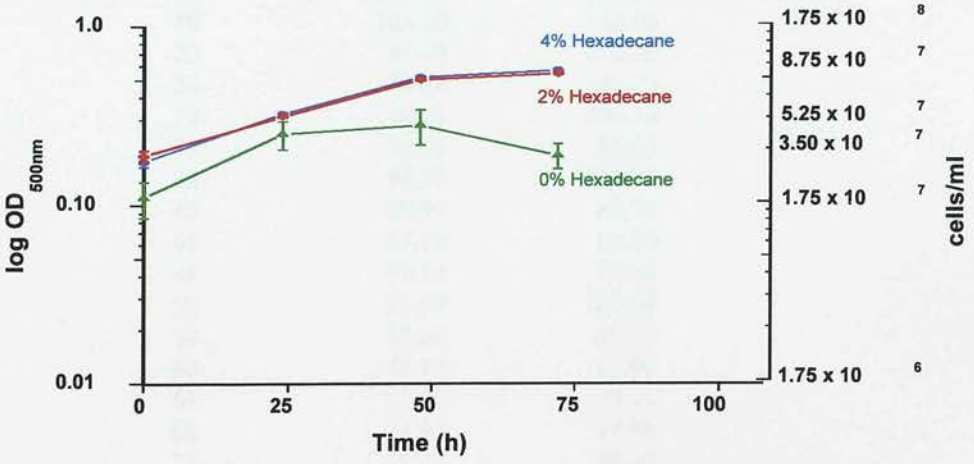
Figures 5.5 and 5.6 show the growth obtained in Ayer's medium, following shaking, with either 0.5ml or 1ml inoculum. If these Figures are used as examples for all minimal medium treatments they demonstrate that the OD_{500nm} increase over time was small and appears to be starting to plateau, suggesting no further growth can be sustained. Initial bacterial number was approximately 1.75×10^7 cells (0.5ml) and

3.0 x 10^7 cells (1ml), giving an OD_{500nm} of 0.09 and 0.185 respectively. If these starting OD_{500nm} values are then used to estimate the cell number produced over time, we find that the cultures increased to 5 x 10^7 and 9 x 10^7 cells after 72 incubation, which is equivalent to just 8h growth in rich media (Figure 5.3).



NB. cells/ml estimated from starting inoculum OD

Figure 5.5: Mean growth of *Pseudomonas fluorescens* isolate P5049 obtained in Ayer's minimal media following shaking with 0.5ml inoculum (with SEM bars)



NB. cells/ml estimated from starting inoculum OD

Figure 5.6: Mean growth of *Pseudomonas fluorescens* isolate P5049 obtained in Ayer's minimal media following shaking with 1ml inoculum (with SEM bars)

Inoculum: As to be expected there was usually a greater OD_{500nm} value obtained after the addition of 1ml bacterial inoculum, compared to 0.5ml, but there was little distinction between the overall OD change obtained with either inoculum (Appendix 1, Chapter 9).

Shaking: Culture agitation improved results slightly.

Hexadecane concentration: There was no significant difference between the two hexadecane concentrations, but both differed considerably from the control without hexadecane, supporting the statement that bacteria were utilising hexadecane for growth.

5.3.2 (v) Contact angle measurement method

Table 5.7: Droplet contact angles obtained over time, in two bacterial isolates: one biosurfactant producer and one non-producer

Time (h)	Mean Contact Angle (θ)	
	P. fluorescens P5064	*E. carotovora* P5067
4	111.90	113.20
8	117.30	108.20
12	114.10	117.30
16	103.30	137.00
20	81.20	108.30
24	89.00	102.70
28	94.10	103.30
32	76.80	94.00
36	96.10	114.70
40	80.97	89.70
44	87.30	89.30
48	99.20	84.30
52	83.90	109.00
56	83.60	86.30
60	80.10	107.00
64	75.20	79.30
68	72.30	70.00
72	78.30	83.30
76	77.70	78.00
80	77.50	59.70
water only	137.00	137.00

ANOVA found no significant difference between the two isolates (F value =2.24; F crit = 4.1 $p<0.05$)
P. fluorescens P5064 = biosurfactant producer
E. carotovora P5067 = non-biosurfactant producer

Table 5.7 shows that no significant difference was found between the contact angles of the two isolates, even though one isolate produces biosurfactants and the other one does not. Therefore if biosurfactants were produced here, this test has failed to detect them. The contact angles appear to decrease over time (although not continually) so a regression analysis was implemented to determine whether time influences the contact angle produced (Figures 5.7 and 5.8).

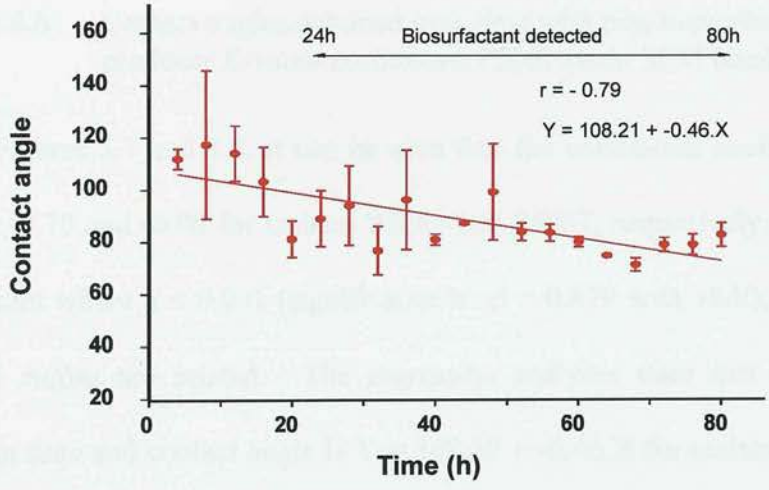


Figure 5.7: Contact angles obtained over time with biosurfactant-producer *Pseudomonas fluorescens* P5064 (with SEM bars)

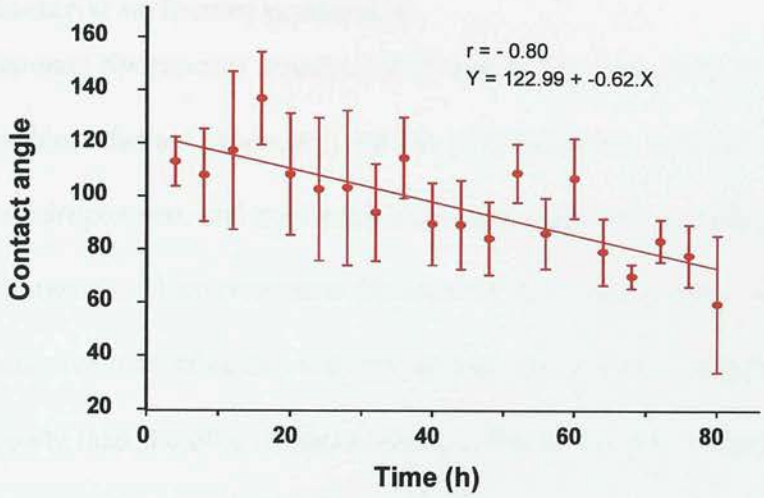


Figure 5.8: Contact angles obtained over time with non-biosurfactant producer *Erwinia carotovora* P5067 (with SEM bars)

From Figures 5.7 and 5.8, it can be seen that the correlation coefficients obtained are r = -0.79 and -0.80 for isolates P5064 and P5067, respectively: Both values are significant where $p < 0.001$ (significance level = 0.679 with 18df), so time and the contact angles are related. The regression analyses state that the relationship between time and contact angle is Y = 108.20 + -0.46.X for isolate P5064 and Y = 122.99 + -0.62.X for isolate P5067 which are both statistically significant where $p < 0.05$. Therefore the contact angles significantly decrease in size over time. This is what would have been expected after the onset of biosurfactant production at 24h, but as the same trend occurred in the non-producing strain, another factor must be influencing the angle obtained. The regression slopes of the two isolates are very similar, although the standard error bars of P5064 (Figure 5.7) are considerably smaller in the latter half of the experiment, than those of P5067 (Figure 5.8), showing that the results of P5064 were less variable.

5.4 Discussion

5.4.1 Bacterial surfactant production

Pseudomonas fluorescens isolates P5049 and P5064 have been shown to be highly effective biosurfactant producers, capable of reducing the surface tension instantly in the water droplet test, and producing a constant supply of biosurfactants from 24h of growth onwards, which represents the onset of stationary growth. Isolate P5038 was a less effective biosurfactant producer because the droplet's surface tension reduced more slowly than the other isolates, taking a few seconds to collapse, and this strain required 28h of growth before biosurfactant activity was detected. These results reflect those found for the production of rhamnolipid biosurfactants by *P. aeruginosa* (Oschner *et al.*, 1994a) and other *P. fluorescens* biosurfactants (Persson *et al.*, 1988) which start in the late exponential phase of growth, due to production being regulated by nitrogen and carbon levels which decrease after exponential growth allowing synthesis to begin (Martin and Liras, 1989). Therefore, these *P. fluorescens* strains may be under similar control. Isolate P5038 showed similar growth characteristics to the other two isolates, so its reduced potency may be inherent of the biosurfactant's effectivity (Chapter 4) or this isolate may produce lower levels of biosurfactant which cannot be detected as early. Hildebrand (pers. comm.) stated that some isolates require more than 48h to produce biosurfactants (i.e. well into stationary phase), so this strain may simply be a later producer than the other two. However, this isolate produces weaker activity in the water droplet test, even after substantial growth, and therefore the quantity of biosurfactant produced requires clarification with a tensiometer. Viscosin has been found to be metabolised in some strains after 48h of growth in liquid culture with only low levels remaining

(Hildebrand, pers. comm.) and although no significant decrease in biosurfactant activity was detected here, for the three strains tested, this may have been due to the sensitivity of the water droplet test. Indeed, the initial time that biosurfactants were detected in this study is dependant on the water droplet test's sensitivity. A quantitative assessment of biosurfactant production should now be implemented to pinpoint the onset of production and allow fluctuations in biosurfactant concentration to be detected.

The three isolates may have produced different biosurfactant types as *P. fluorescens* is known to produce various classes (Laycock *et al.*, 1991; Persson *et al.*, 1988 and Healy *et al.*, 1996), but this could not be determined with the water droplet test. It is already known that isolate P5064 produces viscosin and at least one other biosurfactant type (Harling and Dubickas, 1998 unpublished), so it would be useful to identify the specific biosurfactants produced in all three isolates. Also, it would be interesting to determine if the biosurfactant(s) produced *in vitro* differ from those produced *in planta* because, as the type of biosurfactant produced depends largely on the nutrients available, the plant environment may induce or suppress the production of certain biosurfactants (Georgiou *et al.*, 1992). This is supported by Mo and Gross' (1991) findings that certain biosurfactant-minus mutants of *P. syringae* retained the ability to produce syringomycin *in planta*, due to stimulation by plant metabolites, and as Laycock *et al.* (1991) noted that two additional HPLC peaks were obtained when biosurfactants were extracted from broccoli, as opposed to agar plates, this indicates the possibility of plant-induced surfactant production in head rot.

5.4.2 Biosurfactant assay

5.4.2 (i) Water droplet test

The water droplet test, using inoculum from an agar plate, was found to be the most rapid and sensitive test for detection because the presence of biosurfactants could clearly be detected in 48h old cultures. Inoculum from liquid cultures gave less reliable results and the presence of biosurfactants was missed on a few occasions. Therefore, if some bacterial strains only produce low levels of biosurfactant they may go undetected in liquid culture. This insensitivity may be due to the biosurfactants being diluted in broth, whereas they remain concentrated on agar. This raises doubts over the use of this method with the liquid inoculum and therefore indicates that biosurfactant production may have begun earlier than first detected in the biosurfactant production study (Section 5.3.1). The negative control, using a non-producing *Erwinia* strain, gave no false results. Differences in the rate of droplet collapse were noted between strains, but again this cannot be inferred simply as an effect of the biosurfactant concentration present because it may also be due to the biosurfactant's effectivity. As this test utilised 48h cultures, there was a 24h period for biosurfactants to accumulate, so the test's accuracy may be reduced considerably if younger cultures are used due to the lower biosurfactant concentration present. Threshold levels need to be established to determine the sensitivity of this technique: Purified biosurfactant could be tested and then compared with surface tensiometer results. However, this technique currently has the potential to assess biosurfactant production in bacteria from plate cultures, but as it is not accurately quantitative it should not be used to study biosurfactant production under differing conditions, such as environmental variables, which require a sensitive quantitative assay.

5.4.2 (ii) Blood agar plates

On blood agar, biosurfactant production was not detected. Possibly haemolytic biosurfactants are not produced by these strains or an ingredient in the medium may have inhibited production. There is evidence that *Pseudomonas* species are sensitive to the components in synthetic media, as Quigley and Gross, (1994) found *P. syringae pv. syringae* strains, toxigenic on cherry tissue, were unable to produce syringomycin *in vitro* unless the agar was supplemented with arginine and D-fructose, and other media such as LB or NA fail to stimulate biosurfactant production in these strains (personal observation). However, as glycerol was present (which is the essential carbon source for viscosin production in *P. fluorescens*), bacterial growth was obtained, and the KBA used is known to induce biosurfactant production in these strains (Section 5.3.2i), it seems unlikely that no biosurfactants were produced, but rather none were detected. Siegmund and Wagner (1991) state that biosurfactant production can be inhibited on many complex media used for blood agar, due to catabolite repression, which is the inhibition of many enzymes in the presence of a rapidly metabolising compound such as glucose, until this carbon source is used up, but as the glycerol carbon source of KBA is a slow metabolising substrate this should not have been a problem (Goto, 1992). Possibly there was an insufficient biosurfactant concentration to induce successful results: The biosurfactants syringomycin and tolaasin, require 500ng/ml to cause haemolysis (Hutchison *et al.*, 1995; Knowles and Ellar, 1987, respectively). It is now important to determine whether biosurfactant production was stimulated here, and if any biosurfactants

produced were haemolytic to ascertain whether the methodology or lack of biosurfactant production was responsible for the results obtained.

Not all *P. fluorescens* biosurfactants are haemolytic (Hildebrand, per. communication) so if this applies to strains P5038 and P5049 it may be that a haemolysis assay is not appropriate. Viscosin is known to be haemolytic (Hildebrand, pers. comm.) and P5064 is known to produce viscosin (Harling and Dubickas, 1998 unpublished) so this isolate should now be used as a control to test whether the blood agar test was functioning effectively. Hildebrand successfully used tryptic soy blood agar plates to test for viscosin (Per. communication) and found the blood was cleared around colonies after a few days of growth, so it may be that this medium is more conducive for haemolysis in head rot strains. Alternatively, the sensitivity of the blood agar test may be improved by developing the method of Rainey *et al.* (1991) who used haemolysis to study the biosurfactant, tolaasin, by suspending blood cells in buffer to an absorbance value of 0.09-0.095 at 600nm and then monitoring lysis at this wavelength. This may detect more subtle changes, especially at low concentrations, because it does not rely on a visual assessment. Lavermicocca *et al.* (1997) used a similar system for studying biosurfactant toxins from *P. syringae* pv. *syringae* with 6ml of sterile agarose containing enough red blood cells to give an absorbance value of 0.5 at 500nm after complete lysis, so improvements may be achieved by reducing the number of blood cells present and monitoring OD changes. It is also worth noting that sheep haematocytes were used by these researchers, while horse blood cells were used here and so possibly sheep cells lyse more easily producing more successful results.

5.4.2 (iii) Siegmund and Wagner agar

With the SW agar test, no glycolipid biosurfactant activity was detected (N.B. viscosin is a lipopeptide). Again this may have been due to the medium used: The medium was not nutrient rich and so the *P. fluorescens* strains may have been deprived of essential nutrients for glycolipid production. Also, CTAB, which was present in the medium, is known to inhibit the growth of most bacteria (Siegmund and Wagner, 1991) so its presence may have hindered biosurfactant production, but as growth was obtained on the plates, this seems implausible. Alternatively, these particular *P. fluorescens* strains may not produce glyocolipids or the concentration produced may have been insufficient for detection. Siegmund and Wagner (1991) used purified biosurfactants in this assay, while bacterial cultures were tested here, which may have influenced results. This test should now be repeated with purified biosurfactants for both isolates to determine if glycolipids are produced by these strains and if so, whether a threshold concentration is required to induce results. An isolate, known to produce glycolipids, should also be tested.

5.4.2 (iv) Hydrocarbon utilisation method

The agar plate method was unsuccessful, but the broth method produced a significant change in growth over time, for all treatments, indicating that isolate P5049 is capable of utilising hexadecane as a carbon source. In addition, the growth obtained for the treatments containing hexadecane was greater than those without. Growth in KB broth (excluding glycerol) was far more abundant than that obtained in the two minimal media, but this was later assumed to be due to the presence of an unidentified additional soluble carbon source because growth still occurred even

without hexadecane. The two minimal media showed no significant difference between the levels of growth they generated, which were quite small. Initial bacterial number was approximately 1.75×10^7 cells (0.5ml inoculum) and 3.5×10^7 cells (1ml inoculum) rising to 5×10^7 and 9×10^7 cells, respectively, after 72 incubation, which is equivalent to just 8h growth in rich media. Although growth in a rich medium would be expected to be more abundant, this example illustrates how low the level of growth was in this test. Koch *et al.*, (1991) obtained an OD_{660nm} increase of between 0.2 and 1.2 for growth of *P. aeruginosa* over 44h in Guerras-Santos MM, containing 1% hexadecane, with agitation, which is considerably greater than the mean OD_{500nm} change obtained here (approximately 0.21 to 0.63 over 48h in minimal media) with either 2% or 4% hexadecane. The majority of growth occurred in the first 48h of incubation and as stationary phase appeared to be starting, there would probably be little additional growth obtained if the cultures were incubated for longer. Therefore with such a small OD_{500nm} increase, problems may arise when categorising an unknown isolate.

As expected 1ml bacterial inoculum produced significantly higher OD values than the 0.5ml in most cases, but although the OD_{500nm} value was higher after the addition of 1ml inoculum, there was no apparent advantage of either inoculation volume in terms of the OD_{500nm} increase over time.

Shaking cultures influenced growth only slightly which reflects the results of Koch *et al.* (1991) who successfully carried out this technique under static conditions for *P. aeruginosa*. Increasing the hexadecane concentration failed to improve growth, so

possibly the strain tested is a poor hexadecane utiliser or biosurfactant production was reduced or inhibited in the media tested. Desai *et al.* (1988) described *P. fluorescens* as having remarkable hydrocarbon degrading ability, with biosurfactant production being maximal when 4% gasoline was used as a substrate, so levels of hexadecane tested here should have been adequate. However, this strain may not degrade hydrocarbons efficiently.

As the low level of growth obtained means that errors could be made, especially if biosurfactant concentration was low, improvements are required. This is illustrated by the finding that certain treatments, containing hexadecane, showed no more growth than those without (Appendix 1, Chapter 9). Perhaps another insoluble carbon source may prove to be more compatible with this *P. fluorescens* strain, resulting in greater biosurfactant yield. Desai *et al.* (1988) found different hydrocarbons stimulated different growth responses, with aliphatic hydrocarbons reducing the growth rate significantly, with bacteria producing a low yield of biosurfactant and taking 8 days to complete the growth cycle. In addition, different emulsifier types were produced depending on the hydrocarbon available during growth (Desai *et al.*, 1988) and although they did not test hexadecane, tetradecane was found to stimulate biosurfactant production in *P. fluorescens*. Alternatively the media may have caused poor growth by inhibiting biosurfactant production. Ayer's and Guerra-Santos media could now be tested with a glucose carbon source, to determine whether it was the media or the hexadecane carbon source which led to the low growth levels. In addition to the hexadecane utilisation assay, Koch *et al.* (1991)

recorded the hexadecane uptake of cells by measuring the ^{14}C levels which would have been very helpful here to establish how successful hydrocarbon utilisation was.

The fact that an increase in optical density was observed, over time, indicates the potential this assay has for determining biosurfactant production, following optimisation of various factors including the hydrocarbon, pH and incubation temperature.

5.4.2 (v) Contact angle measurement method

This method failed to detect biosurfactant activity in *P. fluorescens* strain P5064 as no significant difference was found between the contact angles of the biosurfactant producer and the non-producing control. Both isolates showed a gradual reduction in contact angle size over time, starting prior to the onset of biosurfactant production, indicating that a factor, other than biosurfactants, must be influencing the results. Other factors which can influence the contact angle measurements include: The media, temperature and the bacterial cell membrane which has biosurfactant-activity and so, as the bacterial number increases, this alone may have induced a drop in contact angle size. Indeed, the lipopolysaccharides released following cell lysis later on in the growth cycle will be surface active and therefore will contribute to the decrease in contact angle measurements obtained. Bunster *et al.* (1989) found contact angles of approximately 40° for surface active strains and 70° for non-producers and as the biosurfactant producer here produced a lowest value of 72.3°, this indicates that no biosurfactant were produced or the method failed to detect them.

