Know-how for Horticulture™

A Study of post harvest bacterial rots and browning in lettuce and the development of control methods

W.S. Washington
Department of Natural Resources and Environment
Victoria

Project Number: VG98083

VG98083

This report is published by Horticulture Australia Ltd to pass on information concerning horticultural research and development undertaken for Australian Horticulture.

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of the vegetable industry.

ISBN 0 7341 0455 3

Published and distributed by:
Horticultural Australia Ltd
Level 1
50 Carrington Street
Sydney NSW 2000
Telephone: (02) 8295 2300
Fax: (02) 8295 2399
E-Mail: horticulture@horticulture.com.au

Horticulture Australia

Table of Contents

Media summary

Browning and bacterial rots of lettuce and salad vegetables is a major problem in Australia. It appears to be more severe during certain times of the year and on farms that have been in continuous cultivation for a number of years. The rotting of the vegetables leads to problems related to product quality in many ways. Severe rots cause direct losses and mild rots lead to product quality issues with consumers. Even when rots are not a direct problem the bacteria that are responsible may lead to other quality issues during postharvest handling and processing. These include the major quality problem with fresh-cut lettuce, browning of the cut surfaces.

The major group of bacteria that are implicated in the browning of processed lettuce and are the cause of some field and postharvest rots are the fluorescent pseudomonads. These bacteria live on the surface of the plant and are especially found in soils with a high component of fresh or degraded plant material.

Major aims of this project were to:

- Investigate the browning effect on lettuce caused by fluorescent pseudomonads.
- Examine the bacterial loads in soils during cultivation over subsequent growing seasons.
- Investigate the development of control measures to reduce the overall load of fluorescent pseudomonads in the field and after harvest.

This project has shown that fluorescent pseudomonads are capable of either causing a rapid browning reaction on the cut surface of lettuce or exacerbating the problem over a 48-hour period on bought lettuce or lettuce grown under sterile conditions. The natural levels of fluorescent pseudomonads in soils of lettuce growing regions have been shown to vary over the seasons and also vary between crop types. The finding that the after-harvest lettuce waste contained high populations of fluorescent pseudomonads resulted in a recommendation for a time lapse between harvest and re-planting of seedlings. The project has also shown that fluorescent pseudomonad levels on cut lettuce can be reduced, whilst maintaining good quality produce. Also bacterial levels in the field were found to increase with the use of mulches, but the overall yield and quality of the lettuce heads were increased.

The benefit this project will have on the industry is to provide a greater awareness of the quality problems of lettuce that this group of bacteria contribute to. For example, a leading lettuce processor now tests their lettuce for *Pseudomonas* species so that they can better observe and control the quality of the lettuce. Growers and handlers have a greater awareness of the need to maintain good cool chain management to reduce the potential for further browning and rots of lettuce during marketing.

There should be further investigations into the effect of different soil additives such as tea-tree and eucalyptus mulches on bacterial levels and product yield and quality. Further work should also be conducted to develop efficient and rapid detection methods for fluorescent pseudomonads to aid the accurate identification and measurement of the bacteria by industry.

Technical summary

Lettuce (*Lactuca sativa* L.) is an economically important food crop grown worldwide. Demand for quality lettuce in the Australian domestic and export market is high with production worth A$88 million (1998/99) nationally (ABS Agstats). The emergence of the fresh-cut industry over the last few years has seen an increase in the use of salad vegetables such as lettuce. The importance of the fresh-cut industry has been reflected in the Australian retail sales of salad mixes and fresh-cut vegetables with growth from A$15m in 1996 to A$70m in 1999-2000 (Anon, 2000a).

Visual quality of lettuce can be threatened by rots and browning, often leading to reduced shelf life and product quality issues with consumers. Browning of cut lettuce tissue is due to the plant's natural defence mechanisms or enzymatic browning and it can also be increased by the action of bacteria.

Fluorescent pseudomonads have been implicated in browning of lettuce due to the presence of high populations on the leaf surface. This group of bacteria comprise saprophytic and pathogenic bacteria that have contributed to field and postharvest problems. Some strains of the fluorescent pseudomonads possess pectolytic enzymes, which are capable of rapidly degrading plant tissue. Damage during handling or harvesting of the lettuce can be a major entry point for the bacteria prior to microbial spoilage. As the dominant resident on the lettuce it only takes a small amount of damage to initiate the pathogenic effects of the bacteria.

The major aims of the project were to:

a) Examine the bacterial populations during cultivation in order to observe the levels of fluorescent pseudomonads in the field.
b) Further understand the relationship between fluorescent pseudomonads and the browning of cut lettuce.
c) Evaluate control measures designed to reduce the overall load of these bacteria in the field and postharvest.

The investigations were separated into two approaches:

Part 1. Identification and monitoring of bacterial levels

- Monitoring the presence of fluorescent pseudomonads in the soil of lettuce growing properties was conducted using general soil sampling techniques. The quantification and identification of the fluorescent pseudomonads was undertaken using selective media (KB agar) and the LOPAT system of identification (Lelliott *et al.* 1966).

  Other soil based experiments, including the field reduction experiments used similar methods to those described above.

Part 2. Effect of bacteria on browning of cut lettuce

- Analysis of the effect of the browning on cut lettuce leaves caused by the different bacterial species was conducted using either shredded lettuce or lettuce grown in aseptic conditions. Lettuce leaves were inoculated with known bacterial

suspensions and stored at 8°C for 48-hours. Quality of the leaves and the number of cut surfaces exhibiting browning was assessed after 48-hours. The detection of fluorescent pseudomonads for all of the experiments was done using the microbiological methods described above.

Our investigations showed that the natural levels of fluorescent pseudomonads in soils of lettuce growing regions varied over the seasons and also varied between crop types. Detection of fluorescent pseudomonads within these sampling periods also showed a variety of different *Pseudomonas* species. The finding that the after-harvest lettuce waste contained high populations of fluorescent pseudomonads resulted in a recommendation for a time lapse between harvest and re-planting of seedlings.

The rapid browning effect by fluorescent pseudomonads on the cut surface of inoculated lettuce was demonstrated over a 48-hour period in aseptically grown and bought lettuce. These results showed that the browning and subsequent rots caused by the fluorescent pseudomonads were more severe and rapid than the damage caused by other bacteria. The project also showed that fluorescent pseudomonad levels on cut lettuce can be reduced, whilst maintaining good appearance and quality. Even though work showed that the use of mulches increased levels of total aerobic bacteria and fluorescent pseudomonads, the overall yield and quality of the lettuce crop was increased.