The lack of biosurfactant effect may be due to the fact that the bacteria were from broth cultures which gives a much weaker reaction than bacteria from plate cultures in the water droplet test (Section 5.3.2.(i)). In addition, the bacterial suspension was mixed with a drop of water, further diluting the biosurfactant concentration. Therefore results may be more successful by simply measuring a droplet of bacterial suspension over time, as Bunster *et al.* (1989) did, rather than dilute it in water. Bunster *et al.* (1989) assessed droplets from cultures composed of 50% bacterial suspension : 50% KB broth. They then removed a 5µl aliquot to conduct the measurements upon. Here the broth suspension was made from 100ml broth with 100µl of bacterial suspension added (from which 20µl aliquots were removed and added to a 50µl water droplet) which represents a considerable reduction in the cells/ml tested. This may explain why the contact angle change was not greater for the biosurfactant producer. Establishing the threshold concentration required to initiate a change in contact angle is required to establish whether the concentration present in the suspension was too low.

This assay was time consuming and would be more suitable, once optimised, for obtaining detailed information about a specific biosurfactant-producer once the threshold concentration was known, rather than as a screen for testing many biosurfactant producers.

5.4.3 General considerations for biosurfactant detection tests

With optimisation, some of the biosurfactant tests used here may successfully determine biosurfactant production in head rot strains. Further research is required for all tests to determine when (and if) biosurfactant production starts on the test

medium, the quantity produced, and the threshold concentration required for detection. This could be implemented with a surface tensiometer on purified biosurfactants and would determine whether the methodology or biosurfactant concentration was responsible and also whether any false positives were made. This, along with an isolate known to induce the desired response, would pinpoint where the problems had occurred. However, depending on the quantity, type and characteristics of the biosurfactant(s), some tests may not be compatible for the head rot strains, even if they are based on general biosurfactant detection. For example, Koch *et al.* (1991) failed to adapt the thin layer chromatography method of Matsuyama *et al.* (1987) to detect rhamnolipids which they suggest may have been due to an insufficient quantity of biosurfactants present or because rhamnolipids do not remain associated with the cell as do the cyclodepsipeptides of *S. macrescens*.

No test assessed here was accurately quantitative and so for an in-depth study of biosurfactant production under differing environmental conditions, a sensitive quantitative assay would be required. Alternative tests to consider for this purpose include an inhibition assay, where the production of biosurfactants prevents the growth of another organism. This works by plating a biosurfactant-sensitive isolate, with the test strain, on to agar and then measuring regions of inhibited growth where the biosurfactant and organism meet, preventing further growth. This type of assay is quantitative in that, the more biosurfactant produced the less growth is observed. Gladstone (1997) demonstrated that viscosin inhibited growth of the germ tube and spore formation in *Fusarium poae* and *Botrytis cinerea* and slightly inhibited the growth of *Flavobacter*. Several strains of *P. fluorescens* are also known to lyse

fungal zoospores (J. Ligon, Per communication) so the possibility of using an assay based on microbe competition appears to be feasible. Tagg and McGiven (1971) assayed for bacteriocins (bacterial proteins which are lethal to other bacteria) by adding a suspected bacteriocin producer to wells cut in agar, leaving it to allow bacteriocin diffusion and then inoculating it by turning the agar upside down and flooding with an indicator organism. Zones of inhibition could be seen when bacteriocins were present, which correlated in size with the quantity of bacteriocin produced. They claimed that because only a thin layer of agar separated the two organisms, the sensitivity of the test was high. This methodology may be worth adopting for the detection of biosurfactants. Syringomycin has also been quantified using a similar inhibition assay, based on the fungus *Geotrichum candidum,* (Sinden *et al.*, 1971), but this method is less sensitive at detecting syringomycin production than leaf tissue (Gross and DeVay, 1977a). As Hildebrand (1989) and Gladstone (1997) described the development of biosurfactant induced necrotic lesions, maybe a plant based assay could be developed for head rot. However, to accurately quantify small quantities of biosurfactants, HPLC or a surface tensiometer must be used. Both methods are accurate when using a known standard, although it is time consuming to carry out as the biosurfactant requires purification. HPLC analysis was conducted for *P. syringae* toxin production and although laborious, it is a considerably more accurate method (Gross, 1991) and would be suitable for analysing whether production varies *in planta*.

It is inadvisable to rely on one method of testing to study biosurfactants because any weaknesses within the methodology may lead to errors. Koch *et al.* (1991) used four

independent methods to detect rhamnolipids, including a growth inhibition assay with *B. subtilis*; a blood agar plate method containing 5% sheep blood; recording the interfacial tension and measuring the ^{14}C hexadecane uptake to determine whether hydrocarbon utilisation had occurred and therefore this may have been advisable here.

However, the experimentation carried out has demonstrated that: Biosurfactants are produced between 20h and 28h after inoculation in the three strains tested; the water droplet test is currently the most suitable test for biosurfactants, and optimisation is required to enhance the sensitivity of the other four tests analysed.

Chapter 6: Biosurfactant-minus mutants: Production and characterisation

6.1 Introduction

As stated in Chapter 4, Hildebrand (1989) reported that biosurfactants were crucial for head rot development on unwounded heads, and Harling *et al.* (1994) demonstrated that the majority of head rot pathogens produce biosurfactants. Notably however, there are also non-producing pathogenic strains, and it is not known whether these induce disease without the involvement of biosurfactants, or if residual, non-pathogenic biosurfactant producers interact to bring about disease development. Hildebrand (1989) claimed that biosurfactant producers induced watersoaking alone, or soft rot in the presence of pectolytic enzymes, while non-producers could only rot wounded tissue. Although biosurfactants were required to induce disease on non-wounded tissue (Hildebrand, 1989), it is unlikely that biosurfactants act as a pathogenicity factor, because they do not have the potential to bring about disease alone. This chapter examines whether biosurfactants do indeed have a biological role in disease development, by producing biosurfactant-minus mutants of a pathogenic strain which can then be analysed for altered pathogenicity on broccoli tissue. Mutagenesis was carried out using the transposon Tn5, which can translocate into the host's genome and randomly disrupt gene function. A fuller description of Tn5 mutagenesis follows shortly.

6.1.1 Genetic control of pathogenicity and virulence

To determine whether biosurfactants act as pathogenicity/virulence factors in head rot, it is important to be aware of research conducted on genes previously implicated in plant disease development.

Pathogenicity genes determine whether or not disease symptoms develop, whilst virulence genes determine the severity of symptoms (Sigee, 1993). Interestingly, there is increasing evidence that evolutionary pathways leading to pathogenicity and virulence are highly conserved between pathogens causing diverse disease symptoms on various hosts (Sigee, 1993). This is illustrated by *hrp* genes (which cause a hypersensitive response (HR) in incompatible plant tissues, and disease in compatible tissues), because homology has been found between the *hrp* regions of *Pseudomonas solanacearum* and *Xanthomonas campestris*, and although no such homology was found between these species and *Pseudomonas syringae* (Boucher *et al.*, 1987), different *P. syringae* pathovars do show *hrp* sequence similarity (Sigee, 1993). In addition, Todd *et al.* (1990) found pathogenicity genes of the soft rot pathogen *Xanthomonas campestris* pv. *campestris* had conserved functional analogies with several pathovars: *oryzae* (which causes wilting in rice, without any associated rotting); *graminis*; *poae; phlei* and *arrhenatheri*. There is also evidence of avirulence (*avr)* gene homology in different *P. syringae* pathovars (Sigee, 1993), with the pathovars *tomato* and *glycinea* being genetically identical (Heath, 1991).

The examples cited above illustrate the homology between pathogens which differ in host identity, disease type and geographic location (Sigee, 1993). If *P. fluorescens* biosurfactants are involved in pathogenesis, they may share genetic homology with other related pathogenicity/virulence factors. Certainly, *P. syringae pv. syringae* virulence toxins also have biosurfactant-activity (Hutchinson *et al.* 1995), indicating that there may be homology between the genes controlling toxin production and those involved in biosurfactant production.

6.1.2 Analysing pathogenicity/virulence via mutagenesis

Daniels *et al.* (1984) carried out Tn5 mutagenesis on *Xanthomonas campestris*, the causal organism of crucifer black rot, producing 4 classes of pathogenicity mutant: (1) no visible disease symptoms; (2) localised tissue discolouration; (3) partial symptoms or (4) a reduced probability of infection (i.e. inoculation of a group of seedlings gave 10 to 20% showing characteristic wild-type symptoms, whereas the remainder showed either no symptoms or a localised response). This demonstrates the phenotypic diversity of mutants obtained, due to the multiplicity of genes regulating pathogenesis.

Several factors must be noted when carrying out mutagenesis with Tn5. As mentioned previously, Tn5 mutagenesis is random, (i.e. the insertion point cannot be targeted to specific genes) and as a result mutants must be screened after mutagenesis to select those with the desired phenotype (i.e. biosurfactant-minus mutants). Care must be taken to identify auxotrophic mutants, which have a metabolic block that renders them unable to synthesise a particular amino acid, as they fail to grow *in planta*. This leads to secondary avirulence, because the mutated gene does not influence disease primarily (Sigee, 1993). Auxotrophs can be selected against by plating the mutants on to minimal medium, as only prototrophs, without a metabolic defect, can grow (Sigee, 1993).

Tn5 mutagenesis is a particularly useful mutagenesis method. Insertion occurs frequently, but with generally only one mutation per genome, so that the resulting phenotype can be linked with the mutation. However, occasionally several phenotypic effects may occur which appear to be unrelated. This may be due to a

number of factors: (1) regulatory genes which are pleiotrophic in nature (control more than one, usually unrelated, function within a cell) (Lewin, 1994); (2) a gene involved in synthesizing a precursor which is utilised in many biosynthetic processes, and (3) a transposon inserting into an operon (a cluster of related genes and their transcriptional promoter), thus preventing expression of all the operon's downstream genes at once (Gross, 1991). For example, a pleiotrophic gene was mutated in a syringomycin study, and as a result mutants were non-producers, non-pathogenic and severely restricted in the ability to grow *in planta*. This was later identified as being due to an arginine deficiency rather than the loss of toxin production (Quigley and Gross unpublished, cited in Gross, 1991). The arginine deficiency alone could not have accounted for the non-pathogenic phenotype, but if the mutants had not been analysed further, syringomycin may have been inaccurately classed as a pathogenicity determinant necessary for bacterial growth *in planta* (Gross, 1991). Further mutagenesis considerations include the possibility of a secondary mutation occurring unwittingly, or a mutant's phenotype varying *in planta* due to the nutrients available, as found for syringomycin-minus mutants which could only produce the toxin *in planta* (Mo and Gross, 1991).

6.1.3 Mutagenesis carried out by transposon Tn5

Tn5, which was used in this study to produce biosurfactant-minus mutants, was first discovered in bacteriophage λ situated in a strain of *Escherichia coli* (Berg *et al.* 1975) and has since been located in bacterial chromosomes, phages and plasmids (Berg and Berg, 1983). Encoding resistance to the antibiotic kanamycin, Tn5 is a 5.8 kilo base (kb) long transposon with the ability to transpose from a vector molecule

and integrate within a host cell's genome. Structurally, it contains terminal insertion sequences, known as IS50 elements, which are 1533 base pairs (bp) long inverted repeats that generate a 9bp duplication of the target site on insertion. The right IS50 element encodes two proteins, Tnp and Inh, from their respective genes *Tnp* and *Inh*. Tnp is a 476 amino-acid tranposase, while Inh is a 421 amino acid inhibitor protein, which makes insertion of a second Tn5 unlikely (Karcher, 1995). Transposons move at high frequency with one insertion causing a single, stable mutation, which is generally non-leaky (i.e. gene function is completely lost). Due to the kanamycin resistance gene, cells containing Tn5 inserts can be detected by plating them on to agar containing kanamycin and, since plant pathogenic bacteria rarely possess kanamycin resistance, this system is ideally suited to the study of bacterial pathogenesis (Goto, 1992). Therefore Tn5 is a very useful method and is considerably more advantageous than other mutagenesis methods, such as using chemicals or UV light. However, there are several drawbacks: (1) occasionally insertion occurs in a gene operon, at a location where the adjacent sequences combine to form a promoter site causing the increased expression of all downstream genes (Karcher, 1995); (2) the IS50 regions can transpose independently of Tn5 resulting in further gene disruption (Hirschel and Berg, 1982) which cannot be easily detected as they lack kanamycin resistance, and (3) although Tn5 transposes in an almost random manner, there are occasional hot spots of insertion (Karcher, 1995).

6.1.4 Mechanism of Tn5 transposition

For mutagenesis, Tn5 is located in a vector plasmid within a donor bacterial strain, such as *E. coli*. The vector transfers into the recipient cells in a process known as

conjugation: A mating aggregate is formed between the donor and recipient strains, allowing direct cell to cell transfer of the vector through *sex pili* appendages. Once inside the recipient cell, Tn5 transposes from the vector into the recipient's genome, causing a mutation (the recipient cell is now known as a transconjugant). Conjugation requires two genes which are located in the vector plasmid: The *tra* gene, for establishing the mating aggregate, and the *mob* gene, for mobilising the DNA transfer. Conjugative plasmids carry both genes and therefore promote their own transfer from donor to recipient (diparental mating). Non-conjugative plasmids lack the *tra* gene and require a helper containing this gene to transfer (triparental mating). Plasmids are eliminated from the recipient genome, after mutagenesis, either by using a temperature sensitive plasmid, which is not maintained at high temperatures, or a suicide vector which cannot replicate in the host and so is lost after replication (Goto, 1992).

Following transposition, the location of Tn5 within a genome can then be deduced with the following steps - (1) cutting the DNA with restriction enzymes (bacterial enzymes which recognise specific DNA sequences to cleave); (2) Southern blotting; a technique to transfer and 'fix' the DNA on to a membrane (Southern, 1975), and (3) exposing the membrane to a labelled Tn5 probe. The probe will hybridise with any Tn5 copies bound to the membrane allowing the fragment size to be noted.

6.1.5 Aims

Pathogenic *Pseudomonas fluorescens* isolate P5064, which produces large quantities of viscosin in culture (Harling and Dubickas, 1998, unpublished), was used in this study because it resembled the viscosin producing strains implicated in head rot

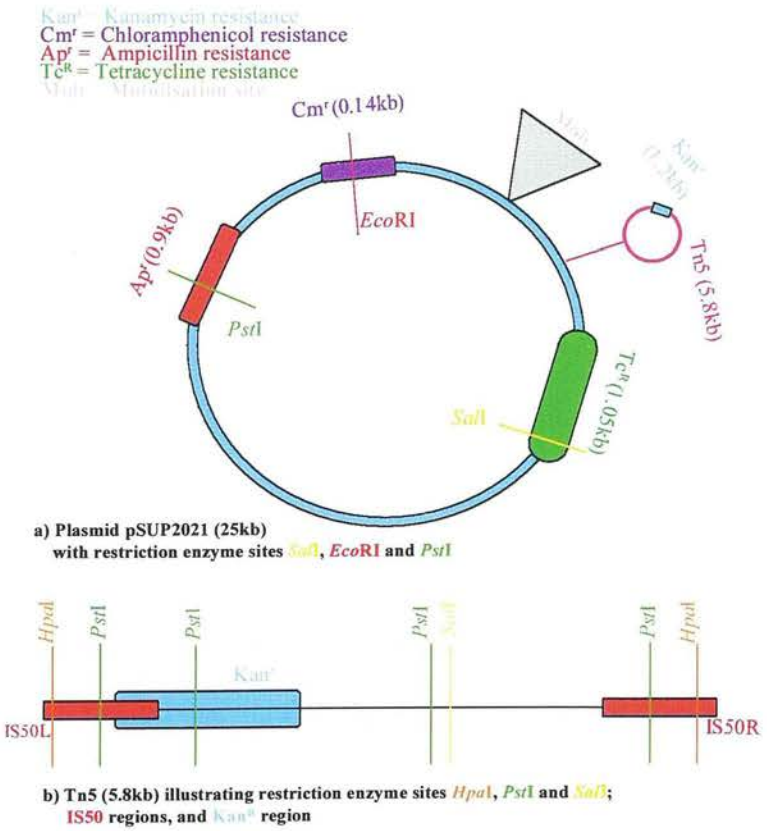
development by Hildebrand *et al.*, (1990). The aim was to produce biosurfactant-minus mutants with Tn5 mutagenesis and then assess them for altered pathogenicity/virulence on broccoli tissue. Two other biosurfactant producers were also mutated: Pathogenic P5049 and non-pathogenic N5024. All transconjugants obtained were screened for loss of biosurfactant production using the water droplet test described in Chapter 5. To ensure that biosurfactant production was the only phenotypic effect influencing pathogenesis, all mutants were compared to the wild-type for growth rate and production of pectolytic enzymes, protease, and fluorescent pigments. These factors were selected as examples of the range of factors which help to identify plant pathogenic bacteria. Pectolytic enzymes are major pathogenicity determinants in head rot (Hildebrand, 1989) and fluorescent pigments easily identify fluorescent Pseudomonads. Whilst the role of protease secretion in phytopathogenic bacteria, such as *Erwinia carotovora* and *Xanthomonas campestris* (Sigee, 1993), is unclear, mutants of *Xanthomonas campestris* pv. *campestris* lacking production show delayed symptom development (Tang *et al.*, 1987). No reports of *P. fluorescens* protease products influencing disease have been published.

Mutants were then assessed for altered pathogenicity on excised broccoli heads, followed by a molecular study of the mutations, via Southern blotting with a labelled Tn5 probe, to determine: (1) where Tn5 had inserted; (2) if different genes were affected, and (3) if any secondary IS50 transpositions occurred.

6.2 Materials and methods

6.2.1 Bacterial strains and plasmids

The pathogenic strains *P. fluorescens* Group IVb P5064 and P5049, and non-pathogenic Group Vb isolate N5024 were used in this study. All are copious biosurfactant producers previously isolated from diseased broccoli heads (Robertson *et al.*, 1993). *Escherichia coli* S17-1 harbouring the Tn5 plasmid vector, pSUP2021, was obtained from Dr R. Simon (Universität Bielefeld, Fakultät für Biologie, Postfach 8640, D-4800 Bielefeld, Germany). The Tn5 derivative confers kanamycin resistance, while the plasmid vector carries resistance to chloramphenicol and tetracycline (Simon *et al.*, 1983) (Figure 6.1).



Not drawn to scale; produced from Boliver, 1977; Simon *et al.*, 1983 and Jorgensen *et al.*, 1979.

Figure 6.1: Restriction map of plasmid vector pSUP2021:
a) plasmid map, and b) a Tn5 map

As this plasmid has a narrow host range it is not stably maintained in *Pseudomonas* species. Another plasmid vector, pMH1701, carried by *E.coli* S17-1 was also used in this mutagenesis study and was obtained from Dr A. Suwanto (Department of Biology, Bogor Agricultural University, Indonesia). It too confers resistance to chloramphenicol and kanamycin (Hynes *et al.*, 1989).

6.2.2 Transposon mutagenesis of *P. fluorescens*

E. coli S17-1 cells carrying pSUP2021 and pMH1701 were cultured separately in LB broth supplemented with chloramphenicol and kanamycin sulphate (50μg/ml and 25μg/ml, respectively), at 30°C, for 12h with shaking at 200rpm. The *P. fluorescens* strains were cultivated in LB broth at 30°C for 16h, again with shaking. Both donor and recipient cultures were washed: 1ml of broth (approximately 1 x 10^9 and 2 x 10^9 cells for the *E. coli* and *P. fluorescens* strains, respectively) was centrifuged at 6000rpm for 1min, then 1ml of sterile 10mM Tris-HCl solution (pH 7.5) was added to the pellet. This was repeated twice before final resuspension in 1ml of the same Tris-HCl solution. Transposon mutagenesis was based on the membrane filtration method of Walter *et al.* (1987). This involved bringing the two bacterial species together in a process known as conjugation, for 12h at 30°C, on 0.45μm nitrocellulose filters (Whatman), situated on LB agar, at a donor:recipient ratio of 1:5. Afterwards, filters were placed in 700μl of 10mM Tris-HCl solution (pH 7.5) and vortexed to remove the bacterial cells. The mating suspension was serially diluted in Tris-HCl solution and plated on to Ayer's MM agar, containing kanamycin at 25μg/ml (auxotrophs and *E. coli* cells fail to grow because of their metabolic block and proline growth dependence, respectively). The individual donor and recipient

cultures were plated on to this agar also to ensure neither grew: Again the donor requires proline and the recipient is not kanamycin resistant. The frequency of transconjugants was calculated by plating the mating suspension on to MM agar, without kanamycin sulphate. Transconjugants were checked for co-integration of the plasmid vector (in addition to Tn5) by plating the cells on to: (1) MM containing kanamycin and chloramphenicol at 25μg/ml and 50μg/ml respectively, and (2) MM with only kanamycin (whole plasmid insertion confers chloramphenicol resistance). All plates were incubated at 30°C (±2°C) for 48h. Mutants were maintained on LB slopes without antibiotics to prevent the development of antibiotic dependence (Meynell and Meynell, 1970).

6.2.3 Selection of mutant bacterial colonies defective in biosurfactant production (surf$^-$)

The water droplet test described in Chapter 5 (Section 5.2.3i) was used to assess transconjugants for biosurfactant production. Following 48h growth on KBA containing kanamycin, bacteria from individual colonies were transferred using a sterile toothpick to a 50μl water droplet situated on a plastic Petri-dish lid. Bacteria which showed no ability to reduce the surface tension were selected as putative biosurfactant-minus mutants (surf$^-$).

6.2.4 Phenotypic characterisation of the surf$^-$ mutants

To determine whether any additional phenotypic effects had been produced, all surf$^-$ mutants were compared with the wild-type for growth rate doubling time, and the ability to produce three extracellular secretions: Protease, pectolytic enzymes and fluorescent pigments.

Mutant growth-rate was compared to the wild-type by assessing isolate doubling time. Twenty millilitres of KB broth, containing kanamycin, was inoculated with 10µl of a 1 x 10^8 cells/ml bacterial suspension. Three replicate flasks were inoculated for each isolate, and incubated at 25°C (±2°C) with shaking at 200rpm. Growth was assessed every 2h at OD_{500nm}, for 16h, then the values plotted on logarithmic paper to allow the doubling times to be extrapolated (where 0.1 represents 1 x 10^8 cells/ml and 0.16 represents 2 x 10^8 cells/ml (Figure 6.3). One mutant was checked for growth beyond the log phase: Mutant 6416 and wild-type P5064 were studied through the growth cycle until late stationary phase. A 100µl aliquot of both isolate suspensions were added to 250ml flasks containing 100ml of KB broth, at 25°C (±2°C), with shaking at 200rpm. The OD_{500nm} was recorded every 4 hours with three replicate flasks assessed for each isolate.

Protease activity was detected by sub-culturing the mutants on to NA (Oxoid) plates supplemented with 5% (w/v) skimmed milk, then observing for regions of hydrolysis after 2 days growth at 25°C (±2°C). Pectolytic enzyme production was assessed on CVP plates, after 6 days incubation at 25°C (±2°C). Areas of pectolysis were noted by flooding the plates in 1% hexadecyltrimethlammonium bromide (which precipitates intact pectin). Pectolysis is then seen as a halo against a dark background (Sands *et al.*, 1980). Mutants were assessed for ability to produce fluorescent pigments by viewing colonies under UV light at 365nm following 2 days growth on KBA at 25°C (±2°C). Colonies which fluoresce are producers.

6.2.5 Pathogenicity testing of the surf⁻ mutants

Surf⁻ mutants were tested for altered pathogenicity by inoculating mature broccoli heads as described in Chapter 2 (Section 2.2.2). Lints were soaked in a 1 x 10^8 cells/ml suspension for each mutant culture, with four replicates for each. Control lints were soaked either in water only (-ve control), or the wild-type pathogen strain P5064 (+ ve control). Results were recorded against the five point subjective scoring scale described in Chapter 2 (Section 2.2.2), then data were subjected to percentile angular transformation using arc sine (Schefler, 1969); thus categories 0 - 4 became 0% (0), 33% (1), 50% (2), 67% (3), 100% (4). To allow the mutant isolates to be easily compared with the wild-type score by extending the range of scores, the water control value (22.5) was deducted from all of the isolates and then each mutant was expressed as a percentage of the wild-types disease score.

6.2.6 DNA extraction and quantification

To characterise the mutants genetically, DNA was extracted using a Puregene DNA extraction kit (Flowgen) according to manufacturer's instructions. Quantification was carried out by mixing 10µl of extracted DNA solution with 90µl of molecular biology grade water (Sigma) and then measuring the OD_{260nm} and OD_{280nm}. An OD_{260nm} of 1 is equivalent to 50µg DNA (Sambrook *et al.* 1989). A control containing 10µl of the kit's elution buffer and 90µl water was used to calibrate the spectrophotometer. The OD_{260nm}/OD_{280nm} ratio estimates sample purity, with a value of 1.8 signifying a pure sample. Lower values indicate protein or phenol contamination, while higher values represent RNA contamination (Sambrook *et al.* 1989).

6.2.7 Gel Electrophoresis

Size separation of DNA fragments was carried out using low melting temperature agarose, prepared using 1 x TAE buffer. DNA samples were mixed with gel loading buffer at a 1:3 ratio, then 15µl was loaded into each gel lane. The agarose concentration, voltage and run times varied. Following electrophoresis, gels were stained in ethidium bromide solution at 50µg/ml for 15 minutes, and the image captured under UV light at 365nm with an image analyser (Flowgen).

6.2.8 Tn5 probe production

The Tn5 probe was obtained by extracting the vector pSUP2021 from *E. coli* S17-1: 1ml of an overnight LB broth culture was centrifuged at 10,000rpm for 1min, then a 'PlasmidPURE miniprep' kit (Sigma Chemicals) was used according to manufacturer's instructions. The plasmid DNA was then quantified as described in Section 6.2.6. The DNA (approximately 3µg) was then cut with 20 units of restriction enzyme *Pst*I, to isolate the Tn5 DNA from the vector. *Pst*I is a 6 base cutter recognising four sites within Tn5 (Rothstein *et al.*, 1980). The reaction was incubated at 37°C, for 2h, then stopped by the addition of 16µl of gel loading buffer. Samples were then run on a 0.8% agarose gel in 1 x TAE buffer for 2h at 80V to separate the fragments. Two Tn5 fragments (1 kb and 0.8 kb), both containing part of the kanamycin resistance gene, were obtained. Either could have been used as probes but the 1000bp fragment was selected for labelling. To obtain sufficient DNA for labelling, DNA from five cutting reactions was run on a gel simultaneously to allow the bands to be extracted and the DNA bulked. The DNA was extracted from the agarose with the 'NucleiClean' kit (Sigma chemicals) according to

manufacturer's instructions and the Tn5 fragments were labelled with a digoxigenin-dUTP DNA labelling and detection kit (Boehringer-Mannheim) according to manufacturer's instructions. The kit detects labelled DNA by enzyme immunoassay using an anti-digoxygenin antibody conjugated to alkaline phosphatase, which is then visualised by the colourimetric substrates NBT/X-phosphate. A control hybridisation was performed to determine the probe concentration.

6.2.9 Southern Blotting

Prior to Southern blotting, the mutants and wild-type P5064 were cultured overnight in LB broth with shaking at 200 rpm. A 1ml aliquot of each suspension was removed and centrifuged at 10,000 rpm for 1min. Genomic DNA was extracted from each pellet using a Puregene DNA extraction kit (Flowgen), according to manufacturer's instructions. The DNA was resuspended in TE buffer overnight at room temperature and then quantified as in Section 6.2.6. Approximately 3µg of DNA was cut with 20 units of restriction enzymes *Eco*RI and *Sal*I (Sigma Chemicals) for each isolate. Both restriction enzymes are 6 base cutters: *Eco*RI does not cut within Tn5, while *Sal*I cuts Tn5 centrally. Digests were incubated overnight at 37°C according to Supplier's instructions (Sigma Chemicals). The reactions were stopped with 16µl of gel loading buffer and then the samples run on a 0.8% low melting temperature agarose gel (15µl sample/lane) for 4h at 50V to separate the fragments. The gel was stained with ethidium bromide and the image captured. Wild-type P5064 acted as a negative control because it does not contain a Tn5 copy.

The gel was then shaken gently in denaturation solution (0.5M NaOH, 1.0M NaCl) for 1h to denature the DNA in preparation for probe hybridisation. This was followed by a second hour in neutralisation solution (0.5M Tris, 1.5M NaCl, pH 7.0) to neutralise the gel's pH (Sambrook *et al.*, 1989). Blotting was carried out according to Karcher (1995). The gel was placed, bottom side up, on a sheet of 10 x SSC soaked Whatman No. 3 chromatography paper (3MM), situated on a raised glass platform, in a reservoir of 10 x SSC solution (Figure 6.2). The gel was covered with a positively charged nylon membrane (Boehringer-Mannheim) which had been cut to size. Three sheets of Whatman No. 3 paper (two soaked in 10 x SSC and one dry) were then added to the membrane, followed by dry paper towelling (forming a 4 inch block). A 400 gram weight was placed on top of the stack and the blot was left to develop overnight. Capillary action draws the 10 x SSC solution through the gel, consequently transferring the denatured DNA from gel to membrane.

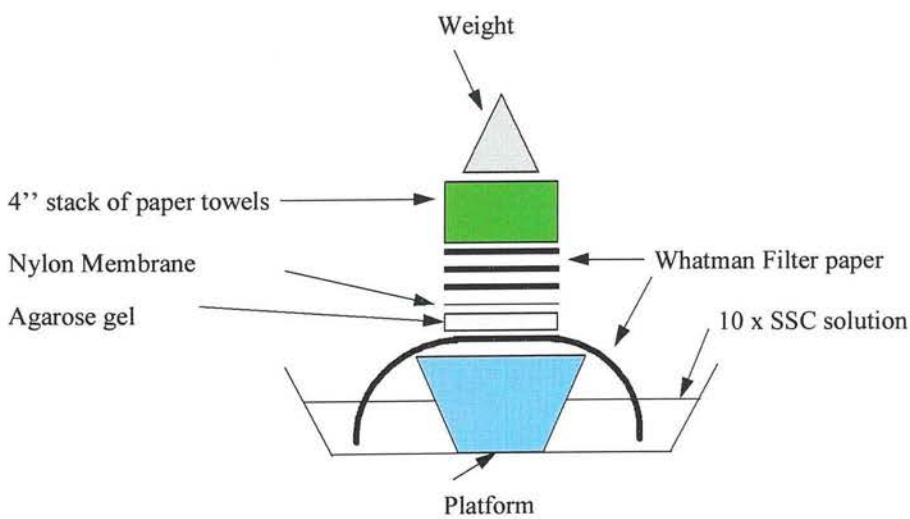


Figure 6.2: Southern blotting apparatus

After blotting, the nylon membrane was soaked in 5 x SSC for 5 minutes, air dried and then baked at 80°C for 2h to 'fix' the DNA on to the membrane. Blots were stored in cellophane at 4°C until ready to probe.

The Tn5 probe was denatured at 68°C (±2°C) for 30min then added to pre-hybridisation buffer (5 x SSC; 0.1% w/v N-lauroylsarcosine; 0.02% w/v SDS and 1% of the kit's blocking reagent) at 25ng/20ml. The 1000bp Tn5 fragment, containing parts of both the IS50 region and kanamycin resistance gene, was the probe used to develop all blots. The membrane was soaked in pre-hybridisation buffer for 30min at 68°C (±2°C), before being incubated overnight in the probe solution, at a volume of 20ml probe solution per 100cm^2 membrane, at 68°C (±2°C), with gentle agitation. Unbound probe was removed by washing in 2 x SSC, 0.1% SDS at room temperature (twice for 5 minutes each), and then 0.1 x SSC, 0.1% SDS at 68°C (twice for 15 minutes each). The bound probe was detected according to the manufacturer's instructions (Boehringer-Mannheim) with the colour reaction being left to develop overnight.

6.3 Results

6.3.1 Mutants and virulence

The transformation frequency obtained was 4.8×10^{-6} and 6.2×10^{-8} Tn5-containing colonies per recipient bacterial cell, for pSUP2021 and pMH1701 respectively (Appendix 2, Chapter 9). Due to the higher transformation frequency, only pSUP2021 mutants were assessed for biosurfactant loss. Thirty-five surf$^-$ mutants were obtained, giving a frequency of 3.64×10^{-6} colonies per Tn5-containing bacterial cell. The mutants were labelled 6401 through to 6435. No co-integration of plasmid vector was detected in the transconjugants obtained. Mutagenesis failed to produce transconjugants in *P. fluorescens* strains P5049 and N5024, even under different mating conditions.

6.3.2 Phenotypic characterisation of the 35 surf$^-$ mutants obtained

Production of fluorescent pigment, pectolytic enzymes and protease production was absent in five of the 35 mutants: 6407, 6416, 6417, 6418 and 6419 (Table 6.1). The remaining 30 mutants retained these properties.

No significant reduction in isolate doubling time was detected (Table 6.1) for the mutants.

Table 6.1 Phenotypic characterisation of 35 *Pseudomonas fluorescens* mutants deficient in biosurfactant production.

Isolate Number	Growth doubling time (minutes)	Fluorescent pigment	Protease	Pectinase
6401	66.6	+	+	+
6402	66.6	+	+	+
6403	66.6	+	+	+
6404	73.3	+	+	+
6405	80.0	+	+	+
6406	60.0	+	+	+
6407	86.6	-	-	-
6408	73.3	+	+	+
6409	80.0	+	+	+
6410	80.0	+	+	+
6411	66.6	+	+	+
6412	73.3	+	+	+
6413	66.6	+	+	+
6414	80.0	+	+	+
6415	73.3	+	+	+
6416	73.3	-	-	-
6417	80.0	-	-	-
6418	73.3	-	-	-
6419	73.3	-	-	-
6420	66.6	+	+	+
6421	86.6	+	+	+
6422	66.6	+	+	+
6423	73.3	+	+	+
6424	66.6	+	+	+
6425	73.3	+	+	+
6426	66.6	+	+	+
6427	86.6	+	+	+
6428	66.6	+	+	+
6429	66.6	+	+	+
6430	66.6	+	+	+
6431	73.3	+	+	+
6432	66.6	+	+	+
6433	73.3	+	+	+
6434	73.3	+	+	+
6435	60.0	+	+	+
Negative control (water)	-	-	-	-
Positive control (P5064 wild-type)	80.0	+	+	+

No significant differences was found between the isolates doubling times in ANOVA

For isolates 6416 and P5064, Figure 6.3 demonstrates that there was little difference between their growth characteristics.

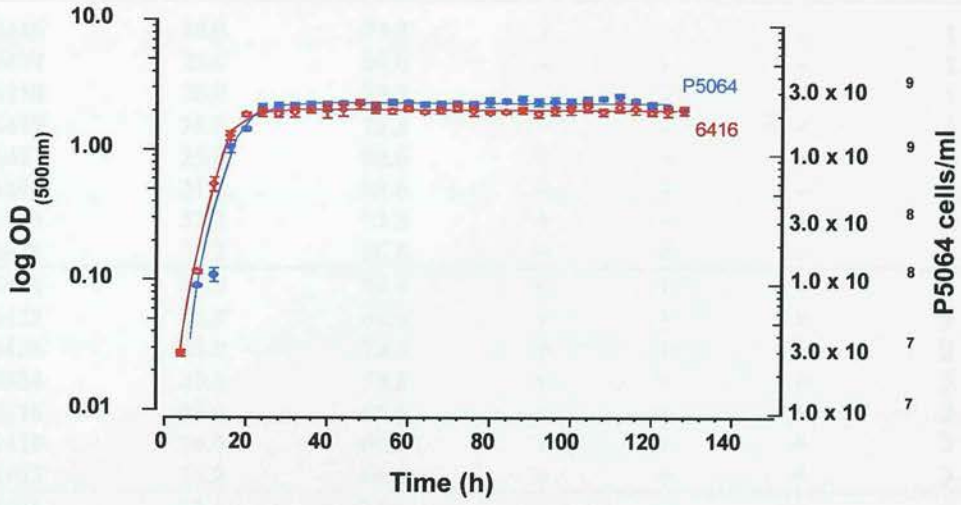


Figure 6.3: Comparison of growth in *Pseudomonas fluorescens* P5064 and mutant 6416 (with SEM bars)

Both isolates show very similar exponential growth, but P5064 continues slightly longer at this rate resulting in a higher bacterial number during stationary phase. Isolate 6416 entered stationary growth at approximately 20h, p5064 at 24h. As the rate of exponential growth (and therefore the doubling time) was similar for both isolates, the doubling times calculated for the mutants appear to be accurate.

So from the phenotypic characterisation we can identify two subgroups: (1) those which lost extracellular products and (2) those which retained them.

6.3.3 Mutant pathogenicity on excised broccoli heads

Table 6.2 illustrates the results of the mutant pathogenicity testing alongside the phenotypic characterisation described in Section 6.3.2.

Table 6.2 Pathogenicity and phenotypic characterisation of 35 *Pseudomonas fluorescens* surf⁻ mutants

Isolate Number	% wild-type disease*	Doubling time (minutes)	Fluorescent pigment	Protease	Pectinase	Subgroup
6416	**18.8**	**73.3**	-	-	-	**1**
6417	**25.0**	**80.0**	-	-	-	**1**
6418	**25.0**	**73.3**	-	-	-	**1**
6419	**25.0**	**73.3**	-	-	-	**1**
6427	**25.0**	**86.6**	+	+	+	**2**
6407	**31.2**	**86.6**	-	-	-	**1**
6415	**31.2**	**73.3**	+	+	+	**2**
6428	**31.2**	**66.6**	+	+	+	**2**
6414	**43.8**	**80.0**	+	+	+	**3**
6422	**43.8**	**66.6**	+	+	+	**3**
6425	**43.8**	**73.3**	+	+	+	**3**
6434	**43.8**	**73.3**	+	+	+	**3**
6426	**50.0**	**66.6**	+	+	+	**3**
6429	**50.0**	**66.6**	+	+	+	**3**
6403	**56.2**	**66.6**	+	+	+	**3**
6404	62.5	73.3	+	+	+	4
6420	62.5	66.6	+	+	+	4
6424	68.8	66.6	+	+	+	4
6431	68.8	73.3	+	+	+	4
6432	75.0	66.6	+	+	+	4
6406	81.2	60.0	+	+	+	4
6409	81.2	80.0	+	+	+	4
6410	81.2	80.0	+	+	+	4
6421	81.2	86.6	+	+	+	4
6433	81.2	73.3	+	+	+	4
6401	87.5	66.6	+	+	+	4
6408	87.5	73.3	+	+	+	4
6412	87.5	73.3	+	+	+	4
6413	87.5	66.6	+	+	+	4
6405	93.8	80.0	+	+	+	4
6435	93.8	60.0	+	+	+	4
6402	100.0	66.6	+	+	+	4
6423	100.0	73.3	+	+	+	4
6411	112.5	66.6	+	+	+	4
6430	112.5	66.6	+	+	+	4
water control	0					
wild-type	100	80.0	+	+	+	
LSD value	**39.4%**	**NS**				

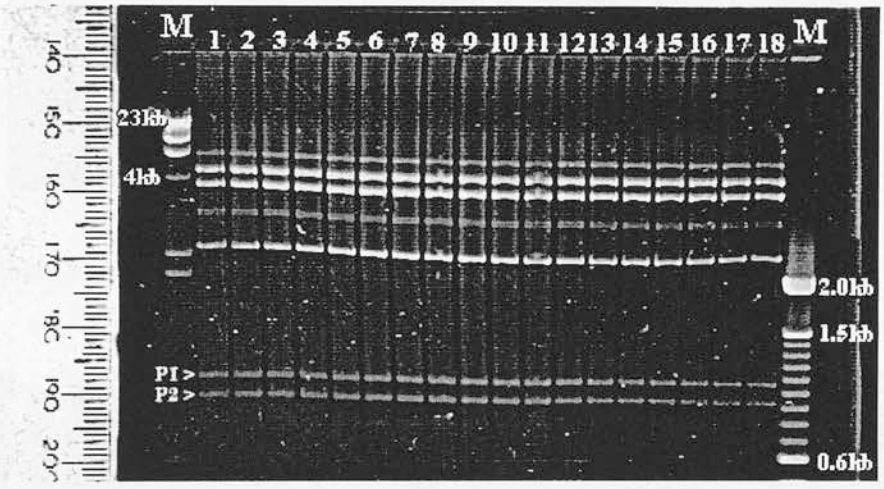
*Data arc sine transformed and presented as % of the wild-type score after deduction of water control value.
Bold: Significantly less disease than the wild-type; NS = No significant differences.
Subgroups:

1 = No disease symptoms (i.e. <39% of wild-type score) and no extracellular products (5 mutants)
2 = No disease symptoms and extracellular product production intact (3 mutants)
3 = Significantly reduced virulence & extracellular product production (7 mutants)
4 = No significant difference in disease symptoms (i.e. >60% of wild-type score)

All mutant isolates, and the wild-type, had the water control value (22.5) removed from their transformed disease scores and then all were expressed as a percentage of the wild-type score. This was to spread the mutant scores across a wider range for analysis. The wild-type controls showed spreading soft-rot (average transformed score obtained was 82.5 before adjustment), while the water controls showed a loss of the waxy bloom (average transformed score was 22.5). In all, 15 mutants produced significantly less disease than the wild-type. Of these, eight produced watersoaking but no rotting (<39.4% of the wild-type score) which meant that the scores were not significantly different from the water control. This was interpreted as the isolates having lost the ability to cause disease and were therefore classed as non-pathogenic (Table 6.2). Five of these non-pathogenic mutants also failed to produce extracellular products (Section 6.3.2) forming subgroups 1 and 2 respectively. The remaining seven mutants which produced significantly less disease than the wild-type (<60% of the wild-type score were classed as reduced virulence mutants (vir $^{-}$) because they still produced significantly more disease than the water controls (>40 - <60%) (subgroup 3). This can be interpreted as mutants retaining their basic compatibility with the host (= pathogenicity) but losing their ability to cause severe disease (= loss of virulence); this interpretation was drawn from Sigee, (1993). Finally, twenty mutants showed no significant difference in the disease level produced compared with the wild-type (>60%) (Subgroup 4).