Investigations are required to further evaluate the browning effects of the fluorescent pseudomonads on the cut surface of lettuce. This would include work on the ecology of the bacteria and the ways in which they enter into or onto the plant. Also to further the identification of methods to remove bacteria from lettuce after harvest, particularly before processing. Future work is needed to capture the potential of the different types of mulches such as tea-tree and eucalyptus, in monitoring the bacterial levels and increasing product yield and quality. In addition, work should be conducted to develop efficient and rapid detection methods for fluorescent pseudomonads to aid the identification and quantification of the bacteria on crops and in soil.

Acknowledgements

The completion of this project would have not have been possible without the assistance of a large number of people.

- Kon Koroneos – K. Koroneos and Co. P/L, Werribee South, Vic.
- Paul Gazzola – L & G Gazzola & Sons P/L, Somerville, Vic.
- Nelson Cox – Cox's Vegetable Farm, Lindenow, Vic.
- Bill Taylor Jnr – Taylor Farms P/L, Boisdale, Vic.
- John Faragher
- Bruce Tomkins
- James Hutchinson
- Julia Behrsing and the other members of the NRE-IHD 'Food safety, quality and nutrition' team

PART 1 Background

Lettuce (*Lactuca sativa* L.) is an economically important crop plant grown worldwide. Demand for quality lettuce in the Australian domestic and export market is high with production worth A$88 million (1998/99) nationally (ABS Agstats). The emergence of the fresh-cut industry over the last few years has seen an increase in the use of salad vegetables such as lettuce. The importance of the fresh-cut industry has been reflected in the Australian retail sales of salad mixes and fresh-cut vegetables with growth from A$15m in 1996 to A$70m in 1999-2000 (Anon, 2000a).

Consumers use visual quality to select vegetables. Visual quality can be threatened by discolouration, rots and browning which are common in lettuce. In fresh-cut products containing lettuce, browning on the cut surfaces may lead to reduced shelf life or even to rejection of the entire batch. Rots in lettuce lead to economic losses with severe rots causing substantial losses and mild rots leading to product quality issues with consumers.

The browning reaction

The amount of discolouration, browning and rots in lettuce vary according to pre and postharvest factors (Laurila *et al.* 1998). Rots and discolouration may initially affect some crops in the field but with postharvest practices such as handling, storage and preparation, rots and discolouration may be further enhanced. Cutting can lead to browning reactions at the cut surfaces and to loss of product quality. Cutting lettuce causes the plant cells at the cut surface to rupture resulting in the release of cellular fluids. The browning reaction observed has been attributed to oxidising enzymes that, in the presence of oxygen, convert phenolic compounds into products which are rapidly polymerised to form brown or black pigments. These pigments are observed at the cut leaf edge (Laurila *et al.* 1998; Castañer *et al.* 1996). Much research has been conducted to study the ways in which browning can be inhibited. This includes the use of chemical inhibitors such as citric acid and ascorbic acid, modified atmosphere packaging, the use of physical treatments such as heat shock application and in the future, the use of genetic engineering to manipulate the plant to produce lesser amounts of the oxidising enzymes (Loaiza-Velarde & Saltveit 2001; Laurila *et al.* 1998; Michelmore 1997). However, these have only reduced browning slightly and not totally inhibited it.

Another possibility is that bacteria associated with the surfaces of lettuce may cause the browning at cut surfaces. Plant pathogenic bacteria, especially those producing pectolytic enzymes, may play a role in the elicitation of the plant's defence mechanisms (Morris & Nguyen-The 1996). As a result of the exposed cell fluid contents after cutting or other damage, bacteria may take advantage of the favourable environment and readily colonise.

Zagory (1999) noted that healthy, intact tissue may be a poor substrate for bacterial growth whilst damaged or physiologically compromised tissues would deteriorate faster and provide a better substrate for growth. Large numbers of bacteria were found in areas where the leaf cuticle was broken and were observed infecting internal plant cells (Zagory 1999). It is interesting to note that the procedures mentioned

previously to reduce or inhibit the browning in lettuce are also important methods for bacterial control.

Fluorescent Pseudomonads – possible contributors to browning?

Both saprophytic and pathogenic bacteria make up the microbial population on many vegetables including lettuce. The genus, *Pseudomonas* is the dominant bacteria, which makes up to 90% of the microbial population on the leaf surface (Zagory 1999). *Pseudomonas* species, notably the fluorescent pseudomonads, comprise both saprophytic bacteria and pathogenic bacteria that produce pectolytic enzymes capable of degrading plant tissues. Fluorescent pseudomonads are named for their ability to produce pigments that fluoresce when exposed to UV light. This ability enables bacterial isolation to be carried out quite easily on media that are selective for these pigments such as King's medium B (King *et al.* 1954). Their natural resistance to antibiotics such as novobiocin, cycloheximide and penicillin also makes isolations from soil, water and produce much easier (Sands & Rovira 1970).

Fluorescent pseudomonads are the main contributors to some diseases and rots in the field and during postharvest storage. Varnish Spot, a disease in which rotting of the inner lettuce leaves occurs, is caused by a variety of fluorescent pseudomonads including *Pseudomonas cichorii* and *Pseudomonas marginalis* (Patterson *et al.* 1986). It can result in an entire lettuce crop being destroyed. Pectolytic strains of *Pseudomonas* are found particularly in soft rots of leafy vegetables after harvest (Brocklehurst & Lund 1981). *Pseudomonas marginalis* causes soft rot in lettuce after physical injury and is a contributor to rots in refrigerated storage where cold temperatures inhibit the growth of other pectolytic bacteria such as *Erwinia carotorvora* (Nguyen-The & Prunier 1989). Plant pathogenic bacteria such as some fluorescent *Pseudomonas* species have been known to enter the plant through natural openings, such as the stomata. However, it is clear that damage during harvesting or handling of the vegetable can be a major entry point for the bacteria prior to microbial spoilage (Nguyen-The & Prunier 1989; Hikichi *et al.* 1996). The infection sources of fluorescent pseudomonads seem to be infected plant debris, water, seeds and soil. They can be spread via aerosols when water is splashed onto soil and plants (Hikichi *et al.* 1996). As a dominant resident on the lettuce it may only take a small amount of damage to initiate the pathogenic effects of the bacteria.