6.3.4 Tn5 Probe production

Figure 6.4 shows the results of the *Pst*I digest on plasmid pSUP2021 DNA and the two Tn5 fragments selected. The other five bands seen (ranging from 9kb to 2.5kb in size) represent further products of the *Pst*I digestion of the vector pSUP2021. The control hybridisation reaction indicated that there was 2ng/µl of each labelled *Pst*I fragment.

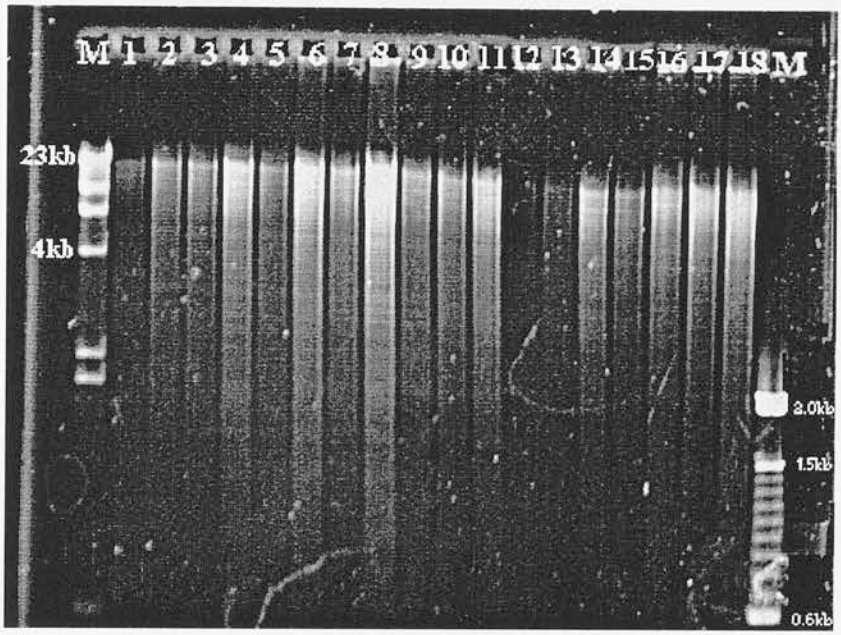


P1 = Probe 1, P2 = Probe 2
M = DNA size markers

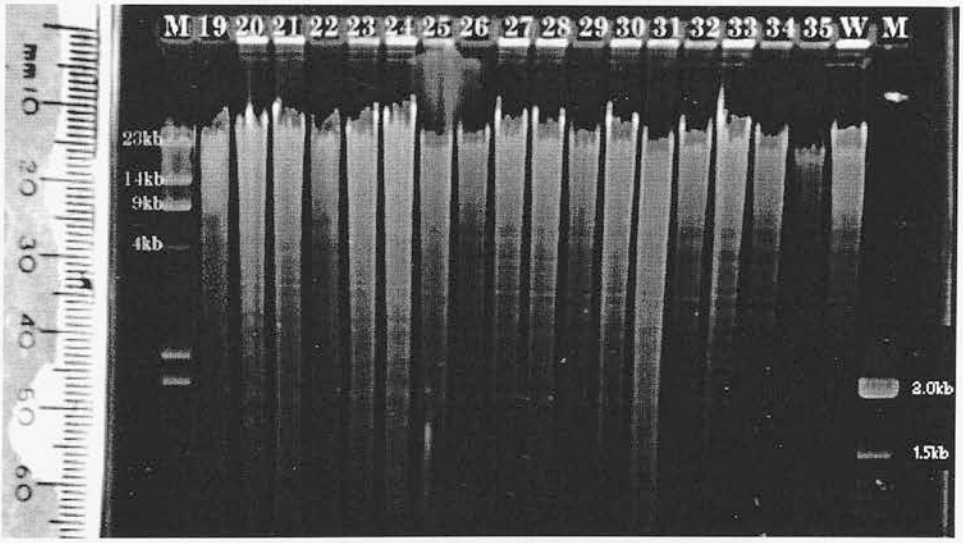
Figure 6.4: Plasmid pSUP2021 DNA cut with 20 units of restriction enzyme *Pst*I

6.3.5 Southern Blotting

Following restriction enzyme digestion of the mutants, *Sal*I generated a wide range of fragment sizes (Figure 6.6), while *Eco*RI produced predominantly fragments larger than 6kb (Figure 6.5). This is demonstrated by the intensity of the white glow representing the location of stained DNA.

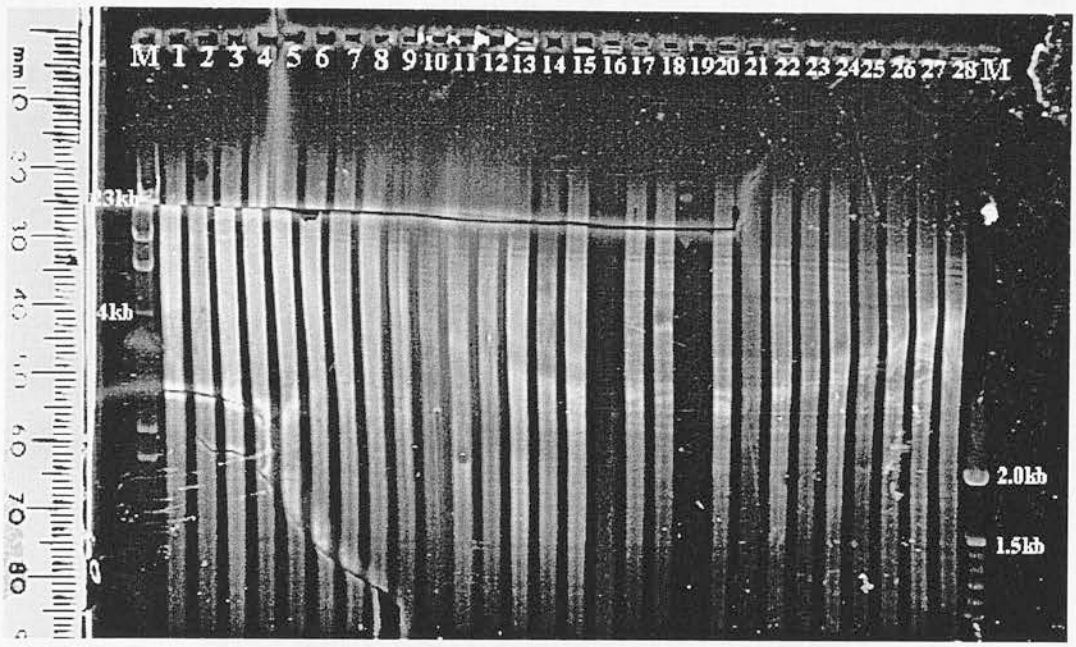


(a) Mutants 1 to 18 (M = DNA size markers)

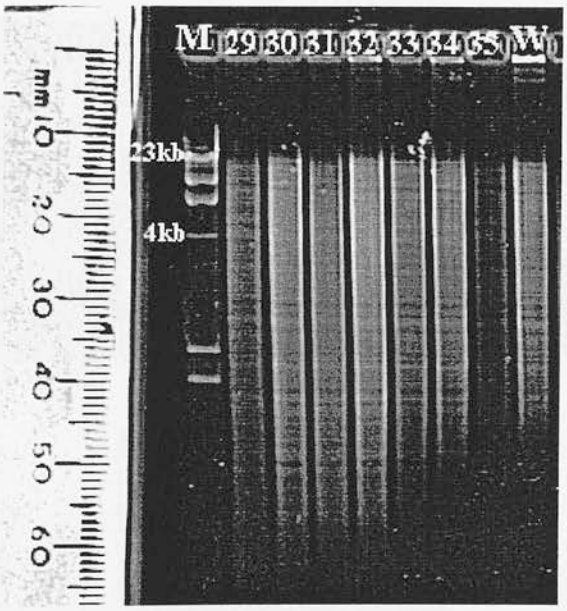


(b) Mutants 19 to 35 and wild-type P5064 (M = DNA size markers; W = wild-type P5064)

Figure 6.5: Examples of the gel electrophoresis results following digestion of the 35 mutants, and wild-type P5064, with 20 units of *Eco*RI



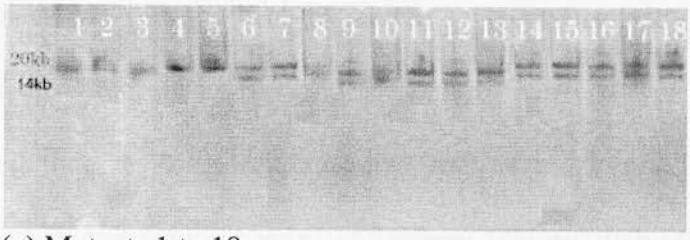
(a) Mutants 1 to 28 (M = DNA size markers)



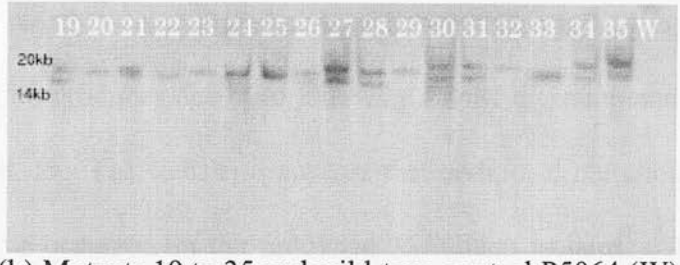
(b) Mutants 29 to 35 and wild-type P5064 (M = DNA size markers; W = wild-type P5064)

Figure 6.6: Examples of the gel electrophoresis results following digestion of the 35 mutants, and wild-type P5064, with 20 units of *Sal*I

Figures 6.7 and 6.8 show the Southern blot results following *Eco*RI and *Sal*I digestion.

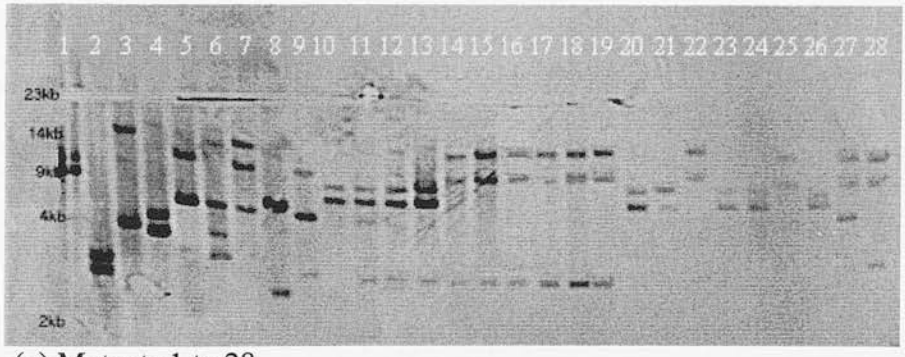


(a) Mutants 1 to 18



(b) Mutants 19 to 35 and wild-type control P5064 (W)

Figure 6.7: Examples of Southern blots carried out on the 35 surf$^-$ mutants, following digestion with *Eco*RI (20 units)



(a) Mutants 1 to 28

(b) Mutants 29 to 35 and wild-type control P5064 (W)

Figure 6.8: Examples of the Southern blots carried out on the 35 surf$^-$ mutants, following digestion with *Sal*I (20 units)

Following Southern blotting the presence of Tn5 hybridised bands was noted in the 35 mutants (Figures 6.7 and 6.8). Some mutants did not blot successfully first time, due to insufficient DNA having been extracted, so several rounds of blotting were carried out and then the results compiled in Table 6.3. The wild-type control gave no band, indicating that no non-specific binding had taken place. *Eco*RI digestion produced one, or two, hybridised bands of between 14kb to 20kb (Table 6.3), while *Sal*I produced mostly two or three bands of between 2kb and 14kb (Table 6.3). As one Tn5 mutation produces one band following *Eco*RI digestion and two bands after *Sal*I (because it cuts Tn5 in half) it appears that additional mutational or insertional events may have occurred, in the following 18 mutant isolates: 6406; 6407; 6409; 6411; 6412; 6413; 6414; 6415; 6416; 6417; 6418; 6419; 6427; 6428; 6430; 6431; 6434 and 6435 (Table 6.3).

Table 6.3: Number of Southern blot bands hybridising with the Tn5 probe for each mutant isolate and the presence of additional insertions

Isolate Number	Phenotypic Subgroup	Band sizes (kb)	Hybridisation subgroups (b1-b4)*	Disease score (%)	Number of hybridising bands following *Eco*RI digestion	Number of hybridising bands following *Sal*I digestion	Additional mutation
6416	**1**	**2.8, 8.0 & 11.0**	**b3**	**18.8**	**2**	**3**	**Yes**
6417	**1**	**2.8, 8.0 & 11.0**	**b3**	**25.0**	**2**	**3**	**Yes**
6418	**1**	**2.8, 8.0 & 11.0**	**b3**	**25.0**	**2**	**3**	**Yes**
6419	**1**	**2.8, 8.0 & 11.0**	**b3**	**25.0**	**2**	**3**	**Yes**
6427	**2**	**4.0, 8.0 & 11.0**	**-**	**25.0**	**2**	**3**	**Yes**
6407	**1**	**4.5, 9.0 &13.0**	**-**	**31.2**	**2**	**3**	**Yes**
6415	**2**	**2.8, 8.0 & 11.0**	**b3**	**31.2**	**2**	**3**	**Yes**
6428	**2**	**3.0, 8.0 &11.0**	**-**	**31.2**	**2**	**3**	**Yes**
6414	**3**	**2.8, 8.0 & 11.0**	**b3**	**43.8**	**2**	**3**	**Yes**
6422	**3**	**8.0 & 11.0**	**b4**	**43.8**	**1**	**2**	**No**
6425	**3**	**8.0 & 11.0**	**b4**	**43.8**	**1**	**2**	**No**
6434	**3**	**3.0, 9.0 & 13.5**	**-**	**43.8**	**2**	**3**	**Yes**
6426	**3**	**5.5 & 7.0**	**b1**	**50.0**	**1**	**2**	**No**
6429	**3**	**9.0 & 13.5**	**-**	**50.0**	**1**	**2**	**No**
6403	**3**	**3.8 & 14.0**	**-**	**56.2**	**1**	**2**	**No**
6404	4	3.6 & 4.0	-	62.5	1	2	No
6420	4	5.5 & 7.0	b1	62.5	1	2	No
6424	4	5.5 & 7.0	b1	68.8	1	2	No
6431	4	4.0, 6.0, 8.0 & 9.0 & 13.5	-	68.8	2	5	Yes
6432	4	6.0 & 8.0	-	75.0	1	2	No
6406	4	3.0, 3.6 &5.0	-	81.2	2	3	Yes
6409	4	2.8 & 4.0	-	81.2	2	3	Yes
6410	4	5.5 & 7.0	b1	81.2	1	2	No
6421	4	5.5 & 7.0	b1	81.2	1	2	No
6433	4	3.8 & 9.0	-	81.2	1	2	No
6401	4	8.5 & 10.0	-	87.5	1	2	No
6408	4	2.6 & 5.0	-	87.5	1	2	No
6412	4	2.8, 5.5 & 7.0	b2	87.5	2	3	Yes
6413	4	2.8, 5.5 & 7.0	b2	87.5	2	3	Yes
6405	4	5.0 & 10.5	-	93.8	1	2	No
6435	4	2.0, 3.5 & 13.0	-	93.8	2	3	Yes
6402	4	2.8 & 3.0	-	100.0	1	2	No
6423	4	5.5 & 7.0	b1	100.0	1	2	No
6411	4	2.8, 5.5 & 7.0	b2	112.5	2	3	Yes
6430	4	3.0, 3.6, 3.8, 6.0, 8.0 & 9.0	-	112.5	2	6	Yes

Phenotypic subgroup 1 = No disease symptoms and no extracellular products
Phenotypic subgroup 2 = No disease symptoms and extracellular product production intact
Phenotypic subgroup 3 = Reduced disease symptoms and extracellular product production
Phenotypic subgroup 4 = No significant difference in disease symptoms
Hybridisation subgroup b1 = Mutants producing two bands at 5.5 and 7.0kb
Hybridisation subgroup b2 = Mutants producing three bands at 2.8, 5.5 and 7.0kb
Hybridisation subgroup b3 = Mutants producing three bands at 2.8, 8.0 and 11.0kb
Hybridisation subgroup b4 = Mutants producing two bands at 8.0 and 11.0kb
*18 mutants had unique banding profiles and were therefore not grouped
Values in Bold = produced significantly less disease than the wild-type

The additional mutations are most likely to be due to IS50 insertions rather than another Tn5 copy, due to the banding number obtained. An additional Tn5 mutation would produce at least four bands following *Sal*I digestion, as found in isolates 6430 and 6431, whereas an IS50 insertion would only create three bands (unless multiple IS50 insertions had occurred). The 18 mutants with an additional band included all eight mutants in phenotypic subgroups 1 and 2 (1 = non-pathogenic, with no extracellular products; 2 = non-pathogenic, with extracellular products); two mutants from subgroup 3 (reduced virulence and extracellular products), and eight mutants from subgroup 4 (same disease level as the wild-type). It is very unlikely that non-specific hybridisation occurred, as the wild-type P5064 showed no bands at all. The bands detected must therefore indicate the presence of non-genomic DNA.

Following *Sal*I digestion 17 mutants could be grouped into four banding profiles (Figure 6.8). These groups were: (b1) 6410, 6420, 6421, 6423, 6424 and 6426 (two bands) (b2) 6411, 6412, 6413 (three bands) (b3) 6414, 6415, 6416, 6417, 6418, 6419 (three bands) and (b4) 6422, 6425 (two bands). The remaining 18 mutants had unique banding profiles, making a total of 22 different profiles, which may suggest that 22 different genes were disrupted. However, the presence of IS50 insertions possibly increased the true profile number: Isolate 6410 has a very similar banding pattern to isolate 6411, with the exception of a third band at approximately 2.8kb. The phenotypes of these two isolates are also similar indicating that the same gene may have been mutated, with the addition of an IS50 mutation occurring in 6411 to create a different banding profile. The large *Eco*RI bands produced (between 14kb to 20kb) meant that *Eco*RI bands could not be distinguished into groups. However,

the presence of two bands of equal size cannot necessarily be regarded as the mutation having occurred within the same genetic loci, because the restriction enzyme may have coincidentally cut two fragments to the same length.

Two of the banding groups (b2 and b4) contained mutants from only one phenotypic grouping (subgroup 4 for hybridisation group b2 and subgroup 3 for hybridisation group b4) following *Sal*I digestion. However, in group b1 some mutants came from subgroup 3 (reduced virulence) and the remainder came from subgroup 4 (no significant difference in disease level induced), whilst in b3 the mutants came from subgroups 1, 2 and 3 (all of which show significantly less virulence than the wild-type) (Table 6.2).

6.4 Discussion

6.4.1 Phenotypic characterisation

The 35 surf$^-$ mutants were categorised into four phenotypic subgroups: (1) five were non-pathogenic (<39.4% wild-type score) and produced no extracellular products; (2) three were non-pathogenic with production of extracellular products; (3) seven were vir$^-$ (>40- <60% wild-type score) and produced extracellular products, and (4) 20 produced the same level of disease as the wild-type (>60% wild-type disease). Therefore in all, 15 produced significantly less disease than the wild-type (of which 10 contained more than one mutational insertion), while the remaining 20 showed no change. Growth was unaffected in all 35 mutants. The range of phenotypes produced is in accordance with other mutagenesis studies (Daniels *et al.*, 1984; Anderson and Mills, 1985). It should be noted that only using one pathogenicity method carries the risk of introducing bias in the mutant classes obtained: A range of techniques would isolate a more representative collection of the biosurfactant genes directly or indirectly associated with disease development (Daniels, 1987). However, as the excised head test is the only successful method for determining head rot pathogenicity (Chapter 2) this was not possible.

As five of the mutants failed to produce pectolytic enzymes, proteases and fluorescent pigments, they may have lost the function of an *out* gene which controls extracellular enzyme export across the cell membrane (Kang *et al.*, 1994). *Out* genes have been reported in *Erwinia chrysanthemi* (He *et al.*, 1991); *Erwinia carotovora* ssp. *carotovora,* where they control pectinase and cellulase (Reeves *et al.*, 1993), and in *Pseudomonas solanacearum* where protease, polygalacturonase and

endogalacturonase enzymes are transported (Kang *et al.*, 1994). Four of these mutants share one banding profile (b3), whilst the remaining one differs, but it is quite feasible that both sets of mutants have lost *out* gene function.