Fluorescent pseudomonads are also widespread in soil. They are distributed unevenly through soil and are largely associated with organic matter at the early stage of decomposition (Rovira & Sands 1971). High numbers of fluorescent pseudomonads, up to 10^4 per gram, are prevalent on recently fallen leaves and in the rhizosphere of living roots (Rovira & Sands 1971; Cuppels & Kelman 1973). The soil populations and types of fluorescent pseudomonads vary widely depending on the state of decomposition of organic matter (Rovira & Sands 1971; Sands & Hankin 1975). Previous studies have found that the fluorescent pseudomonads isolated from soil were usually the saprophytic *Pseudomonas fluorescens* (biovar V) which do not produce pectolytic enzymes (Sands & Hankin 1975; Stanier *et al.* 1966). Soil isolates, which are pectolytic, may represent a residual population incorporated into the soil on plant material or in water and are not typical soil inhabitants.

Previous research into the levels of fluorescent pseudomonads in soil has shown that relatively low numbers were found. Sands and Rovira (1971) reported that fluorescent pseudomonads represented <1% of the total bacterial population in the soil and rhizosphere. Similarly Sands *et. al.* (1972) found that the fluorescent pseudomonads comprised no more than 0.27% of the total bacterial population.

The major aims of the project were to:

a) Examine the bacterial populations during cultivation in order to monitor the levels of fluorescent pseudomonads in the field.
b) Further understand the relationship between fluorescent pseudomonads and the browning of cut lettuce.
c) Evaluate control measures designed to reduce the overall load of these bacteria in the field and postharvest.

The benefit this project will have on the industry is to provide a greater awareness of the quality problems on lettuce that this group of bacteria can contribute to. Growers and handlers will have a greater awareness of the need to maintain good cool chain management to reduce the potential for further rots of lettuce during marketing.

Along with improved growing and handling practices to avoid unnecessary damage, minimisation of the fluorescent pseudomonads may lead to reduced rots in the field and during postharvest storage and importantly, reduced browning in cut lettuce used in the fresh-cut vegetable industry.

PART 2 Browning at the cut surface of lettuce

2.1 Introduction

Browning of damaged tissues of fresh vegetables is a main cause of quality loss. The browning process decreases the marketability of vegetables leading to economic losses for the growers (Castañer *et al.* 1996). The amount of discolouration, browning and rots in lettuce vary according to pre and postharvest factors (Laurila *et al.* 1998). Rots and discolouration can initially affect some crops in the field but with postharvest practices such as handling, storage and preparation, these problems may be further enhanced. Damage caused by cutting can lead to browning reactions at the cut surfaces and to loss of product quality. Cutting lettuce causes the plant cells at the cut surface to rupture resulting in the release of cellular fluids (Couture *et al.* 1993).

Bacteria associated with the surfaces of lettuce such as the group of fluorescent pseudomonads also cause the browning at cut surfaces. Plant pathogenic bacteria, especially those producing pectolytic enzymes, play a role in the elicitation of the plant's defence mechanisms (Morris & Nguyen-The 1996). Their presence on the leaf surface and plant roots of the susceptible vegetables at the time of harvest enable them to penetrate through natural openings, cut surfaces or through the destruction of the plant barrier by the pectolytic enzymes (Jay 1996). As a result other non-pectolytic bacteria are able to enter the plant tissue and help bring about further tissue damage and browning.

The aims were to:
a) observe the level of fluorescent pseudomonads on cut lettuce in postharvest conditions
b) evaluate the influence of bacteria on the browning of the cut surface of lettuce
c) assess the browning reaction and quality of the lettuce caused by fluorescent pseudomonads in postharvest conditions.

2.2 Method

2.2.1 Effect of postharvest conditions on fluorescent pseudomonads on harvested lettuce

2.2.1.1 Preparation of lettuce and postharvest conditions
Crisphead lettuce (cultivar unknown) was harvested from a property in Werribee South and transferred immediately to the laboratory. The outer most leaves were removed and excess dirt washed off. A lettuce was placed into a plastic snaplock bag. Two coolrooms of each temperature (4°C, 8°C & 12°C) were set up. In each room 3 blocks were organized. In each block the 4 removal times (day 2,7,12 and 14) were randomly assigned to the 4 bags of lettuce. There were 6 replicates of each of the 12 treatments. On removal, the fluorescent pseudomonad levels and the quality of the lettuce was measured.

2.2.1.2 Assessment of fluorescent pseudomonads

Leaves were shredded, mixed thoroughly and 50 g was placed into a sterile bag and stomached for 2 min in 225mL of sterile peptone buffer containing 0.1% buffered peptone. Serial dilutions were made in peptone buffer (0.1%). These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The detection and quantification of the fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies as an indicator (refer to figure 2.1).

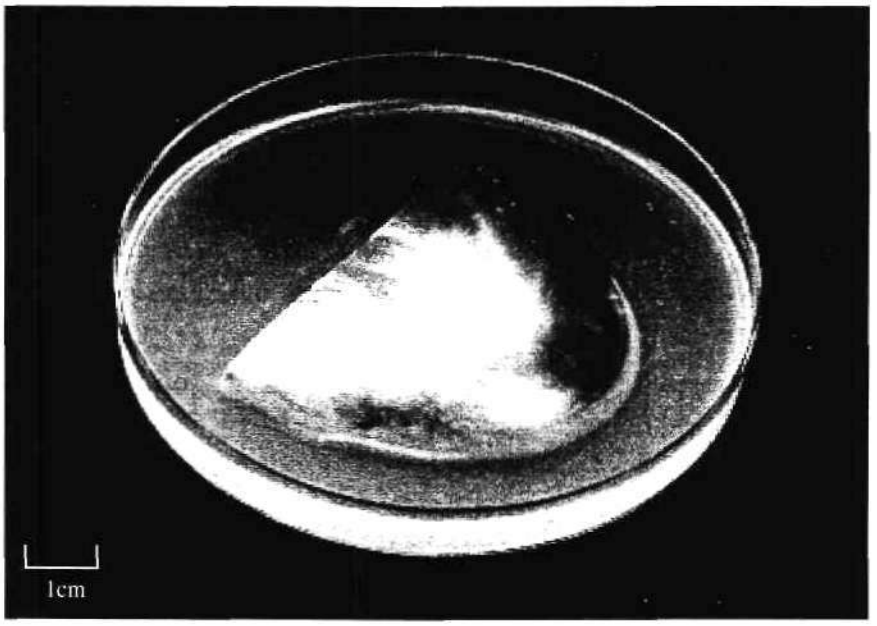


Figure 2.1 UV fluorescence of colonies of fluorescent pseudomonads

2.2.1.3 Evaluation of the quality of the lettuce

Visual quality rating was according to a 1-9 score described by Kader *et al.* (1973), where 9= excellent, 5 =limit of salability and 1 =extremely poor.

2.2.1.4 Data analysis

Analysis of variance (GenStat 5.4.1) was performed on the data produced from the experiments. Log counts of bacteria were analyzed to meet the assumption of constant variance. The least significant difference (LSD) was determined to compare treatments. All tests were at the 5% significance level, unless otherwise stated. Bars on graphs are 1x LSD (P=0.05)). Where several LSD were generated, the maximum LSD has been shown.

2.2.2 Effect of bacteria on the cut surface of market lettuce and aseptically grown lettuce (tissue culture)

2.2.2.1 Preparation of bacterial suspensions
The bacteria used in this study were *Pseudomonas syringae* (isolated from celery, CHS OR/207/2), *Pseudomonas marginalis* (isolated from lettuce), *Pseudomonas fluorescens* (isolated from lettuce, CHS VG/207/96), *Erwinia sp.* (isolated from lettuce, CHS VG/205/96), *Erwinia carotovora sp. carotovora* (isolated from potato, CHS VG/987/17) *and Xanthomonas campestris* (isolated from cauliflower, CHS AG/112/11). The bacteria were cultured each week on king's B medium (KB). For each experiment, one loopful of culture was inoculated into a flask of nutrient broth (Difco) and incubated with shaking at 27°C for 48 hours. The concentration of this stock suspension was confirmed by making serial dilutions in peptone buffer containing 0.1% buffered peptone (Merck, Aust.) in deionised water. These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and incubated for 48 hours at 27°C.

2.2.2.2 Effect of the bacteria on the cut surface of tissue culture cos lettuce plants
Seeds of cos lettuce (cultivar Verdi (Henderson seeds, Aust.) were surface sterilized with calcium hypochlorite solution (1% active chlorine (Chem-supply)) and rinsed three times in sterile deionised water. Seeds were placed onto Murashige and Skoog (MS) medium (Sigma, USA) (Murashige & Skoog 1962) and placed into the growth room at 24°C for 3 weeks. Upon germination, seedlings were transferred to vessels containing MS medium for a further 8 weeks. When the lettuce leaves were approximately 6-10 cm in length they were removed and cut aseptically. Cut leaves were placed into sterile stomacher bags (SafetyPlus, Aust.). Approximately 5 mL of the appropriate inoculation suspension at a concentration of 10^4 to 10^5 was aliquoted into the bag of lettuce and mixed thoroughly for 3-5 minutes. The bag was drained of excess inoculation suspension, sealed and stored at 8°C for 48 hours. Two control treatments were prepared using cut, but uninoculated leaves and cut, but peptone water (diluent) treated leaves (Table 2.1).

Experimental design of blocks was organized based on the size of the plants. Eight plants were arranged in each of the 6 blocks. Treatments were randomly assigned to each container within each block. Inoculation treatments were conducted to a block at a time.

Table 2.1 Inoculation treatments of tissue culture lettuce

Treatment No.	Inoculation
1	Control – no inoculation
2	Inoculation with diluent only
3	Inoculation with *Pseudomonas fluorescens*
4	Inoculation with *Pseudomonas marginalis*
5	Inoculation with *Pseudomonas syringae*
6	Inoculation with *Erwinia sp*
7	Inoculation with *Erwinia carotovora*
8	Inoculation with *Xanthomonas campestris*

Bacterial counts on the tissue-culture lettuce were conducted prior to bacterial inoculations to assess the presence of bacteria on the lettuce grown under aseptic conditions.

2.2.2.3 Assessment of the quality and bacterial confirmation of the tissue culture lettuce

Visual quality of the cut lettuce was assessed after 48 hours. Assessments were conducted by the Kader *et al.* (1973) visual quality rating and on the number of cut surfaces exhibiting browning. Bacterial confirmation for each treatment was conducted using Petrifilm TAC plates and KB medium for colony type.

2.2.2.4 Data analysis

Analysis of variance (GenStat 5.4.1) was performed on the data produced from the experiments. Average quality scores and numbers of cut surfaces exhibiting browning were analyzed to meet the assumption of constant variance. The least significant difference (LSD) was determined to compare treatments. All tests were at the 5% significance level, unless otherwise stated. Bars on graphs are LSD (P=0.05)).

2.2.2.5 Observation of the bacteria on the cut surface of market lettuce

Iceberg lettuce (*Lactuca sativa* L.) was obtained from a local market (unknown cultivar). Lettuce wrapper and core leaves were discarded and the rest of the leaves were trimmed and cut. Cut leaves were rinsed twice in double deionised water (as it has been shown that chlorinated water was not particularly effective at eliminating E. *coli* cells inoculated onto broccoli or lettuce (Behrsing *et al* 2000)) and centrifuged with a manual salad spinner to remove excess water. Pieces were randomly mixed and 50 g was each placed into two plastic snaplock bags. An inoculation solution of the different bacteria; *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Erwinia carotovora*, *Erwinia sp.*, and *Xanthomonas campestris*, was prepared by dilution of the stock suspension in peptone buffer (0.1%) to make a concentration of 10^4 and the concentration was determined. Approximately 5 mL of the inoculation suspension was aliquoted into each bag of lettuce and mixed thoroughly for 3-5 minutes. The bags were drained of excess inoculation suspension and stored at 8°C for 48 hours.

2.2.2.6 Evaluation of the quality of the cut lettuce

Visual quality rating was according to a 1-9 score described by Kader *et al.* (1973), where 9= excellent, 5 =limit of salability and 1 =extremely poor.

2.2.3 Analysis of fluorescent pseudomonads and the quality of cultivars of fresh-cut lettuce harvested from several locations

2.2.3.1 Locations and cultivars

Crisphead cultivars, Toronto, Ponderosa, Silverado and Target were harvested from a property at Somerville and Maffra.

2.2.3.2 Lettuce preparation

The lettuces were shredded, washed in chlorinated water (5 to 10mg/l Nylate® solution) for 5 minutes, spun dry and placed into snaplock plastic bags. Four bags of each lettuce cultivar were stored at 4°C for 7 days.

2.2.3.3 Assessment for fluorescent pseudomonads and quality

Samples of lettuce were obtained pre-wash, post-wash and after 7 days storage. Cut lettuce samples of 50 g were placed into sterile bags and stomached for 2 min in 225mL of sterile peptone buffer containing 0.1% buffered peptone. Serial dilutions were made in peptone buffer (0.1%). These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The assessment for the presence of fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies (refer to figure 2.1). Visual quality of the cut lettuce was assessed after 7 days. The rating was conducted according to a 1-9 score described by Kader *et al.* (1973), where 9= excellent, 5 =limit of salability and 1 =extremely poor.