In all, 18 of the mutants contained an additional mutational insertion, including: All those in subgroups 1 and 2; two mutants from subgroup 3 and eight from subgroup 4. As no non-specific hybridisation was found with the wild-type control, the additional bands must have been Tn5-related. The secondary mutations may have arisen from independent IS50 insertions, because *Sal*I digestion of the mutants produced only one extra band than expected (an additional Tn5 insertion would have generated two extra bands). This could be confirmed by re-probing the blots with the 800bp *Pst*I probe (Section 6.2.8) which does not contain IS50 sequences and so would only detect complete Tn5 inserts. Only mutants 6430 and 6431 could feasibly contain a second Tn5 copy because they possess 6 and 5 *Sal*I bands respectively. Indeed, it would appear that the additional mutations in these isolates were Tn5 induced because they have produced two *Eco*RI bands, while IS50 insertions would have generated three bands (unless the additional IS50 regions inserted within the same EcoRI site which is less probable). This is unusual because the *Inh* gene works to limit Tn5 insertions to one per genome, but it is possible. Alternatively, the extra insertions may have resulted from insufficient digestion with *Sal*I (with some Tn5 copies being full cut into small fragments, whilst others were not cut sufficiently and remain in larger fragments). However, the mutant DNA was digested overnight and the spread of DNA fragment sizes obtained was good (Figure 6.6) which means that all sites should have been cut successfully. Furthermore, it has been reported that

*Sal*I may cut at secondary restriction sites under non-ideal digestion conditions and therefore that may have been responsible for the additional bands produced. However, as the *Eco*RI digests, which did not show a good spread of fragment sizes (Figure 6.5), produced extra bands corresponding to the extra bands found following *Sal*I digestion, it would be unlikely that insufficient digestion had occurred in the DNA from the same mutants when using two different enzymes, and so it indicates that IS50 insertions appear to be that main culprit for the additional bands. Therefore it seems more probable that extra IS50 insertions have occurred in this study. New Tn5 plasmid vectors are available which contain disabled IS50 elements (Harling, pers. communication), so preventing the occurrence of additional insertions. It may be useful to repeat this work with one of these vectors. Additional mutations mean that the observed disease phenotype may not actually result from the gene controlling biosurfactant loss, so these mutants are not desirable. However, they may still provide valuable information concerning the genetic control of biosurfactants, provided that care is taken to link each mutation with its phenotypic effect. Independent IS50 insertions have been found in other *Pseudomonas* species mutated with Tn5 (Anderson and Mills, 1985; Boucher *et al.*, 1985). Boucher *et al.* (1985) reported that four of the 12 avirulent *P. solanacearum* mutants they studied had IS50 inserts, so the number found here appears to be high. Staskawicz *et al.* (1983) claimed that a 1.5kb *Eco*RI fragment of *P. solanacearum* acted as a hotspot for IS50 insertions - possibly a similar region is present here, accounting for the high number of secondary transpositions.

Spontaneous mutations may also have occurred in the 35 mutants. If so, some may have been responsible for the loss of biosurfactant production or altered symptom expression obtained. However, as the mutants phenotypes all appeared to be stable over time, they are probably Tn5-derived rather than spontaneous. Nonetheless, an indication of the wild-type's spontaneous mutation rate would be useful - P5064 could be plated on to agar containing an antibiotic to determine how many become resistant. Staskawicz *et al.* (1983) found spontaneous avirulent *P. solanacearum* mutants occurred at a rate of 10^{-2} per Tn5 mutant, which indicates that the likelihood of spontaneous mutations affecting results is relatively large.

To determine whether biosurfactants are influential in disease we need to be able to link biosurfactant loss with the mutated gene (and resulting phenotype), which means analysing the mutants with only one mutation. Of the 17 mutants with one insert, there were two subgroups (corresponding with the previous subgroups, 3 and 4): (1) five mutants were vir $^-$, and (2) 12 mutants showed no change in disease level. Therefore 29.4% of mutants (containing only one insert) produced less symptoms following the loss of a biosurfactant related gene. This may signify that biosurfactants act as a virulence factor. However, as the majority of mutants (70.6%) did not show any virulence change following biosurfactant loss, it is more likely that biosurfactants are not involved in virulence. The most likely reason for the five mutants' vir $^-$ phenotype would be due to the mutated gene being pleiotrophic in nature, as found for the syringomycin-minus mutants which contained only one Tn5 insert in the *syr*A gene - the mutants were non-pathogenic and severely restricted in ability to grow because the gene was involved in syringomycin production and

arginine prototrophy (Xu and Gross, 1988b). This possibility is quite feasible here, because *P. fluorescens* biosurfactants are thought to be produced by a thiotemplate mechanism, and so it is likely that many of the genes involved have secondary activities. Alternatively, the gene may be regulatory; involved in the synthesis of a common cell precursor, or part of a gene operon which controls different cell processes. Nonetheless, further work is essential before biosurfactant involvement can be ruled out, because: (1) the mutations in the 12 mutants showing no change in virulence may have increased production of another virulence factor, minimising the effect of biosurfactant loss; (2) some of these mutants may still be producing low levels of biosurfactants, undetected by the water droplet test, or (3) production may only be stimulated *in planta* where specific metabolites are present, as reported for *in vitro* syringomycin-minus mutants which retained toxin production in a cherry pathogenicity test (Quigley and Gross, 1994). Interestingly, 90% of *P. syringae* isolates were reported to produce larger quantities of syringomycin in the presence of plant signal molecules (Quigley and Gross, 1994), so this may also be occurring here, especially because viscosin and syringomycin share structural homology. Possibly the type, toxicity and quantity of biosurfactants produced by wild-type P5064 alters *in planta.* If so, the pathogenic, non-producing, bacteria classified by Robertson *et al.* (1993) may in fact be producing biosurfactants on broccoli tissue. This would support the claim of Hildebrand (1989) that biosurfactants are an essential prerequisite for disease. Additional evidence for the theory that biosurfactant production may alter *in planta* comes from Laycock *et al.* (1991) who analysed biosurfactant extracts taken from either broccoli heads, *Pseudomonas* agar F (PAF)

or synthetic liquid medium. They reported that broccoli tissue induced two additional HPLC peaks to the *in vitro* media, indicating the possibility of specific plant metabolites inducing biosurfactant production in head rot. If this plant induced stimulation is proven to occur in all biosurfactant producers, biosurfactants must have an important function on plant tissue, even if not as a virulence factor. Indeed, as the majority of pathogenic strains are producers, there must be a selective pressure to retain these genes. Gross (1991) reported that the activation of virulence genes, on perception of specific chemicals or physical stimuli, imparts order and balance to pathogenesis that will optimise the bacterium's chances for long-term survival. Therefore considerable research is required before any accurate conclusions can be made concerning the role of biosurfactants *in planta*.

As biosurfactant loss did not influence growth in culture, *P. fluorescens* biosurfactants presumably are not necessary for *in vitro* growth. This reflects the results of Gladstone (1997) who found no growth enhancement of selected mutants when co-inoculated on to broccoli heads with viscosin, and syringomycin is known not to stimulate bacterial growth (Gross, 1991). The non-biosurfactant toxin coronatine, produced by *Pseudomonas syringae* pv. *tomato*, is the only toxin known to induce higher bacterial population numbers *in planta*, although growth was not influenced *in vitro* (Bender *et al.*, 1987). This is surprising because if biosurfactants aid colonisation and establishment *in planta* (Bunster *et al.*, 1989), producing strains would be expected to have a growth advantage. The work of Bender *et al.* (1987) found that the toxin coronatine influenced *in planta* (but not *in vitro*) bacterial growth, and so this may also be the case for the biosurfactants of *P. fluorescens*. The

35 mutants isolated were all prototrophic and therefore should grow well *in planta*, but this should now be confirmed. The kanamycin resistance gene conferred within Tn5 would make this straightforward to study for the mutants, but the wild-type strain would require another marker. Spontaneous antibiotic resistance may be the most direct method to obtain this marker, although care must be taken to ensure that no disease or growth related genes are affected.

As any auxotrophic surf $^-$ mutants produced were discarded, valuable genetic information on biosurfactant regulation may have been lost. This also applies to surf $^+$ prototrophs which produced a greater or lesser quantity of biosurfactants than the wild-type. Such syringomycin mutants have been termed hyperproducers and hypoproducers, respectively, with 2.8% of mutants being reported to be hyperproducers (Xu and Gross, 1988a). Although it is likely that such mutants were produced here, they could not be isolated because their detection requires a sensitive quantitative assay. This was demonstrated by Peet *et al.* (1986) who found, after modifying the bioassay used, that the *P. syringae* pv. *phaseolicola* mutants labelled toxin-minus, were in fact, phaselotoxin hypoproducers. Such mutants are important for obtaining a fuller picture of biosurfactant action - studying mutants with increased production may help to clarify the contradictory surf $^-$ results obtained.

The Southern blot results illustrated that 17 of the mutants could be placed into four groups based on their hybridisation profile (after Southern blotting). Two of these groups (b2 and b4) contained mutants from the same phenotypic subgroup. Where this occurred, we can infer that the mutants have all been affected in the same gene,

which was responsible for that particular phenotype. The other subgroups (b1 and b3) contained mutants from different phenotypic subgroups, so different genes must have been affected which were coincidentally cut to the same size following *Sal*I digestion.

6.4.2 Influence of these results on previous studies

The large number of surf$^-$ virulent mutants produced here would appear to indicate that biosurfactants are not a virulence factor in head rot, although further research is required to elucidate why the other 29.4% of mutants were vir$^-$. In support of this claim, Harling *et al.* (1994) found pathogenic non-producers (and in particular, one pathogenic isolate which produced neither pectolytic enzymes nor biosurfactants), and most convincingly, Robertson *et al.* (1993) reported that non-producing *Erwinia carotovora* strains rotted unwounded tissue. These *Erwinia* strains are important because, as the bacterial genus does not produce biosurfactants, there is no possibility of production being stimulated *in planta,* as with *Pseudomonas* mutants. So it would appear that biosurfactants are not involved in virulence. However, there is considerable research which opposes this view. Harling *et al.* (1994) also isolated pathogenic producers which did not produce pectolytic enzymes. Although the biosurfactants could not account for the pathogenicity, they may be important in enabling this strain to attack the difficult-to-wet plant tissues. In addition to Hildebrand's (1989) claim that biosurfactants are a key factor in head rot aetiology of wounded heads, biosurfactants are also known to aid bacterial establishment and colonisation *in planta* (Bunster *et al.*, 1989). If this also applies to head rot strains, it may enhance the opportunity for disease development and would be much more

useful to the bacterium under field conditions where competition for nutrients is fierce. This would also explain why biosurfactant loss reduces disease virulence in head rot, but non-producers can still initiate disease. Such non-pathogenic factors are important to bacterial growth and survival outside the host and will determine the bacterium's response to different physical and biological environmental situations (Sigee, 1993).

More detailed support has been provided by Gladstone (1997) who reported that co-inoculating purified viscosin (obtained from Sigma Aldrich) with surf $^-$ isolate 6415 (produced here), led to a significant increase in disease symptoms, with a 15μg/ml^{-1} concentration producing the maximum increase (from 50 to 68% of the maximum disease score). This suggests that biosurfactant was enhancing the ability of this mutant to cause disease, but it must be remembered that in artificial situations, artefacts are possible, particularly when high concentrations are used (Sigee, 1993). When co-inoculating surf $^-$ isolate 6415 with biosurfactant extracted in the laboratory from culture filtrates of *P. fluorescens* strain P5064, the effect on disease production was less, which Gladstone (1997) explained as a concentration difference. The extracted biosurfactant may also have been a mixture of viscosin and other (possibly less phytotoxic) biosurfactants, or they may have been chemically unstable as this is often a problem with secondary metabolites (Sigee, 1993). Inoculating extracted biosurfactant, without bacteria, induced similar or higher disease levels than the corresponding concentration of extract plus isolate 6415. This may be due to the bacterial suspension diluting the concentration of the biosurfactant extract (the bacterial suspension was mixed 50:50 with the biosurfactant suspension) or it may

represent an artefact of the strong biosurfactant concentration used. It should be noted that isolate 6415 was in subgroup 2: Non-pathogenic with extracellular product production intact. However, it also contained a secondary mutation, making it unsuitable for this study because the reduced virulence cannot be directly linked to biosurfactant production. In addition, the mutated gene's function was not known, so it may be pleiotrophic or responsible for a major pathogenicity determinant which would prevent any quantity of biosurfactants stimulating disease. Therefore it is important to be cautious in the interpretation of results.

The most convincing evidence for biosurfactant involvement in disease is the necrotic, biosurfactant-induced, stomatal lesions reported by Hildebrand (1989). Gladstone (1997) also found microscopic lesions on the stomata of broccoli heads inoculated with both purified viscosin and P5064. Biosurfactant concentrations as low as 1.5μg/ml stimulated this response, which was explained as being partially due to additional biosurfactants from saprophytes. This evidence, and the fact that synthetic surfactants induce lesions on plant tissue (Knoche *et al.*, 1992), depending on their chemical structure (cationic ones are more phytotoxic than anionic ones) (Falk *et al.*, 1994), suggests that biosurfactants may well be influential in disease. Maybe chemical structure is the key factor in pathogenesis whether bacterial or synthetic. Possibly non-pathogenic, biosurfactant producers, produce the wrong type of biosurfactants for disease virulence. However, it must also be noted that there have been reports of synthetic surfactants enhancing plant growth (Kumar *et al.*, 1992), so surfactant phytotoxicity may be concentration dependant. If the researchers cited here (i.e Hildebrand (1989); Kumar *et al.*, (1992)), used strains

which produced different biosurfactant types, this may explain the conflicting results obtained. It would be interesting to test whether the pathogenic, non-biosurfactant, strains isolated by Harling *et al.* (1994) can also induce lesions. This would help to clarify (if no biosurfactants are produced *in planta*) if biosurfactants are toxic to broccoli tissue.

Gladstone also found that in addition to forming lesions, purified viscosin, isolate p5064 and surf ¯ 6423 degraded the floret's wax crystals to leave the cell walls exposed: Viscosin damaged all floret tissue, while P5064 and 6423 caused regional damage only (Gladstone, 1997). Isolate 6415 and the water control did not damage the wax layer in any way. Therefore one surf ¯ mutant (6423) damages the wax, while the other (6415) does not. It would appear that either 6423 still produces low levels of biosurfactant *in planta*, or the wax damage is not biosurfactant-related. Gladstone (1997) concluded that viscosin and the extracted biosurfactant from p5064 were phytotoxic. This contradicts Hildebrand *et al.* (1990) who found no effect on the wax, but supports the findings of Bussotti *et al.* (1997) and Schreiber *et al.* (1995) who, respectively, found that the synthetic surfactants Sodium Alkylbenzene Sulphonate (SABS) and Brij30 induce changes in plant cuticular wax.

If biosurfactants are proven to be phytotoxic, why are there reports of non-producing pathogens, such as the *Erwinia* pathogens which do not produce biosurfactants at all? The most feasible explanation would be the effect residual bacteria are having on the pathogenicity test results. The number of residual bacteria may well have varied considerably between research groups depending on the cultivars used and the

production conditions, especially as the different groups were located world-wide. While there were approximately 2.4×10^3 cfu residual bacteria on the heads used here (Section 2.3.1, Chapter 2), the number of biosurfactant producers has not been established. Stanghellini and Miller (1997) reported that rhamnolipid producers were readily isolated from the roots and leaves of field grown plants, and although not quantified, Bunster *et al.* (1989) and Hildebrand (1989) claimed that high numbers of biosurfactant producers were obtained from plant tissue. If residual bacteria are influencing *in vitro* results, they will surely be more influential in the field where their numbers are higher and competition between isolates is great. For this reason, biosurfactants may have a more significant effect under field conditions than *in vitro*.

Possibly the importance of biosurfactants depends on the other pathogenicity/virulence factors produced: If a pathogen produces copious other virulence factors, biosurfactants may not be necessary, but they may provide the necessary help for less virulent pathogens. This may explain why some pectolytic enzyme-producing bacteria were only pathogenic when they were co-inoculated with biosurfactant-producing strains (Hildebrand, 1989), yet other non-producing strains induce disease (Robertson *et al.*, 1993). Ideally isolates from all research groups should be tested under the same laboratory conditions to allow for variation in factors such as residual bacteria, environment and cultivar susceptibility to be eliminated. This, in addition to testing greater numbers of biosurfactant producers, should help to clarify the situation. Unfortunately non-pathogenic isolate N5024, and pathogen P5049, failed to mutate in this study, even after altering the experimental conditions, but another vector may be more successful.

6.4.3 Relationship between head rot biosurfactants and related virulence toxins

Although further work is required before the function of biosurfactants can be elucidated, analysing the homology present between head rot biosurfactants, such as viscosin, and the lipopeptide surface-active toxins syringomycin, syringopeptin and tolaasin, may provide clues to viscosin's biological role. The lipopeptide toxins are the only plant toxins to produce necrosis in plant tissues via plasmalemma disruption (Sigee, 1993). This therefore adds weight to the reports of head rot biosurfactants causing necrotic lesions in broccoli (Hildebrand, 1989; Gladstone, 1997). It is the toxins' lipopeptide structure which enables them to insert into the lipid membrane layers (Takemoto, 1992), forming pores which allow ions to travel across the plasmalemma. Therefore in theory, this activity should be feasible for viscosin. The toxins cause hyperpolarization, rapid K^+ efflux and stimulation of a proton pump ATPase (Gross, 1991) with prolonged ion transport effects being lethal to affected cells (Mott and Takemoto, 1989). The export of K^+ ions activates leaf stomata closure (similar to the way abscisic acid regulates stomatal closure) (Mott and Takemoto, 1989); leads to the accumulation of sucrose, amino acids and inorganic ions within intercellular spaces, and raises the acidic pH of intercellular fluid to almost neutral making the environment more conducive to bacterial establishment (Gross, 1991). Non-toxin biosurfactants have also been described as enhancing bacterial colonisation and establishment on plant tissues (Bunster *et al.*, 1989). For tolaasin, which facilitates the spread of the pathogen on mushroom caps by lowering surface tension of water, Zn^{2+}-sensitive ion channels are formed at a concentration of 200ng/ml (Brodey *et al.*, 1991; Rainey *et al.*, 1991). However, syringomycin is the

most widely studied biosurfactant toxin, and many similarities can be observed between it and the head rot biosurfactant viscosin.

Highly virulent *P. syringae* strains produce 10-80μg/ml of syringomycin *in vitro* (Gross and DeVay, 1977b). This correlates with the level of viscosin that Gladstone (1997) found produced the highest increase in disease symptoms. Syringomycin lowers the surface tension of water to 31mN/m (Hutchison *et al.*, 1995), viscosin to 27mN/m, and they require 1.25mg/ml (Hutchison *et al.*, 1995) and 4-9mg/ml (Laycock *et al.*, 1991), respectively, to form the critical micelle concentration. Syringomycin apparently accentuates the disease process by killing a large number of host cells during pathogenesis as reflected by larger lesion size (Gross, 1991). The lesions produced by syringomycin-minus mutants, affected in genes *syrB* or *syrC*, are only about 60% of the wild-type size (Mo and Gross, 1991). It would be interesting to determine whether pathogenic and non-pathogenic head rot strains induce different sized lesions. It is known that *syrB* is transcriptionally activated within 24h of bacterial penetration of host tissue (Mo and Gross, 1991), which may be similar to the genes induced in *P. fluorescens* to produce additional HPLC peaks. Both syringomycin and viscosin lyse erythrocytes: The dynamics have not been studied for viscosin, but syringomycin does so at a threshold of 500ng/μl, via ion-channels of between 0.6 and 1nm in size (Hutchison *et al.*, 1995). Both biosurfactants are synthesised in the stationary phase of growth (Mo and Gross, 1991; this work), and may be regulated via a thiotemplate mechanism (Mo and Gross, 1991) because of the presence of D-amino acids, unusual or modified amino acids, and cyclised carbon skeletons (Sigee, 1993). The unusual structure makes syringomycin resistant to

inactivation by non-specific degradative enzymes and as viscosin also has D-amino acids this may also apply, thereby enabling producers to resist competition from other microbes.

Although it can be seen that both biosurfactants share many properties, the role of syringomycin's biosurfactant activity in the plant-pathogen interaction has not been fully characterised. Several theories have been proposed for the biosurfactant activity of syringomycin: (1) Syringomycin, like tolaasin, may facilitate the spread of bacteria on plant surfaces by reducing the contact angle of water to new areas of the host surface where nutrients concentrate (Hutchison *et al.*, 1995); (2) water films on plant surfaces may concentrate the toxin as evaporation occurs, thereby enhancing detergent activity (Hutchison *et al.*, 1995); (3) Che *et al.* (1992) suggested that its detergent-like action was largely responsible for the inhibition of H^+-ATPase activity in mung bean cells, and (4) micelle formation may have a critical role in the pore formation because of the propensity amphipathic syringomycin molecules have to partition the host plasma membrane. In addition, Hutchison *et al.* (1995) proposed a model for the biosurfactant action of syringomycin as a virulence factor: If the *in planta* syringomycin concentration rises above the CMC, micelles predominate and solubilise the plasma membrane, while at concentrations near the CMC a few micelles form and fuse with the plant plasma membrane forming functional pores.