2.2.3.3 Data Analysis

Analysis of variance (GenStat 5.4.1) was performed on the data produced from the experiments. Average quality scores and log counts of the bacteria were analyzed to meet the assumption of constant variance. The least significant difference (LSD) was determined to compare treatments. All tests were at the 5% significance level, unless otherwise stated. Bars on graphs are LSD (P=0.05)).

2.3 Results

2.3.1 Observation of fluorescent pseudomonads on harvested lettuce in postharvest conditions

Levels of fluorescent pseudomonads were present on the lettuce leaves stored at the three temperature ranges of 4, 8 and 12°C from early on during the storage trial. Figure 2.2 shows the levels of fluorescent pseudomonads found on lettuce when stored at the different temperatures and analyzed on different days during the trial.

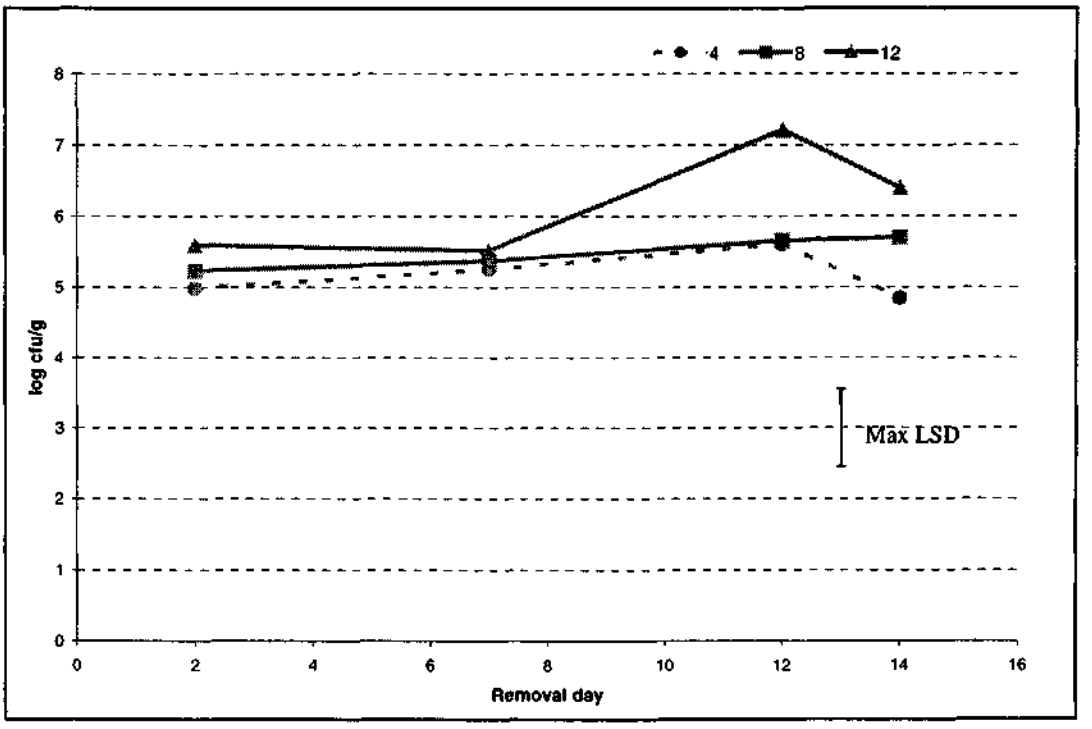


Figure 2.2 Fluorescent pseudomonad levels on lettuce leaves stored at 4°C, 8°C and 12°C for 2 weeks [bars are maximum LSD (P=0.05)]

No significant differences of fluorescent pseudomonad levels on lettuce were observed between temperatures up to a week in storage. During the second week, differences in fluorescent pseudomonad levels were observed (Figure 2.2). On day 12, lettuce stored at 12°C showed significantly higher levels of pseudomonads than lettuce stored at 4 and 8°C. Also on day 14, the levels of fluorescent pseudomonads were significantly higher at 12 °C compared to 4°C (P=0.013).

The average quality of the lettuce decreased over time and in response to the different temperatures. Figure 2.3 shows the average quality rating of lettuce stored at different temperatures and assessed over two weeks. Quality of the lettuce stored at most temperatures remained at a fairly good level for the two-week period. For the first week, lettuce stored at all three temperatures remained at a good quality rating (average above 6) with only slight browning present on some of the leaves. No significant differences between quality were noted at the different storage

temperatures (P=0.072). During the second week the lettuce stored at 4 and 8°C showed no objectionable browning problems. However lettuce stored at 12°C showed quite poor quality on assessment days 12 and 14 (average score of 3 and 2 respectively) (Figure 2.3). Lettuces showed brown blemishes on the midribs and leaves and often deterioration to rotting. Figure 2.3 showed that the average quality of the lettuce at 12°C on day 12 and 14 were significantly different from the average quality of the lettuce stored at 4 and 8°C (P=0.007).

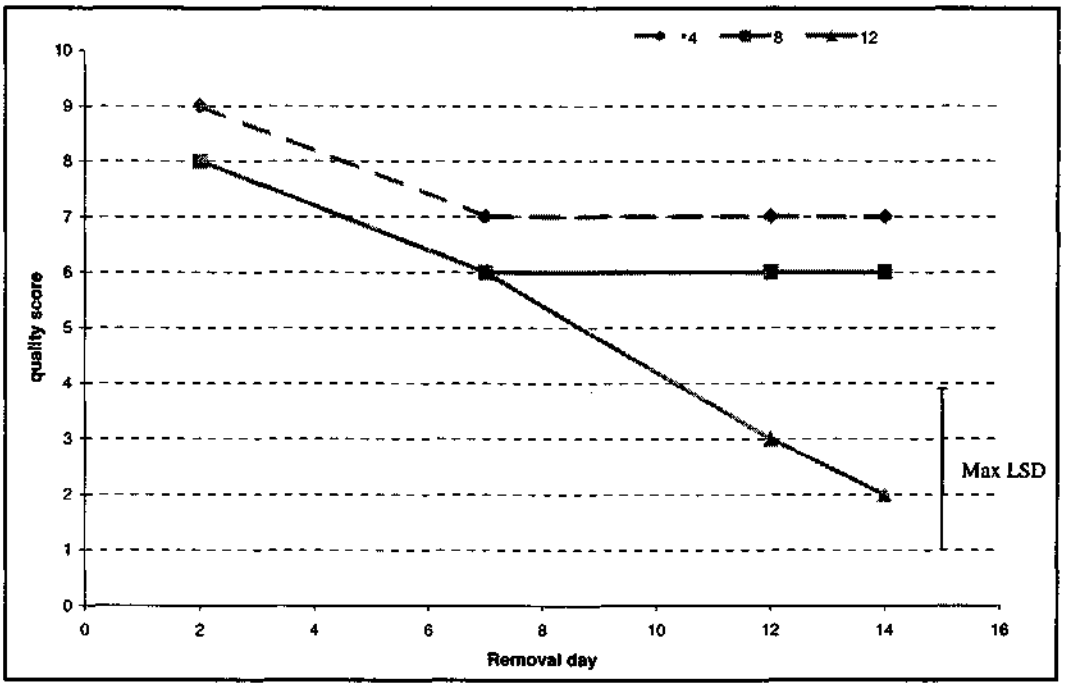


Figure 2.3 Average quality of cut lettuce stored at 4°C, 8°C and 12°C for 2 weeks [bars are maximum LSD (P=0.05)](Quality scores: 1=very poor to 9=very good)

Overall observations showed that storage temperature and length of storage impacted on the quality of the lettuce and the amount of fluorescent pseudomonads on the leaves. Preferable storage temperatures of below 4°C and 8°C would be necessary to maintain good quality lettuce, reduce browning and aid the retardation of the growth rate of fluorescent pseudomonads on the leaves of the plant.

2.3.2 Effect of bacteria on the cut surface of market and aseptically grown lettuce

2.3.2.1 Effect of the bacteria on the cut surface of tissue culture cos lettuce plants

Browning was observed on the cut surface of the leaves of lettuce inoculated with different bacteria. Figure 2.4 shows the results of the number of cut surfaces exhibiting browning and the average quality of the leaves 48 hours after bacterial inoculation. Lettuce plants were tested for bacteria prior to the inoculations and were found to be completely free, therefore any browning or damage was a reflection of the inoculated bacteria.

Control lettuce showed good quality with a rating score of 9 and had no cut surfaces exhibiting browning (Figures 2.4 & 2.5). The average quality of control lettuce and the peptone (diluent) treated lettuce were significantly higher than the inoculated lettuce. The peptone treated lettuce showed a few cut surfaces with browning however the intensity of the browning was not as obvious as the bacterial treated lettuce. The appearance of browning in this treatment may have been due to the plant's reaction to a foreign chemical substance on the cut surface.

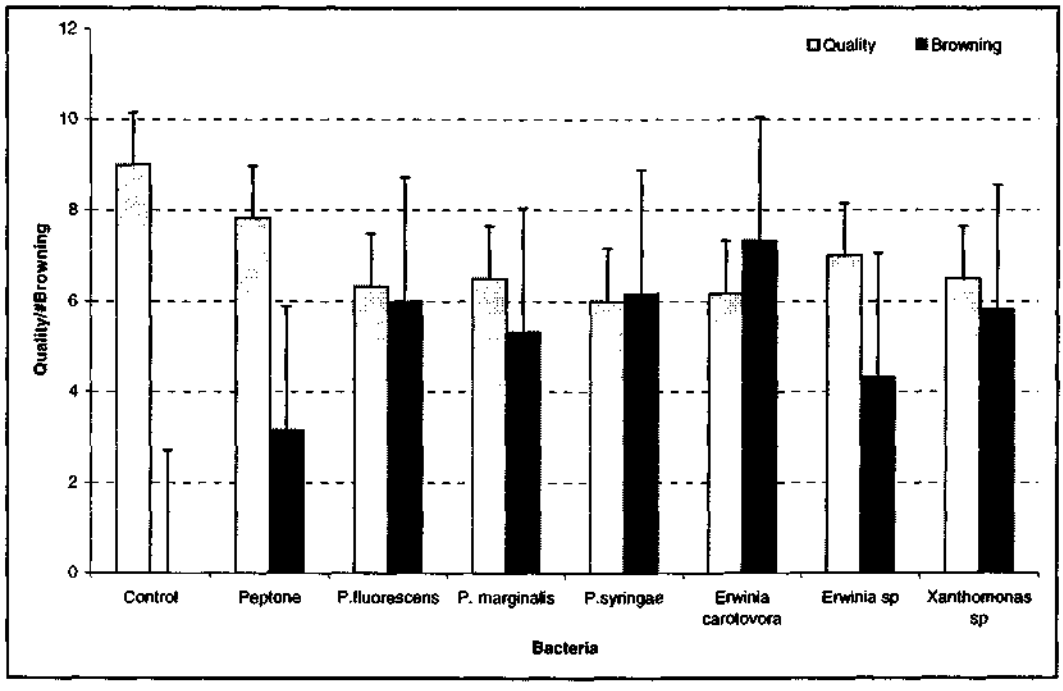


Figure 2.4 Number of tissue culture cut lettuce surfaces exhibiting browning and average quality of lettuce 48 hours after inoculation [bars are LSD (P=0.05)]

All lettuce inoculated with the bacteria showed some degree of browning and quality loss. Lettuce inoculated with *Erwinia carotovora* showed the highest number of brown cut surfaces however they were not significantly higher than the other inoculated lettuce, except the other *Erwinia* species (Figure 2.4). Lettuce quality of the inoculated lettuce also showed no significance between treatments (Figure 2.4). Upon observation of the extent of browning of the cut lettuce, the *Pseudomonas* species produced a greater impact on the cut surface than the *Erwinia* and *Xanthomonas* species. *Pseudomonas fluorescens* and *Pseudomonas marginalis* produced browning usually along the entire region of the cut or wound, whilst *Erwinia carotovora* mainly produced browning along the midrib region (Figure 2.5). Penetration of browning into the conducting vessels and surrounding internal tissues of the leaf occurred more with the Pseudomonad species, especially *Pseudomonas fluorescens*, than with the other species used. Rotting of the leaf surface was evident in some leaves inoculated with *Pseudomonas marginalis* and *Erwinia carotovora* (Figure 2.5). This was to be expected, as both possess pectolytic enzymes. Observation of some of the bacterial damage can be seen in figure 2.5.