Therefore, like head rot biosurfactants, the biosurfactant activity of syringomycin is hard to define. However, whatever its function, the biosurfactant activity of syringomycin must be vital to the bacterium's fitness, because a relatively large

proportion of the genome (over 100kb) is dedicated to its synthesis (Caponero *et al.*, and Gross *et al.* both in press, cited in Hutchison and Gross, 1997) - as biosurfactant production is complex, it would not have been maintained if they were not significant in the long term survival of the bacterial species (Gross, 1991). If syringomycin's biosurfactant activity does influence virulence, it would be likely that viscosin does also because of the homology they share.

6.4.4 Could biosurfactants reduce plant disease?

Although 57% of mutants produced here were unaffected in virulence, there is abundant contradictory research supporting the involvement of biosurfactants in disease. Conclusions can only be drawn after the genes mutated here are assessed further. However, interestingly, there may be another function for biosurfactants - as biocontrol agents.

As *P. fluorescens* is saprophytic on the majority of its hosts, the biosurfactants it produces cannot be toxic towards all its hosts. Broccoli may be more susceptible to the phytotoxic effect of *P. fluorescens* biosurfactants than other plant species, or it may be one of the few hosts which stimulates *in planta* biosurfactant production. However, biosurfactants such as viscosin, are known to lyse cells of other microbes (Groupe *et al.*, 1951; Gladstone 1997). Indeed, syringomycin and syringopeptin display different spectra of antimicrobial activities from each other, which may be important to the epiphytic survival of the bacterium. In terms of head rot, this may be influential in disease aetiology by eliminating competitors and allowing the pathogen to colonise and induce disease. It would be interesting to determine whether head rot strains are more resistant to the harsh effect of industrial surfactants

in pesticide sprays because they themselves are producers. If so, they may remain to initiate disease, while more susceptible non-pathogenic strains are eliminated. Alternatively, *P. fluorescens* strains, other than those involved in head rot, may potentially act as biocontrol agents, as they lyse many micro-organisms yet are non-pathogenic on the majority of plants. Stanghellini and Miller (1997) describe how between 5-30μg/ml of rhamnolipid biosurfactants from *P. aeruginosa* successfully lyse zoospores by destroying the plasma membrane permeability (Stanghellini and Tomlinson, 1987). Although other biosurfactants, including a glycolipid, a sophorose lipid, a trehalose lipid and the lipopeptide, surfactin, did not have the same biocontrol properties, it could be possible to find a biosurfactant producing strain which could reduce the occurrence of head rot. It was claimed that the possibility of rhamnolipid-insensitivity developing is low because it would require a major structural change in the chemical make up of the plasma membrane (Stanghellini and Miller, 1997). There is a dichotomy between biosurfactant's biocontrol and phytotoxic properties, in plant pathogenesis, and if both are in action at once, competitors will be reduced and disease will be initiated, giving the pathogen a significant competitive edge over other micro-organisms.

Therefore biosurfactant activity is diverse, depending on its structure, concentration and host which makes it difficult to elucidate their role.

6.4.5 Conclusions and further work

Further research is required before any firm decision can be made about the involvement of biosurfactants in disease. The identity of the mutated genes must be established: Each fragment containing a Tn5 insert should be: (1) cloned and

sequenced, and (2) used as a probe to locate the wild-type gene. Sequence homology may be found with other genes in a genebank database (especially syringomycin), but if not, gene function could be ascertained via an expression vector to identify the translated proteins. This would allow for more detailed work into the control of biosurfactants: mRNA expression could be analysed in the presence of specific plant metabolites to determine what factors influence production. The mutants containing IS50 insertion should also be studied. To determine whether the reduced virulence in the mutants with additional mutations, was due to Tn5 or the secondary IS50 inserts, the interrupted genes could be located and used to transform the wild-type strain and if they become avirulent, this demonstrates a high linkage between Tn5-encoded Kan^r determinant and the mutated virulence gene. (Boucher *et al.*, 1985).

Following this, further characterisation of vir^- examples 6422, 6426 and 6403 and vir^+ 6410, and 6423, which all possess only one mutation, should provide information about whether biosurfactants have virulence properties. For example, they could be analysed *in planta* to determine whether production increases or changes; whether residual bacteria may be influencing their disease potential, and if producers can colonise more successfully than non producers. Mutagenesis should ideally be carried out on other biosurfactant producers - non pathogenic and pathogenic, to eliminate doubts over whether P5064 was atypical.

We have seen that in relation to plant-bacteria interactions, biosurfactants may possess the ability to: (1) form lesions in plant tissue; (2) affect cuticular wax; (3) be

potential biocontrol agents; (4) aid bacterial colonisation and establishment on plant tissue; (5) spread bacteria around the plant surface to where nutrients are present; (6) concentrate other pathogenic factors by evaporation of water droplets, and (7) enhance the non pathogenic survival of producing bacteria. Whatever the outcome of future research, their involvement in bacterial fitness must be vital, due to the large genomic loci devoted to their synthesis. The influence of residual bacteria can not be underestimated in the results, especially as they are abundant under field conditions. Further analysis of the genes affected in the surf $^{-}$ mutants produced here and the production of mutants in other bacterial strains should clarify whether there is a biosurfactant involvement or not.

Chapter 7: General discussion and suggestions for future research

The research conducted in this project has tackled a variety of topics within the broad areas of bacterial pathogenicity and host susceptibility to head rot disease, and the results have provided novel information about the disease process upon which future research can now build with the ultimate aim of developing effective control strategies.

7.1 Summary of the results obtained in this study

To recap on the results, a successful pathogenicity test has been developed, based on the inoculation of mature broccoli heads with inoculum soaked lint. This technique can accurately differentiate between the disease susceptibility levels of the broccoli cultivars tested, and reflect those found in the field by Robertson *et al.* (1993) (with one exception, Shogun, which is thought to be becoming more susceptible to disease in the field (Harling, per communication)). In addition, this test is rapid, induces strong disease symptoms, and the inoculation procedure does not inflict wounding on the plant tissue. It has proven to be invaluable for the work carried out during this project and should also aid future research into the disease process.

A seedling pathogenicity test, based on wounding the seedling's hypocotyl with a needle containing bacterial inoculum from an agar plate colony, failed to generate reproducible results, successful disease symptom expression, or accurate cultivar disease susceptibility ratings. It was speculated that the test may benefit from the inoculation of older seedlings, or the use of seedlings raised under a different nutrient source (because plant age and nutrient source are important factors in the development of disease). Unfortunately altering the inoculation procedure did not

generate more reliable results, but it did confirm (for both the excised head and seedling pathogenicity tests) that tissue wounding results in greater symptom development, and in the case of the excised head test, that free water availability was considerably more important than the inoculum concentration in the outcome of disease.

Head size and head doming were found to be the most important morphological factors in determining cultivar disease susceptibility. Bud number and stomatal number also produced high correlations (but not at a significant level) indicating that they may have a weak role in influencing cultivar susceptibility, but bud prominence was not related to disease susceptibility. As discussed in Chapter 3, these results have important implications for the development of more resistant cultivars by plant breeders, and represent the most directly practical results produced during this thesis.

When studying the growth, *in planta*, of one pathogenic and one non-pathogenic bacterial strain, the results indicated that the pathogenic strain (inoculated on to broccoli tissue using the protocol of the excised head pathogenicity test) multiplied no more vigorously than the non-pathogenic strain inoculated (hence, growth rate did not give rise to its pathogenic phenotype). Moreover, other observations suggest that the growth rate of both bacterial isolates was equivalent on both susceptible and resistant tissue (implying that cultivars susceptibility was not influenced by the rate of pathogen multiplication and spread), and when both strains were inoculated together no competition was detected between the two bacterial isolates (so the pathogens' disease phenotype did not arise from it having the ability to out-compete other

bacteria). Unfortunately, though, variability was too great to be certain about the results - however, it provides a hypothesis for future experiments. Therefore these results have provided useful tentative information about the growth dynamics of two bacterial strains *in planta* (which may not be common to all head rot pathogens). It would now be interesting to expand this experiment, with a much greater number of replicates (at least 20/treatment) and to encompass a greater number of head rot pathogens and broccoli cultivars, as a sound knowledge regarding the *in planta* survival of head rot pathogens is crucial in the development of control methods.

When developing a biosurfactant detection test to study the role of biosurfactants in the disease process, a water droplet technique was found to be the most sensitive and rapid method (of five tested). The other four methods tested failed to detect biosurfactants, indicating that they were not sensitive, either to the quantity or type of biosurfactants produced. Further work is now required to elucidate the exact nature of the biosurfactants produced, in terms of their chemical structure, quantities produced and how many different types the pathogen produces. This may help determine why certain tests failed and provide a better insight into biosurfactant production by head rot pathogens.

To analyse the role of head rot biosurfactants in disease, 35 *Pseudomonas fluorescens* biosurfactant-minus mutants were produced using Tn5 mutagenesis. The mutants all grew at an equivalent rate to their wild-type *in vitro*, but 15 were found to be significantly reduced in their pathogenic status. However, Southern blotting (utilising a Tn5 probe) illustrated that 18 of the mutants apparently contained more

than one insert which was speculated to have arisen from either independent IS50 insertions or insufficient digestion with the restriction enzyme *SalI*. Consequently the resulting phenotypes of the 18 mutants may not have been directly due to the loss of biosurfactant production. Of the 17 mutants with only one Tn5 insert, five showed reduced virulence and 12 showed no change in disease phenotype and therefore, until further work is conducted to elucidate the exact nature of the mutations, it was tentatively concluded that biosurfactant production is unlikely to be a factor in head rot pathogenicity or virulence. Although incomplete, this research has been invaluable in expanding our knowledge of this important, yet poorly understood, process.

Therefore, as discussed, the research conducted during this project has given rise to an extensive base of knowledge about head rot which will ensure that the most appropriate research routes are taken in the future to derive at the main characteristics of the disease's aetiology.

7.2 Suggestions for future research

As mentioned previously, future research would benefit from a detailed study of the biosurfactants produced by various head rot pathogens to obtain a better understanding of their role in bacterial fitness. Even though the results obtained in this project indicate that biosurfactants may not be involved in the disease process, previous research into the lipodepsipeptide biosurfactant, syringomycin, indicates that a considerable amount of the bacterium's genome is concerned with the production of these biosurfactants (Gross, 1991)) and so they must be extremely important in terms of bacterial survival. If biosurfactants only influence non-pathogenicity factors,

such as establishment on plant tissue or the ability to compete with other microbes, these factors may still indirectly sway the outcome of disease.

To research this topic in greater detail, it is important to develop a more sensitive, and fully quantitative, detection test. Possibly an agar plate microbe inhibition assay would prove to be suitable method, as this works well for the lipodepsipeptide syringomycin (Sinden *et al.*, 1971) and viscosin has been shown to antagonise other microbes (Gladstone, 1997). A surface tensiometer could be utilised, alongside the development of a test method, to confirm accurately when biosurfactants are produced. To analyse the biosurfactants produced, it would be useful initially to conduct thin layer chromatography (TLC) on the cell free extracts of biosurfactant producing strains (both pathogenic and non-pathogenic), taken at various time points after the inoculation with bacterial isolates of both liquid broth, and broccoli tissue. This would determine both when biosurfactants are produced (*in vitro* and *in planta*) and which types of biosurfactants are produced by each strain tested. Of course, negative controls would be particularly important in this type of analysis to ensure that the results were not subject to artefacts - non-biosurfactant producing strains and un-inoculated test samples would provide sufficient verification.

Furthermore, the biosurfactant mutants produced during this project should now be analysed fully, at the molecular level, to determine the function of the mutated genes, and whether the additional bands obtained in 18 mutants, following Southern blotting, were due to IS50 insertions or insufficient digestion. To identify whether poor digestion was responsible, the mutant's DNA could be re-digested with *Sal*I and

Southern blotted to determine whether the same hybridisation pattern was produced, after probing. Then cloning, and sequencing, could be conducted on the DNA corresponding to the hybridised bands to determine the partial genetic sequence information available from the mutated genes (the full sequence will be interrupted by Tn5). To determine fully the role of biosurfactants, the gene(s) responsible for production need to be isolated, and then mutated (or deleted) and placed back into the bacterium, to test for the effect. To obtain the full gene sequence for any mutation, the partial genetic information (described above), would have to be labelled and used as a probe to 'fish-out' the corresponding wild-type gene in the non-mutated parental strain. Once the wild-type DNA was located, it could be cloned into an expression vector to produce abundant quantities of the gene's protein product, or alternatively particular domains of the protein product's function could be studied by fusing part of the proteins coding sequence with a gene of known function, producing a recognisable fusion product (Alberts *et al.*, 1992), which can then be used to study gene expression *in planta* (i.e. Loper and Henkels, 1997). Alternatively, the gene's mRNA could be isolated and used as a probe to study the transcriptional activity of the gene in bacteria growing *in planta*. It would also be useful to compare the sequence information against a genebank such as the European Molecular Biology Laboratory (EMBL) database, based at Hinxton Hall near Cambridge (http://www.ebi.ac.uk), to analyse for homology with previously identified genes, because this may provide an insight into the gene's function. It is particularly likely that certain mutated genes will share a high degree of homology

with some of the genes responsible for syringomycin production, especially because both viscosin and syringomycin share a similar lipodepsipeptide structure.

The putative independent IS50 insertions could be located by re-probing the Southern blots produced in Chapter 6 with a probe which does not contain IS50 information (probe 2) - if one band is no longer detected, this would clarify that an independent IS50 insertion had occurred, rather than due to insufficient digestion. If IS50 insertions are responsible, the mutation could then be analysed further by cloning and sequencing, as discussed above. If insufficient digestion was responsible for the additional bands obtained (and this was not detected by re-digestion), this would be identified when the bands were sequenced, as different hybridisation bands would be found to be duplicate copies of the same genetic mutation after sequencing.

However, there are areas other than those studied during this project which could be explored. Various bacterial pathogenicity factors, well studied in other plant-pathogen interactions, are currently untested in head rot. Siderophores are a good example of this. These water soluble, rapid diffusing compounds, which chelate iron are identified as either pyoverdines or pseudobactins (Sigee, 1993), and include the fluorescent pigments produced by many *Pseudomonas* species. Siderophore production is an important aspect of the growth and ecology of both pathogenic and non pathogenic organisms (iron is a biologically important metal), but it is of interest in plant pathology for two reasons: (1) the possible association with virulence, and (2) the implications for biological control due to iron competition between microbes. For example, these iron-chelating agents are essential for pathogenesis in bacteria

such as *Erwinia chrysanthemi*, towards *Saintpaulia*, and *Erwinia amylovora* in fireblight, by chelating iron in the iron-limited conditions which prevail *in planta* (Mourgues *et al.*, 1998). Furthermore, in bacterial seedling blight of rice caused by *Pseudomonas plantarii* a siderophore-like substance, troporone, causes yellowish blight of leaves and the inhibition of root elongation at concentrations of 3-25ppm (this toxicity is suppressed by the application of iron) (Goto, 1992). In terms of biocontrol, Buysens *et al.*, (1996) stated that the production of either pyoverdin or pyochelin was necessary for the wild-type level of protection against *Pythium*-induced post-emergence damping-off. In addition, during antagonistic interactions between different micro-organisms, specific strains of the *P. fluorescens-putida* complex exert their plant growth-promoting activity by depriving native microflora which rely on iron nutrition (Goto, 1992).

Therefore, possibly siderophores are influential in the pathogenesis of broccoli tissue, by aiding the pathogenic strains to out-compete other bacterial strains and fungi on broccoli. This is especially relevant because the major head rot pathogens are siderophore producing (fluorescent) Pseudomonads. The ability of a *Pseudomonas* strain to produce siderophores can be demonstrated *in vitro* by their ability to grow on an iron deficient medium such as King's B agar, (1954) and viewing the plates under UV light to detect the presence of any yellow-green fluorescent (siderophore) pigments (although this does not imply that they produce them *in planta*), or by the chrome azurol S assay which is a universal test (Berraho *et al.*, 1997), that would detect non-fluorescent siderophores. Siderophore production from *Pseudomonas fluorescens* has been analysed with the use of a fusion between a Fe-regulated

promoter to an ice nucleation reporter gene (inaZ) (Loper and Henkels, 1997). They found that expression was maximum within 12-24h following inoculation onto bean roots and typically decreased during the following 4 days. In the case of head rot pathogens, the influence of siderophores in disease development could be analysed by producing mutants which can no longer produce siderophores (as described in Chapter 6 for the research carried out into biosurfactants). The use of mutants in biological research is invaluable and should not be underestimated. This would also be more straightforward with siderophores, than biosurfactants, because many siderophore genes (and their regulators) have now been sequenced. Alternatively, a DNA database search could be implemented to determine whether there is any genetic homology with the siderophores already known to induce disease in other species.

The role of ice nucleating activity (INA) in disease has been equally well documented. INA bacteria that reside on plant surfaces induce quick freezing of plant cells above normal freezing point, resulting in substantial damage to plant cells or entry and multiplication of pathogenic bacteria in ice-damaged tissues (Billing, 1987). Strains of both pathogenic and non-pathogenic bacteria may induce rapid freezing at temperatures above -10°C, with both environmental conditions (production is more efficient on solid growth medium, and medium containing polyalchohols such as mannitol, sorbitol and glycerol), and the physiological state of the bacteria affecting ice nucleating activity (Sigee, 1993). It would therefore be interesting to determine whether the ice nucleating activity of head rot strains aids bacterial entry into broccoli tissue, especially because, like INA, head rot is

dependant on environmental conditions. In *P. fluorescens* the ice nucleating activity (ina^+) phenotype is known to be imparted by a single gene, *ina*W, which encodes the 180kda ice nucleating protein inaW. This gene shares close homology with the *ina*Z gene of *P. syringae* (Warren *et al.*, 1986), which is involved in the induction of blossom blast of pear (Sigee, 1993). It would be useful to determine if all head rot strains are ina^+ by placing 10 droplets of bacterial suspension on a hydrophobic surface at a specified freezing temperature and noting the number of droplets freezing within a three minute period - the proportion of droplets freezing at decreasing temperatures can then be plotted to reveal whether the bacterial strain is ice nucleating active (Sigee, 1993), or by synthesising a DNA probe (or PCR primers) to the *ina*W gene and testing all head rot strains to determine if they possess this gene. It would then be possible to conduct mutagenesis to determine if this activity is important in disease. Alternatively, in strains containing the *inaW* gene, the *inaW* gene's mRNA product could be probed for (with a synthesised oligonucleotide probe), from *in vitro* and *in planta* samples, to confirm when transcription of the ice nucleating gene occurs. This would allow the influence of INA on plant tissue to be studied in relation to disease development. Alternatively, INA could be analysed by stimulating over expression of the *inaW* gene and recording the effect this has on disease development.

It would also be interesting to determine whether head rot pathogenicity genes are located on plasmids (circular closed molecules of DNA), which are important for encoding a wide range of functions that are important in plant-microbe interactions (Billing, 1987). For example, genes such as *avr*, *hrp* and those encoding the

production of toxins (e.g. coronatine) and plant hormones are present on bacterial plasmids (Sigee, 1993). Furthermore, the tumour inducing (Ti) and root-inducing (Ri) plasmids of *Agrobacterium tumefaciens*, have been well reported which are responsible for crown gall and hairy root symptoms, respectively (Agrios, 1988). In this species, growing the bacteria at high temperatures results in the loss of the plasmid which consequently causes loss of pathogenicity. Plasmids may also be involved in encoding non-pathogenicity factors such as bacteriocin production and antibiotic resistance (Sigee, 1993), which could be useful to study in terms of obtaining a biological control agent against head rot pathogens. Plasmid based genes can easily be isolated and inserted into a non-pathogenic bacterium (altering its phenotype) to produce a strain which may be able to function as a biocontrol agent. Alternatively, if the head rot pathogenicity genes are plasmid based, the plasmid could be cured to produce a strain which is no longer pathogenic, but which may still retain its ability to successfully colonise plant tissue (and therefore may act as a control against the wild-type pathogen). For analysis of this subject, plasmid DNA can be isolated from chromosomal DNA by centrifugation. After digestion and electrophoresis, the plasmid DNA could then be analysed for the presence of known pathogenicity genes using the methods described above, or plasmid-cured cells could be inoculated onto broccoli tissue to determine whether there was any alteration in the bacterium pathogenicity phenotype due to the loss of the plasmids.

Whatever research route is chosen, the ultimate aim of head rot research is to arrive at a level of knowledge which will facilitate the development of a reliable control method, and therefore this topic will be discussed in the next section.

7.3 Control of head rot

Bacterial diseases are often difficult to control. This is due to several reasons. Plants only have primitive circulation systems to aid the systemic movement of a control agent, and consequently the agent may not reach sites where the pathogen persists. In addition, they have no immune system to support any control which is achieved, and so the control agent needs to be applied every season. Additionally, because the plant is continually growing, repeat applications may be necessary during a season (Billing, 1987). Moreover, whilst many potential control agents have little penetrative power, the phytotoxicity of others precludes their use (Billing, 1987). As discussed, there are currently no satisfactory control methods for head rot and there are no completely resistant cultivars. Therefore the development of a biological control agent may provide the answer.