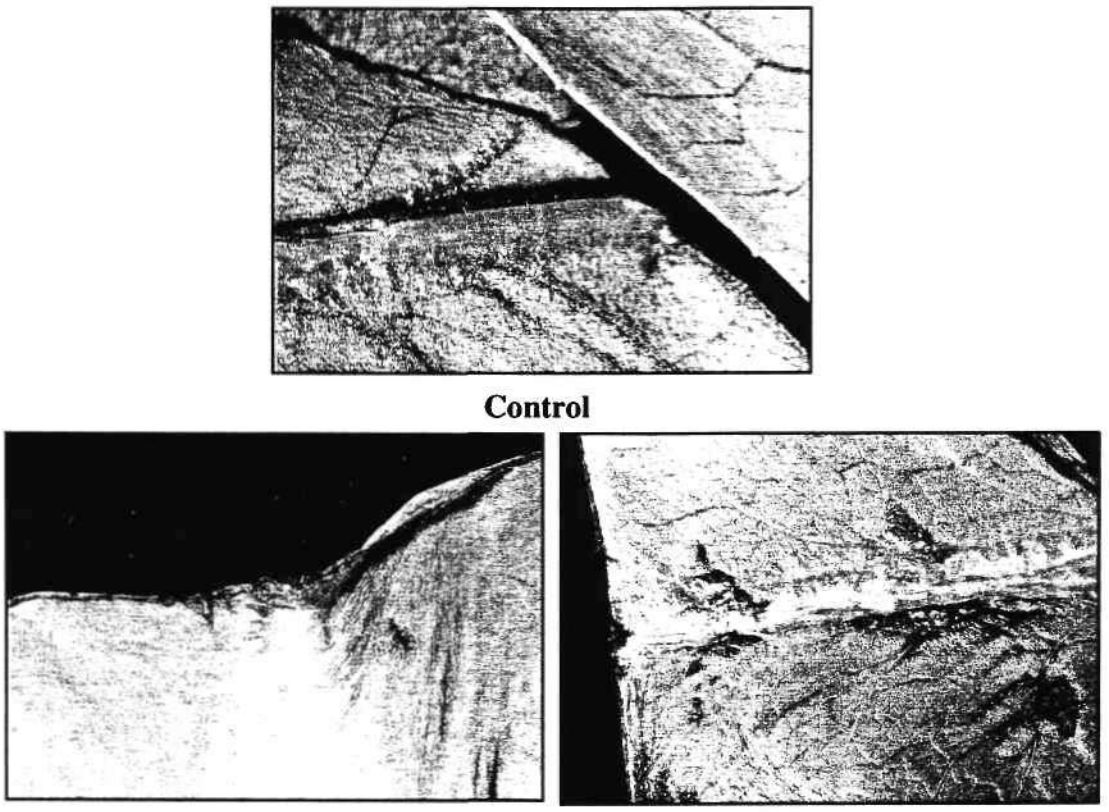


Figure 2.5 Browning on cut leaf surfaces caused by bacteria (after 48 hours) (magnification x20)

The results obtained showed that bacteria affected the browning of the cut lettuce. As shown, when the lettuce was free of bacteria (control), browning did not occur and average quality remained high. When bacteria were present, browning occurred along the cut surface and into the conducting vessels and aided in reducing the average quality of the leaf.

2.3.2.2 Effect of the bacteria on the cut surface of market lettuce

The results from this experiment were purely observational. No statistical analysis was conducted on the quality data. Natural populations of fluorescent pseudomonads were present on the lettuce leaves (average of $3.6x10^4$cfu/g lettuce) prior to inoculation with the different bacteria.

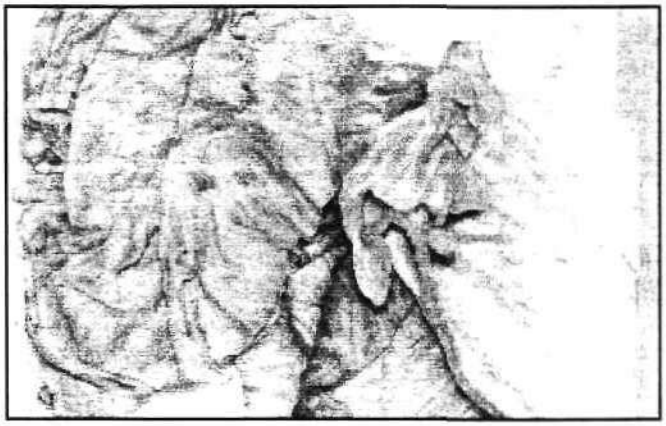
Lettuce inoculated with the different bacteria exhibited browning and quality loss. *Pseudomonas fluorescens* and *Erwinia carotovora* mainly compromised the quality of the lettuce with quality values in the poor and extremely poor range of the scale (Table 2.2). When compared with the control (un-inoculated) lettuce, the inoculated lettuce showed browning along the entire cut surfaces of the lettuce and good penetration into the conducting vessels. Visual analysis of the lettuce showed that lettuce inoculated with *Pseudomonas fluorescens* had progressed to the rotting stage

within the 48-hour period. Figure 2.6 shows the differences between the browning of lettuce when inoculated with *Pseudomonas fluorescens, Erwinia carotovora and Xanthomonas campestris.*

Table 2.2 Average quality of cut lettuce after 48 hours (Kader *et al.* 1973)

Inoculation treatment	Score
Control (no inoculation)	9
Pseudomonas syringae	6
Pseudomonas fluorescens	1
Erwinia sp.	6
Erwinia carotovora	2
Xanthomonas campestris	5

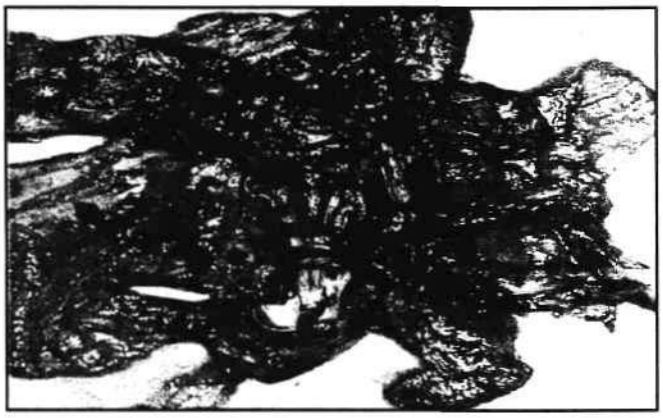
*No statistical analysis conducted



Control

Xanthomonas campestris

Erwinia carotovora



Pseudomonas fluorescens

Figure 2.6 Inoculation of cut lettuce by different bacteria

This brief study showed that the quality of cut lettuce appeared to be reduced by several bacteria in a 48-hour period. Even though the browning was evidently influenced by most of the bacteria, the browning caused by *Pseudomonas fluorescens* was more rapid and severe than the other bacteria used.

2.3.3 Analysis of fluorescent pseudomonads and the quality of cultivars of fresh cut lettuce harvested from several locations

Fluorescent pseudomonad levels varied between different crisphead cultivars as well as between different growing regions (Figure 2.7). Initial (pre-wash) levels of pseudomonads were observed on the surface of all lettuce cultivars, however no significant differences were noted between any pre-wash levels (P=0.138) (Figure 2.7). Washing had no significant effect on the pseudomonad populations that increased during storage (P=0.829).