Biological control agents may be naturally occurring or genetically engineered strains. It is important to ensure that, for example, a particular agent is not ice nucleation active or itself phytopathogenic. The use of genetically engineered antagonists has implications for the introduction and spread of new genes in the general microflora, with unknown and possibly unpredictable results. However, in spite of these limitations, biological control is generally considered a more ecologically acceptable approach when compared to chemical control, and it is more suited to modern concepts of integrated pest management (IPM) (Sigee, 1993).

Fluorescent Pseudomonads, such as *P. fluorescens*, are particularly important as biological control agents in the rhizosphere since they are widely occurring, usually non-pathogenic, and highly effective at eliminating competing microbes.

Pseudomonas spp., *Bacillus subtilis* and *Enterobacter cloaceae* are amongst the bacterial organisms which have been reported to reduce both damping off caused by *Pythium, Rhizoctonia solani* and *Sclerotium rolfsii*, and root rot caused by *Fusarium oxysporum, Sclerotium rolfsii* and *Thielaviopsis basicola* in several vegetable crops when applied as a seed treatment or a soil drench (Punja, 1997). In addition, the application of a bacterial antagonist has even been reported to increase plant growth yield of mature celery, cucumber, onion and tomato under both glasshouse and field conditions. For example, McCullagh *et al.* (1996) reported that applying *P. fluorescens* increased the growth of cucumber plants and their fruit, and provided a yield increase of 18% compared with untreated plants infected with *Pythium aphanidermatum*. Whilst in field trials, wheat yields have been increased by 27% by inoculation of seeds with *P. fluorescens*, and similar treatment of potato seed tubers has been found to increase tuber yields by as much as 70% (Lindsey and Jones, 1989). Such bacteria are called plant growth promoting rhizobacteria (PGPRs).

For effective biological control, the antagonist must survive and grow under natural field conditions to successfully compete on a long term basis with the phytopathogen. Biocontrol using bacterial antagonists is postulated to enhance plant growth and reduce disease by utilising a number of different mechanisms. These include the production of antibiotics and toxins which reduce pathogen growth and infection potential (and may include the biosurfactant discussed in Chapter 6); competition for infection sites or nutrients required by the pathogen to penetrate the host; stimulation of plant growth and vigour; and induction of resistance mechanisms in the host that prevent or slow pathogen ingress. Therefore the production of siderophores,

bacteriocins, antibiotics, and INA activity may all be influential in microbe antagonism. In addition, the biosurfactants produced by non-pathogenic biosurfactant producers which are known to inhibit certain isolates may also result in the development of a biocontrol agent. Although Canaday *et al.* (1987) found that applying bacterial antagonists or a water surfactant was ineffective in reducing disease incidence and severity, it does not mean that other strains will not be able to stop disease.

The potential of developing a biocontrol method to reduce the incidence of head rot is attractive, but would require considerable research to achieve satisfactory results. To reach commercialisation several criteria must be satisfied and data obtained to demonstrate aspects of efficacy, survival, adaptability and scale up potential (Punja, 1997). These include initially assessing the market potential and needs; developing methods for isolating potential biocontrol strains from nature; screening the isolates in laboratory and glasshouse assays for biocontrol activity; studying the effect of environmental factors on biocontrol activity; studying methods of survival, multiplication and spread; studying the mode of action of biocontrol; conducting full field evaluation trials over different years and environments; assessing formulations to maintain activity; assessing scale up required for inoculation production; registering the method for crop use and initially conducting commercial utility/efficacy monitoring (Punja, 1997). Therefore the development of a head rot biocontrol agent may also be hindered because the environmental parameters under which broccoli is produced cannot be manipulated, and so the efficacy of a biocontrol organism may be reduced. However, because various biocontrol agents are also tolerant to chemical

control methods, a reasonable level of control may be achieved by utilising both a chemical, and biocontrol, agent.

Other theoretical methods for controlling diseases such as head rot would be to genetically engineer resistance in the plant, by placing a non-host gene (conferring a resistant trait) into the susceptible plant (transformation). This methodology would significantly reduce the time required to introduce resistance from wild species into commercial cultivars by conventional breeding, but again, because head rot research is limited it will take considerable time to isolate genes which will confer resistance. Furthermore, a fundamental problem with breeding for resistance is that a resistant plant may not be viable commercially, either for cultural or for cropping reasons, or because the consumer or processor finds it unsatisfactory (Billing, 1987). However, in certain diseases there are few successful alternatives available. For example, diseases such as bacterial leaf spot of bell pepper (*Capsicum annuum*), caused by *Xanthomonas campestris* pv. *vesicatoria*, are difficult to control either chemically or biologically, and so genetic control is the only feasible approach of control at present (Sigee, 1993). The first stage in developing genetic control of disease is to identify varieties of plant that are resistant to infection (primary sources of resistance). These may then either be cultivated directly, or their resistance genes combined with other genotypes in a breeding programme which may involve sexual hybridisation, somatic hybridisation or molecular genetics (Sigee, 1993).

Examples of the proposals for such transformations in other crop diseases include producing antibacterial proteins within plants, such as the insect cecropin B gene

(produces a lytic peptide which forms pores in bacterial membranes). Florack *et al.* (1995) found that this approach failed to confer resistance to *Ralstonia solanacearum* or *P. syringae* in transgenic tobacco, as the protein cecropin was degraded by plant proteases, but more recently a stable analogue has been expressed in tobacco and no necrosis was found upon inoculation with *P. syringae* (Huang *et al.*, 1997). Another possibility is to inhibit bacterial pathogenicity and virulence factors. For example, *P. syringae* pv *tabaci* defends itself against the tabtoxin it produces with the gene *ttr*, and this gene has now been expressed in transgenic tobacco plants to provide resistance against the toxin. In addition, the expression of monoclonal antibodies by transgenic plants has successfully protected plants against viral infections, and engineering plants with transferrins (iron scavengers from vertebrates) deprives bacteria of the iron they require to initiate disease and therefore may induce disease resistance. Another approach would be to enhance natural plant defences by increasing the production of elicitors which are recognised by plant cells to trigger defence mechanisms, as this may generate less susceptible plants. This was tested in transgenic tubers producing the pectinolytic enzymes of *Erwinia carotovora* (known to release elicitors). In addition, the expression of cloned resistance genes, such as those involved in the production of phytoalexins, and enhanced production of reactive oxygen species (which cause local hypersensitive cell death, direct antimicrobial activity and reinforce plant cell walls), may increase plant resistance to disease. Finally, the introduction of artificial programmed cell death at the infection site may lead to successful resistance. To avoid the deleterious effects of a general hypersensitive response, these strategies depend on transgenic induction being

restricted to the site of infection, and a key factor in the success of this is the choice of promoter. The systems which have been proposed so far include the expression of the bacterial ribonuclease gene (barnase) driven by a pathogen-inducible promoter (prp 1-1) and an inhibitor of barnase (barstar) driven by a constitutive promoter to avoid deleterious effects from background barnase activity. This system has successfully been used to protect transgenic potato from *Phytophthora infestans* (late potato blight). However, no commercial applications have yet been achieved (Mourgues *et al.*, 1998). Present limits to commercial applications of bioengineering for plant resistance include the fact that to create bacterial resistance in plants on a commercial scale would require efficacy, durability, absence of toxicity and low environmental impact criteria to be met. Antibacterial strategies are difficult to evaluate for efficacy prior to plant transformation. In addition to transgenic integration, efficacy depends on the expression level which can sometimes be affected by homology-dependant gene silencing. This is a serious threat for strategies using homologous sequences (e.g. R genes, phytoalexin genes etc.). Furthermore, proteins must be synthesised, exported and transported to their desired location sufficiently quickly, and without major modification, and be stable at their destination, avoiding degradation by plant proteases. The durability of such systems is even more difficult to anticipate because it is dependant on the ability of bacteria to overcome novel resistance e.g. *avr* genes show frequent mutations, where as lytic enzymes do not. There are also ethical questions over the use of transgenic techniques in the production of crops, in terms of whether the genetic insert can become integrated into wild species and in terms of potential effects on the health of consumers. In

terms of the development of head rot resistance, these approaches, will require years of research to fully implement, but they may be the most suitable route to take in the long term.

There are many routes available for head rot research and due to its economic importance, it is vital that research continues to take us one step closer to our goal.

Chapter 8: Bibliography

Agrios GN, 1988. Plant Pathology. 3rd Edition. Academic Press Inc. London.

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD, 1992. Molecular biology of the cell. Second Edition. Garland Publishing Inc., New York.

Anderson DM and Mills D, 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. *Phytopathology,* **75** (1): 104-108.

Anon., 1984. Calabrese. ADAS Booklet 2371. M.A.F.F., London.

Anon., 1993. Basic Horticultural Statistics for the U.K., Calender and Crop Years 1983-1992. M.A.F.F., London.

Asaka O and Shoda M, 1996. Biocontrol of *Rhizoctonia solani* damping off of tomato with *Bacillus subtilis* RB14. *Applied and Environmental Microbiology*, **62**: 4081-4085.

Ayers SH, Rupp P and Johnson WT, 1919. *A study of the alkali-forming bacteria in milk.* Washington, USA: United States Department of Agriculture Bulletin: **782**.

Bender CL, Stone HE, Sims JJ and Cooksey DA, 1987. Reduced pathogen fitness of *Pseudomonas syringae* Tn5 mutants defective in coronatine production. *Physiological and Molecular Plant Pathology*, **30** (2): 273-283.

Berg DE, Davies J, Allet B and Rochaix JD, 1975. Transposition of R factor genes to bacteriophage λ. *Proceedings of the National Academy of Sciences of the United States of America*, **72** (9): 3628-3632.

Berg DE and Berg CM, 1983. The prokaryotic transposable element Tn5. *Biotechnology,* **1**: 417-434.

Bergey DH , 1974. Bergey's manual of determinative bacteriology. Eighth Edition. Williams and Wilkins Co, Baltimore.

Berraho EL, Lesuera D, Diem HG and Sasson A, 1997. Iron requirement and siderophore production in *Rhizobium ciceri* during growth on an iron-deficient medium. *World Journal of Microbiology and Biotechnology*, **13** (5): 501-510.

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

Billing E, 1987. Bacteria as Plant Pathogens. Aspects of Microbiology, **14**. Van Nostrand Reinhold (UK) Co. Ltd.

Boliver F, 1978. Construction and characterisation of new cloning vehicles III. derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. *Gene*, **4**: 121-136.

Boucher CA, Barberis PA, Trigalet AP and Demery DA, 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-induced avirulent mutants. *Journal of General Microbiology* **131:** 2449-2457.

Boucher CA, Van Gijsegem F, Barberis PA, Arlat M and Zischek C, 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *Journal of Bacteriology*, **169**: 5626-5632.

Brodey CL, Rainey PB, Tester M and Johnstone K, 1991. Bacterial blotch disease of the cultivated mushroom is caused by an ion-channel forming lipodepsipeptide toxin. *Molecular Plant Microbe Interactions*, **4**: 407-411.

Bunster L, Fokkema NJ, Schippers B, 1989. Effect of surface-active *Pseudomonas* spp. on leaf wettability. *Applied and Environmental Microbiology* **55**: 1340-1345.

Burd G and Ward OP, 1996. Physicochemical properties of PM-factor, a surface-active agent produced by *Pseudomonas marginalis*. *Canadian Journal of Microbiology*, **42** (3): 243-251.

Burke TR (Jnr), Knight M and Chandrasekhar B, 1989. Solid-phase synthesis of viscosin, a cyclic depsipeptide with antibacterial and antiviral properties. *Tetrahedron Letters*, **30** (5): 519-522.

Bussotti F, Bottacci A, Grossoni P, Mori B and Tani C, 1997. Cytological and structural changes in *Pinus pinea* L. needles following the application of an anionic surfactant. *Plant Cell and Environment*, **20** (4): 513-520.

Buysens S, Heungens K, Poppe J and Hofte M, 1996. Involvement of pyochelin and pyoverdin in suppression of *Pythium* induced damping off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Applied and Environmental Microbiology*, **62** (3): 865-871.

Campbell LJ, Harling R, Chard J and Sutton M, 1995. Calabrese: Factors controlling symptom development in bacterial spear rot (FV/104). *Final Report of Project FV/104, Horticultural Development Council*, Bradbourne House, Stable Block, East Malling, Kent, ME19 6D2, U.K.

Canaday CH, Mullins CA, Wyatt JE, Coffey DL, Mullins JA and Hall T, 1987. Bacterial soft rot of broccoli in Tennessee. *Phytopathology*, **77** (12): 1712 (Abstr).

Canaday CH, 1989. Identification of horticultural traits for predicting broccoli cultivar susceptibility to bacterial soft rot. *Phytopathology*, **79** (10): 1160 (Abstr.).

Canaday CH, Wyatt JE and Mullins CA, 1991. Resistance in broccoli to bacterial soft rot caused by *Pseudomonas marginalis* and fluorescent *Pseudomonas* species. *Plant Disease*, **75** (7): 715-720.

Canaday CH and Wyatt JE, 1992. Effects of nitrogen fertilisation on bacterial soft rot in two broccoli cultivars, one resistant and one susceptible to the disease. *Plant Disease*, **76** (10): 989-991.

Che F-S, Kasamo K, Fukuchi N, Isogai A and Suzuki, A, 1992. Bacterial phytotoxins, syringomycin, syringostatin and syringotoxin, exert their effects on the plasma membrane H^+-ATPase partly by a detergent action and partly by inhibition of the enzyme. *Physiologia Plantarum*, **86** (4): 518-524.

Conn KL, Tewari JP and Dahiya JS, 1988. Resistance to *Alternaria brassicae* and phytoalexin elicitation in rapeseed and other crucifers. *Plant Science*, **56**: 21-25.

Coupland D, Zabkiewicz JA and Ede FJ, 1989. Evaluation of three techniques used to determine surfactant phytotoxicity. *Annals of Applied Biology*, **115** (1): 147-156.

Csonka LN, 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiological reviews*, **53** (1):121-147.

Cuppels D and Kelman A, 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. *Phytopathology* **64**: 468-475.

Daniels MJ, Barber CE, Turner PC, Cleary G and Sawczyc MK, 1984. Isolation of mutants of *Xanthomonas campestris* pv. *campestris* showing altered pathogenicity. *Journal of General Microbiology*, **130**: 2447-2455.

Daniels MJ, 1987. Molecular genetics of pathogenicity of *Xanthomonas campestris*: technical aspects. *In*: Genetics and Plant Pathogenesis. Edited: Day PR and Jellis GJ. Blackwell Scientific Press.

Denny MW, 1993. Air and Water. The biology and physics of Life's Media. Princeton University Press, Chichester.

Desai AJ, Patel KM and Desai JD, 1988. Emulsifier production by *Pseudomonas fluorescens* during the growth on hydrocarbons. *Current Science*, **57** (9): 500-501.

Desai AJ, Patel RM and Desai JD, 1994. Advances in the production of biosurfactants and their commercial applications. *Journal of Scientific and Industrial Research*, **53**: 619-629.

Dowson WJ and Dillon-Weston WAR, 1937. Soft rot of broccoli. *The Gardener's Chronicle*, July Issue.

Duncan JM and Torrance L, 1992. Techniques for the rapid detection of plant pathogens and diagnosis of plant disease. First Edition. Blackwell Scientific Publication, Oxford.

Falk RH, Guggenheim R and Schulke G, 1994. Surfactant-induced phytotoxicity. *Weed Technology*, **8** (3): 519-525.

Farr SB and Kogoma T, 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiological reviews*, **55** (4): 561-585.

Fett WF, 1984. Accumulation of isoflavanoids and isoflavone glucosides after inoculation of soybean leaves with *Xanthomonas campestris* pv. *glycines* and pv. *campestris* and a study of their role in resistance. *Physiological Plant Pathology*, **24**: 303-320.

Fett WF and Jones SB, 1984. Stress metabolite accumulation, bacterial growth and bacterial immobilisation during host and non-host responses of soybean to bacteria. *Physiological Plant Pathology*, **25**: 277-296.

Fiechter A, 1992. Biosurfactants: moving towards industrial application. *Trends in Biotechnology*, **10**: 208-217.

Florack D, Allefs S, Bollen R, Bosch D, Visser B and Stiekema W, 1995. Expression of giant silkmoth cecropin B genes in tobacco. *Transgenic Research*, **4** (2): 132-141.

Georgiou G, Lin S-C, and Sharma MM, 1992. Surface-active compounds from microorganisms. *Biotechnology*, **10:** 60-65.

Gladstone E, 1997. The role of biosurfactants in head rot of broccoli. BSc Honours project, University of Edinburgh.

Goodman RN, Kiraly Z, Wood KR, 1986. The biochemistry and physiology of plant disease. University of Missouri Press, Columbia.

Goto M, 1992. Fundamentals of Bacterial Plant Pathology. First Edition. Academic Press Inc., London.

Gross DC and DeVay JE, 1977a. Population dynamics and pathogenesis of *Pseudomonas syringae* in maize and cowpea in relation to the *in vitro* production of syringomycin. *Phytopathology*, **67**: 475-483.

Gross DC and DeVay JE, 1977b. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. *Physiological Plant Pathology*, **11**: 13-28.

Gross DC, 1991. Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annual Review of Phytopathology*, **29**: 247-278.

Groupe V, Pugh LH, Weiss D and Kochi M, 1951. Observations on antiviral activity of viscosin. *Proceedings of the Society of Experimental Biologists and Medicine*, **78**: 354-358.

Guerra-Santos LH, Kappeli O and Fiechter A, 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. *Applied Microbiology and Biotechnology*, **24**: 443-448.

Haferburg D, Hommel R, Kleber HP, Kluge S, Schuster G and Zschiegner HJ, 1987. Antiphytoviral activity of rhamnolipid from *Pseudomonas aeruginosa*. *Acta Biotechnologica*, **7**: 353-356.

Harling R, Kellock LJ and Chard J, 1994. Pathogenicity of bacteria causing spear (head) rot of calabrese (broccoli) in Scotland. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent,* **59**: 1189-1191.

He SY, Schoedel C, Chatterjee AK and Collmer A, 1991. Extracellular secretion of pectate lyase by *Erwinia chrysanthemi out* pathway is dependant upon sec-mediated export accross the inner membrane. *Journal of Bacteriology*, **173** (14): 4310-4317.

Healy MG, Devine CM and Murphy R, 1996. Microbial production of biosurfactants. *Resources Conservation and Recycling*, **18**: 41-57.

Heath MC, 1991. The role of gene-for-gene interactions in the determination of host species specificity. *Phytopathology*, **81**:127-130.

Heggie L, 1995. Disease incidence / severity relationships and phytoalexin elicitation in *Brassica oleracea* var. *italica* infected with various spear rot bacteria. BSc Honours project, University of Strathclyde.

Helenius A and Simons K, 1975. Solubilisation of membranes by detergents. *Biochimica and Biophysica Acta*, **415**: 29-79.

Hildebrand PD, 1986. Symptomatology and etiology of head rot of broccoli in Atlantic Canada. *Canadian Journal of Plant Pathology*, **8:** 350 (Abstr.).

Hildebrand PD, 1989. Surfactant-like characteristics and identity of bacteria associated with broccoli head rot in Atlantic Canada. *Canadian Journal of Plant Pathology*, **11** (3): 205-224.

Hildebrand PD, Laycock MV and Thibault P, 1990. Biosurfactant production by pectolytic fluorescent *Pseudomonas* and its role in broccoli head rot. *Canadian Journal of Plant Pathology*, **12**: 334 (Abstr.).

Hirschel BJ and Berg DE, 1982. A derivative of Tn5 with direct terminal repeats can transpose. *Journal of Molecular Biology*, **155:** 105-120.

Huang Y, Nordeen RO, Di M, Owens LD and McBeath JH, 1997. Expression of an engineered cecropin gene cassette in transgenic tobacco plants confers resistance to *Pseudomonas syringae* pv. *tabaci*. *Phytopathology,* **87** (5): 494-499.

Hutchison ML and Johnstone K, 1993. Evidence for the involvement of the surface active properties of the extracellular toxin tolaasin in the manifestation of brown blotch disease symptoms by *Pseudomonas tolaasii* on *Agaricus bisporus*. *Physiology and Molecular Plant Pathology*, **42**: 373-384.

Hutchison ML, Tester MA and Gross DC, 1995. Role of biosurfactant and ion-channel forming activities of syringomycin in transmembrane ion flux: a model for the mechanism of action in the plant pathogen interaction. *Molecular Plant Microbe Interactions*, **8** (4): 610-620.

Hutchison ML and Gross DC, 1997. Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae:* comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. *Molecular Plant-Microbe Interactions*, **10** (3): 347.

Hynes MF, Quandt J, O'Connell MP and Puhler A, 1989. Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sac*B gene. *Gene*, **78:** 111-120.

Ingram DS, 1977. Applications in Plant Pathology. *In*: Plant tissue and cell culture. Edited by Street HE. Second Edition. Blackwell Scientific Publications.

Jin H, Hartman GL, Nickell D and Widholm JM, 1996. Phytotoxicity of culture filtrate from *Fusarium solani* the causal agent of sudden death syndrome of soybean. *Plant Disease,* **80** (8): 922-927.

Johnson DR, Wyse DL and Jones KJ, 1996. Controlling weeds with phytopathogenic bacteria. *Weed Technology*, **10** (3): 621-624.

Jorgensen RA, Rothstein SJ and Reznikoff WS, 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Molecular and General Genetics*, **177**: 65-72.

Kang YW, Huang JZ, Mao GZ, He LY and Schell MA, 1994. Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer-membrane. *Molecular Plant-Microbe Interactions* **7**: 370-377.

Karcher SJ, 1995. Molecular Biology: A Project Approach. First Edition. Academic Press, Inc. USA.

King EO, Ward MK and Raney DE, 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* **44:** 301-307.

Klotz MG, 1993. The importance of bacterial growth phase for *in planta* virulence and pathogenicity testing: Coordinated stress response regulation in fluorescent pseudomonads? *Canadian Journal of Microbiology*, **39**: 948-957.

Kluge B, Vater J, Salnikow J and Eckart K, 1988. Studies on the biosynthesis of surfactin a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. *FEBS Letters*, **231** (1): 107-110.

Knoche M, Noga G, Lenz F, 1992. Surfactant-induced phytotoxicity: evidence for interaction with epicuticular wax fine structure. *Crop Protection* **11**: 51-56.

Knowles BH and Ellar DJ, 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of Bacillus thuringiensis δ-endotoxins with different insect specificity. *Biochimica and Biophysica Acta*, **924**: 509-518.

Koch AK, Kappeli O, Fiechter A and Reiser J, 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *Journal of Bacteriology*, **173**: 4212-4219.

Kumar V, Laouar L, Davey MR, Mulligan BJ, Lowe KC, 1992. Pluronic-F-68 stimulates growth of *Solanum dulcamara* in culture. *Journal of Experimental Biology*, **43** (249): 487-493.

Lambalot RH, Gehring AM, Flugel RS, Zuber P, Lacelle M, Marahiel MA, Reid R, Khosla C and Walsh CT, 1996. A new enzyme superfamily - the phosphopantetheinyl transferases. *Chemistry and Biology*, **3** (11): 923-936.

Lang S and Wagner F, 1993. Biological activities of biosurfactants. *In* Biosurfactants, production, properties and applications. Edited by Kosaic N. Marcel Dekker Inc, New York. Surfactant Series Volume **48**.

Lavermicocca P, Santelacobellus N, Simmaco M and Graniti A, 1997. Biological properties and spectrum of activity of *Psuedomonas syringae* pv. *syringae* toxins. *Physiological and Molecular Plant Pathology*, **50**: 129-140.

Laycock MV, Hildebrand PD, Thibault P, Walter JA, and Wright JLC, 1991. Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*. *Journal of Agricultural Food Chemistry* **39**: 483-489.

Leifert C, Morris CE and Waites WM, 1994. Ecology of microbial saprophytes and pathogens in culture and field grown plants-reasons for contamination problems *in vitro*. *Critical Reviews in Plant Sciences*, **13** (2): 139-183.

Lelliot RA and Stead DE, 1987. Methods for the diagnosis of bacterial diseases of plants, Methods in Plant Pathology. Volume 2. Series Editor Preece TF. Blackwell Scientific Publications, UK.

Lewin B, 1994. Genes V. First Edition. Oxford University Press, Oxford.

Liao CH, McCallus DE and Wells JM, 1993. Calcium-dependent pectate lyase production in the soft-rotting bacterium *Pseudomonas fluorescens*. *Phytopathology*, **83** (8): 813-818.

Lindeberg M and Collmer A, 1992. Analysis of eight *out* genes in a cluster required for pectic enzyme secretion by *Erwinia chrysanthemi*: Sequence comparison with secretion genes from other gram negative bacteria. *Journal of Bacteriology*, **174** (22): 7385-7397.

Lindsey K and Jones MGK, 1989. Plant biotechnology in agriculture. Wiley Biotechnology Series. John Wiley and Sons, New York.

Lipmann F, 1980. Bacterial production of antibiotic polypeptides by thiol-linked synthesis on protein templates. *Advances in microbial physiology*, **21**: 227-260.

Loper JE and Henkels MD, 1997. Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Applied and Environmental Microbiology*, **63** (1): 99-105.

Ludy RL, Hemphill DD (Jnr) and Powelson ML, 1989. Sprinkler irrigation effect on head rot (*Erwinia carotovora* subsp. *carotovora*) and yield of broccoli. *Phytopathology*, **79** (8): 910 (Abstr.).

Ludy RL, Powelson ML and Hemphill DD (Jnr), 1997. Effect of sprinkler irrigation on bacterial soft rot and yield of broccoli. *Plant Disease*, **81** (6): 614-618.

Lund B, 1982. Post Harvest Vegetable Quality. *In*: Bacteria and Plants. Edited by Rhodes-Roberts ME and Skinner FA. The Society for Applied Bacteriology Symposium Series, **No 10.** Academic Press, London.

Martin JF and Liras P, 1989. Organisation and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. *Annual Review of Microbiology*, **43**: 173-206.

Matsuyama T, Sogawa M and Yano I, 1987. Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. *Applied and Environmental Microbiology*, **53** (5): 1186-1188.

McCullagh M, Utkhede R, Menzies JG, Punja ZK and Paulitz TC, 1996. Evaluation of plant growth-promoting rhizobacteria for biological control of *Pythium* root rot of cucumbers grown in rockwool and effects on yield. *European Journal of Plant Pathology*, **102:** 747-755.

Meynell GG and Meynell E, 1970. Theory and practice in experimental bacteriology. Second Edition. Cambridge University Press.

Mithen RF, Lewis BG, and Fenwick GR, 1986. *In vitro* activity of gluconsinolates and their products against *Leptosphaeria maculans*. *Transactions of the British Mycological Society*, **87**: 433-440.

Mithen RF, Lewis BG, Heany RK and Fenwick GR, 1987. Resistance of leaves of *Brassica* species to *Leptosphaeria maculans*. *Transactions of the British Mycological Society*, **88**: 525-531.

Mo Y-Y and Gross DC, 1991. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *Journal of Bacteriology*, **173** (18): 5784-5792.

Mo YY, Giebel M, Bonsall RF and Gross DC, 1995. Analysis of sweet cherry (*Prunus Avium* L) leaves for signal molecules that activate the *syr*B gene required for synthesis of the phytotoxin, syringomycin, by *Pseudomonas syringae* pv. *syringae*. *Plant physiology*, **107** (2): 603-612.

Morris CE and Nguyen-the C, 1996. The role of plant surface bacteria in the hygenic and market quality of minimally-processed vegetables. *In*: *Aerial Plant Surface Microbiology*. Edited by Morris CN, Nicot PC and Nguyen-the C. Plenum press, New York.

Mott KA and Takemoto JY, 1989. Syringomycin, a bacterial phytotoxin, closes stomata. *Plant Physiology*, **90**: 1435-1439.

Mourgues F, Brisset M-N and Chevreau E, 1998. Strategies to improve plant resistance to bacterial diseases through genetic engineering. *Trends in Biotechnology*, **16**: 203-210.

Mulligan CN, Cooper DG and Neufeld RJ, 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. *Journal of Fermentation Technology*, **62**: 311-314.

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

Nakano MM, Marahiel MA and Zuber P, 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. *Journal of Bacteriology*, **170** (12): 5662-5668.

Nakano MM, Corbell N, Besson J and Zuber P, 1992. Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Molecular and General Genetics*, **232**: 313-321.

Neuval JJ, de Moel CP, van der Broek RCFM and Osinga KJ, 1996. Control of head rot disease in broccoli by choice of variety and foliar treatments. *Publicatie - Proefstation voor de Akkerbouw en de Groenteteelt in de Vollegrond, Leyystad*. **No 81B**: 118-122.

Oschner UA, Fiechtner A and Reiser J, 1994a. Isolation, characterisation and expression in *Eschericia coli* of the *Pseudomonas aeruginosa rhl*AB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *Journal of Biological Chemistry*, **269**:19787-19795.

Oschner UA, Koch AK, Fiechtner A and Reiser J, 1994b. Isolation and characterisation of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology*, **176**: 2044-2054.

Oschner UA and Reiser J, 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America*, **92**: 6424-6428.

Peet RC, Lindgren PB, Willis DK, Panopoulos NJ, 1986. Identification and cloning of genes involved in phaseolotoxin production by *Pseudomonas syringae* pv. '*phaseolicola*'. *Journal of Bacteriology*, **166:** 1096-1105.

Persson A, Osterberg E and Dostalek M, 1988. Biosurfactant production by *Pseudomonas fluorescens* 378: growth and product characteristics. *Applied Microbiology and Biotechnology*, **29:** 1-4.

Platt HW and Bollen GJ, 1993. Use of tissue culture potato plantlets for investigation of diseases of subterranean plant parts. *Plant Disease,* **77** (11): 1112-1113.

Punja ZK, 1997. Comparative efficacy of bacteria, fungi and yeasts as biological control agents for diseases of vegetable crops. *Canadian Journal of Plant Pathology*, **19**: 315-323.

Quigley NB and Gross DC, 1994. Syringomycin production among strains of *Pseudomonas syringae* pv *syringae*: conservation of the *syrB* and *syrD* genes and activation of phytotoxin production by plant signal molecules. *Molecular Plant-Microbe Interactions*, **7**: 78-90.

Rainey PB, Brodey CL and Johnstone K, 1991. Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen *Pseudomonas tolaasii*. *Physiology and Molecular Plant Pathology*, **39**: 57-70.

Reeves PJ, Whitcombe D, Wharam S, Gibson M, Allison G, Bunce N, Barallon R, Douglas P, Mulholland V, Stevens S, Walker D and Salmond GPC, 1993. Molecular cloning and characterisation of 13 *out* genes from *Erwinia carotovora* ssp. *carotovora*: genes encoding members of a general secretion pathway (GSP) widespread in gram-negative bacteria. *Molecular Microbiology*, **8** (3): 443-456.

Rentschler I, 1971. The wettability of leaf surfaces and the submicroscopic structure of their wax. *Planta*, **96**: 119-135.

Robertson S and Brokenshire T, 1992. Calabrese: Control of spear rot and downy mildew, *Horticultural Development Council Report*, Scottish Agricultural College, Edinburgh.

Robertson S, Brokenshire T, Kellock LJ, Sutton M, Chard J, and Harling R, 1993. Bacterial spear (head) rot of calabrese in Scotland: Causal organisms, cultivar susceptibility and disease control. *Proceedings Crop Protection in Northern Britain, 1993*: 265-270.

Rosenberg, 1986. Microbial surfactants. *CRC Critical Review in Biotechnology*, **3**: 109-132.

Rothstein SJ, Jorgensen RA, Yin JC-P, Yong-DI Z, Johnson RC and Reznikoff WS, 1980. Genetic organisation of Tn5. *Cold Spring Harbour Symposia on Quantitative Biology*, **45**, 99-105.

Sambrook J, Fritsch EF and Maniatis T, 1989. Molecular Cloning: a Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, New York U.S.A.

Sands DC, Schroth MN and Hildebrand DC, 1980. Pseudomonads. *In*: Laboratory guide for identification of plant pathogenic bacteria. p36. Edited by Schaad NW. American Phytopathology Society, Minnesota, USA.

Scheck HJ, Canfield ML, Pscheidt JW and Moore LW, 1997. Rapid evaluation of pathogenicity in *Pseudomonas syringae* pv. *syringae* with a lilac tissue culture bioassay and syringomycin DNA probe. *Plant Disease*, **81** (8): 905-910.

Schefler WC, 1969. Statistics for the biological sciences. Addison-Wesley Publishing Co.

Schreiber L, Bach S, Kirsch T, Knoll D, Schalz K and Riederer M, 1995. A simple photometric device analysing culticular transport physiology - surfactant effect on permeability of isolated culticular membranes of *Prunus laurocerasus*. *Journal of Experimental Botany*, **46**: 1915-1921.

Siegmund I and Wagner F, 1991. New Method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnology Techniques*, **5** (4): 265-268.

Sigee DC, 1993. Bacterial plant pathology cell and molecular aspects. Cambridge University Press, Cambridge.

Sigma plant tissue culture brouchure, 1990. Sigma Chemical Co., Sigma-Aldrich Co. Ltd.

Silcox D and Holloway PJ, 1986. The use of potasium leakage to assess potential phytotoxic effects of surfactants. *In Biochemical and Physiological Techniques in Herbicide Research*: *Aspects of Applied Biology* **11**: 149-158.

Simon R, Priefer U and Puhler A, 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Biotechnology*, **1**: 784-791.

Simpson RA, 1993. Pathogenicity and host response in bacterial spear rot of calabrese. Honours Research Project, University of Edinburgh.

Simpson EC, 1994. An investigation of phytoalexin elicitation in *Brassica oleracea* var. *italica* inoculated with various spear rot bacteria. BSc. Honours project, University of Strathclyde.

Sinden SL, DeVay JE and Backman PA, 1971. Properties of syringomycin, a wide spectrum antibiotic and phytotoxin produced by *Pseudomonas syringae*, and its role in the bacterial canker disease of peach trees. *Physiological Plant Pathology*, **1**: 199-213.

Southern EM, 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98:** 503-517.

Stanghellini ME and Tomlinson JA, 1987. Inhibitory and lytic effects of a non-ionic surfactant on various asexual stages in the life cycle of *Pythium* and *Phytophtora* species. *Phytopathology*, **77**: 112-114.

Stanghellini ME and Miller RM, 1997. Biosurfactants, their identity and potential efficacy in the biological control of zoosporic plant pathogens. *Plant Disease*, **81** (1): 4-12.

Staskawicz B, Dahlbeck D, Miller J and Damm D, 1983. Molecular analysis of virulence genes in *Pseudomonas solanacearum*. *In*: Molecular genetics of bacteria-plant interactions. pp345-352. Edited by A Puhler. Berlin and Heidelberg: Springer Verlag.

Tagg JR and McGiven AR, 1971. Assay system for bacteriocins. *Applied Microbiology*, **21** (5): 943.

Takasugi M, Katsui N and Shirata A, 1986. Isolation of three novel sulphur-containing phytoalexins from chinese cabbage *Brassica campestris* L. ssp. *pekinensis* (Cruciferae). *Journal of the Chemical Society Chemical Communications*, No. 14: 1077-1078.

Takemoto JY, 1992. Bacterial phytotoxin syringomycin and its interaction with host membranes. *In*: Molecular signals in Plant Microbe Communications pp247-260. Edited by: Verma DPS.

Tang JL, Gough CL, Barber CE, Dow JM and Daniels MJ, 1987. Molecular cloning of protease gene(s) from *Xanthomonas campestris* pv. *campestris*: Expression in *Escherichia coli* and role in pathogenicity. *Molecular and General Genetics*, **210**: 443-448.

Todd GA, Daniels MJ and Callow JA, 1990. *Xanthomonas campestris* pv *oryzae* has DNA sequences containing genes isofunctional with *Xanthomonas campestris* pv *campestris* genes required for pathogenicity. *Physiology and Molecular Plant Pathology*, **36**: 73-87.

Tomiyama K, Sakuma T, Ishizaka N, Sato N, Katsui N, Takasugi M and Masamuni T, 1968. A new antifungal substance isolated from potato tuber tissue infected by pathogens. *Phytopathology*, **58:** 115-116.

Ullrich C, Kluge B, Palacz Z and Vater J, 1991. Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*. *Biochemistry*, **30** (26): 6503-6508.

Walter MV, Porteous A, Seidler RJ, 1987. Measuring genetic stability in bacteria of potential use in genetic engineering. *Applied and Environmental Microbiology* **53**: 105-109.

Warren G, Corotto L and Wolber P, 1986. Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas*. *Nucleic Acid Research*, **14**: 8047-8060.

Wimalajeewa DLS, 1985. Head rot of broccoli in Victoria, Australia, caused by *Pseudomonas marginalis*. *Plant Disease* **69**: 177 (Abstr.).

Wimalajeewa DLS, Hallam ND, Hayward AD, Price TV, 1987. The etiology of head rot disease of broccoli. *Australian Journal of Agricultural Research,* **38**: 735-742.

Wyman JG and Van Etten HD, 1978. Antibacterial activity of selected isoflavanoids. *Phytopathology,* **68**: 583-89.

Xu G-W and Gross DC, 1988a. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of *Pseudomonas syringae* pv *syringae* defective in syringomycin production. *Applied and Environmental Microbiology*, **54**: 1345-1353.

Xu G-W and Gross DC, 1988b. Physical and functional analyses of the *syrA* and *syrB* genes involved in syringomycin production by *Pseudomonas syringae* pv *syringae*. *Journal of Bacteriology*, **170**: 5680-5688.

Yang Y, Wilson LT, Makela ME, Marchetti MA and Krausz JP, 1997. Effect of leaf age and nodal position on receptivity of rice leaves to infection by *Pyricularia grisea*. *Journal of Phytopathology*, **146** (4): 157-204.

Yessad S, Manceau C and Luisetti J, 1992. A detached leaf assay to evaluate virulence and pathogenicity on strains of *P. syringae* pv. *syringae* on pear. *Plant Disease*, **76** (4): 370-373.

Young DH and Kauss H, 1984. Adhesion of *Colletorichum lindemuthianum* spores to *Phaseolus vulgaris* hypocotyls and to polystyrene. *Applied and Enviromental Microbiology*, **47**: 616-619.

Zajic JE and Seffens W, 1984. Biosurfactants. *Critical Reviews in Biotechnology*, **1** (2): 87-107.

Zhang JH, Quigley NB and Gross DC, 1995. Analysis of the *syrB* and *syrC* genes of *Pseudomonas syringae* pv. *syringae* indicates that syringomycin is synthesised by a thiotemplate mechanism. *Journal of Bacteriology*, **177**: 4009-4020.

Chapter 9: Appendices

9.1 Appendix 1 (for Chapter 5, Section 5.3.2 (iv) b)

Table 9.1 shows the mean OD_{500nm} values obtained in the hydrocarbon utilisation flask experiment (Chapter 6, Section 5.3.2 (iv) b).

Table 9.1: Mean OD_{500nm} values obtained in the hydrocarbon utilisation flask experiment, at each time point, for *Pseudomonas fluorescens* P5049

		Hexadecane concentration	2%	4%	0%	2%	4%	0%
		Inoculum concentration	0.5ml (1.75×10^9 cfu)			1ml (3.5×10^9 cfu)		
			Mean OD_{500nm} recorded					
Medium		**Time (h)**			Control (no hexadecane)			Control (no hexadecane)
Ayer's	**shaking**	**zero**	0.08	0.09	0.09	0.23	0.22	0.18
		24	0.16	0.16	0.11	0.31	0.32	0.25
		48	0.25	0.25	0.18	0.50	0.52	0.28
		72	0.29	0.27	0.14	0.55	0.57	0.19
	static	**zero**	0.12	0.08	0.05	0.19	0.18	0.11
		24	**0.11**	0.13	0.06	0.28	0.28	0.17
		48	0.18	0.17	0.04	0.33	0.32	0.24
		72	0.25	0.24	0.05	0.43	0.44	0.21
G.-Santos	**shaking**	**zero**	0.10	0.10	0.08	0.24	0.22	0.18
		24	0.15	0.16	0.09	0.34	0.30	0.22
		48	0.23	0.22	0.13	0.47	0.50	0.27
		72	0.27	0.28	0.13	0.49	0.51	0.31
	static	**zero**	0.07	0.10	0.08	0.17	0.18	0.12
		24	0.09	0.14	0.06	0.23	0.25	0.23
		48	0.15	0.19	0.07	0.30	0.33	0.21
		72	0.20	0.24	0.08	0.39	0.45	0.25
King's B	**shaking**	**zero**	0.14	0.16	0.80	0.12	0.17	0.90
		24	0.89	1.20	0.90	1.70	2.00	1.40
		48	0.90	1.40	1.10	1.98	2.19	1.60
		72	1.10	1.60	1.30	2.00	**2.18**	1.80
	static	**zero**	0.13	0.11	0.80	0.13	0.15	0.80
		24	0.67	0.90	0.70	1.59	1.50	1.10
		48	0.71	1.04	0.65	1.64	1.76	1.07
		72	0.77	1.12	0.74	**1.53**	**1.62**	0.90

Bold = value drops from that recorded previously

9.2 Appendix 2 (for Chapter 6, Section 6.3.1)

Calculation of transformation frequency and frequency of surfactant minus mutants

Transformation Frequency = $\frac{\text{cells on plates containing antibiotics}}{\text{cells on plates with no antibiotics}}$

pSUP2021 = $\frac{4.8 \times 10^{6}}{1.0 \times 10^{13}} = 4.8 \times 10^{-6}$

pMH1701 = $\frac{6.2 \times 10^{6}}{1.0 \times 10^{14}} = 6.2 \times 10^{-8}$

Frequency of surfactant-minus mutants = $\frac{\text{Number of surf}^{-}\text{ mutants obtained}}{\text{Number of transconjugants obtained}}$

= $\frac{17}{4.8 \times 10^{6}} = 3.45 \times 10^{-6}$

N.B. Two rounds of transformations were carried out to obtain 35 surf^{-} mutants.