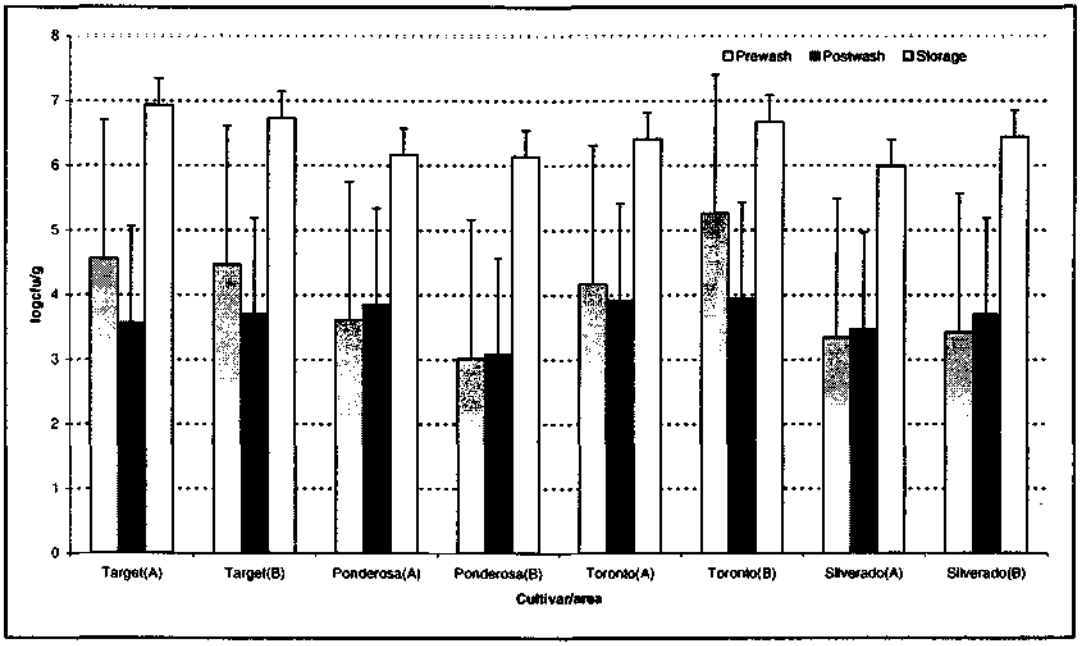


Figure 2.7 Levels of fluorescent pseudomonads on different lettuce cultivars grown in region A (Somerville) and B (Maffra) at each stage of processing and storage (7 days at 4°C) [bars are LSD (P=0.05)]

Significant differences were noted between the fluorescent pseudomonad levels on different cultivars after storage for 7 days at 4°C (Figure 2.6). Cultivar Target (both regions) and Toronto (Maffra) showed higher pseudomonad levels than the other two cultivars, Ponderosa and Silverado. The later cultivars showed significantly lower numbers of fluorescent pseudomonads (P<0.001).

Differences in average visual quality between some cultivars were observed after 7 days storage at 4°C (Figure 2.7).

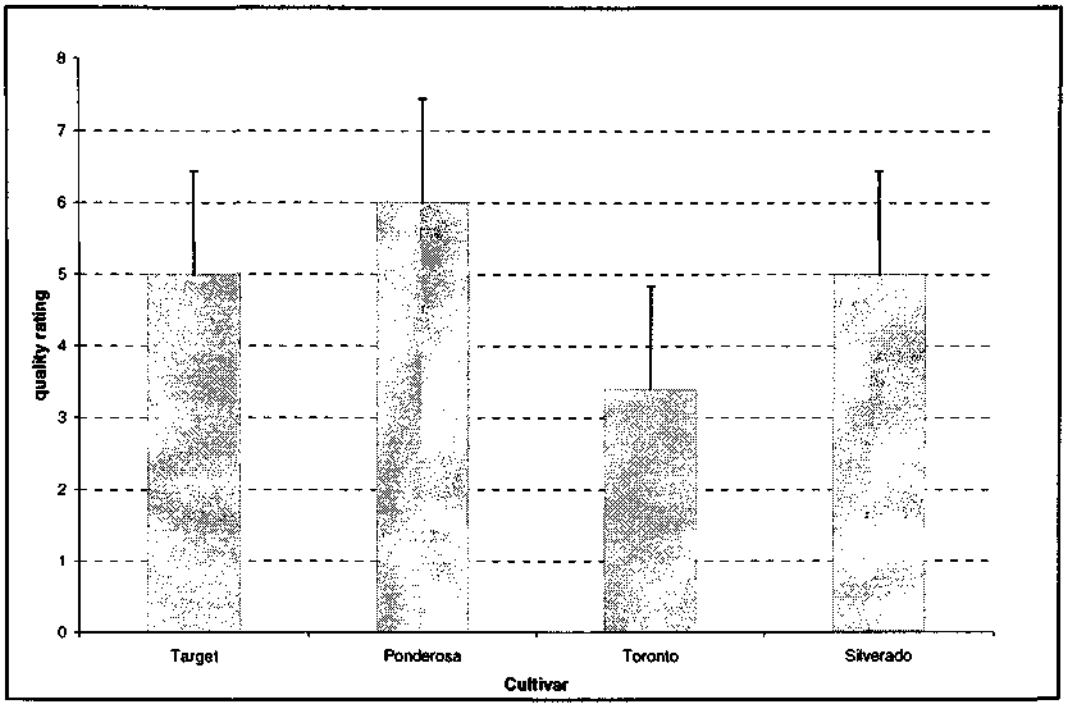


Figure 2.7 Average visual quality of lettuce cultivars after 7 days of storage (4°C) [bars are LSD (P=0.001)]

The cultivar, Ponderosa showed the least amount of browning whilst Toronto showed the highest level of browning (P=0.009) (Figure 2.7 and 2.8). The quality of lettuce harvested from Somerville also showed less browning than the lettuce harvested from Maffra (P=0.024). This could be due to the differences between the two growing regions including soil type, level of pseudomonad species in the soil, climate or different cultural practices.

The lower degree of browning observed in some of the cultivars might be a reflection of several factors including, the initial levels of fluorescent pseudomonads on the surface of the lettuce or differences in the rate at which fluorescent pseudomonads grow and the amount of damage they cause on the leaf surface. As shown in figures 2.6 and 2.7 the cultivars with the lower levels of pseudomonads possessed a better average visual quality after 7 days storage. While the cultivars with the higher levels of pseudomonads had lower quality and more browning.

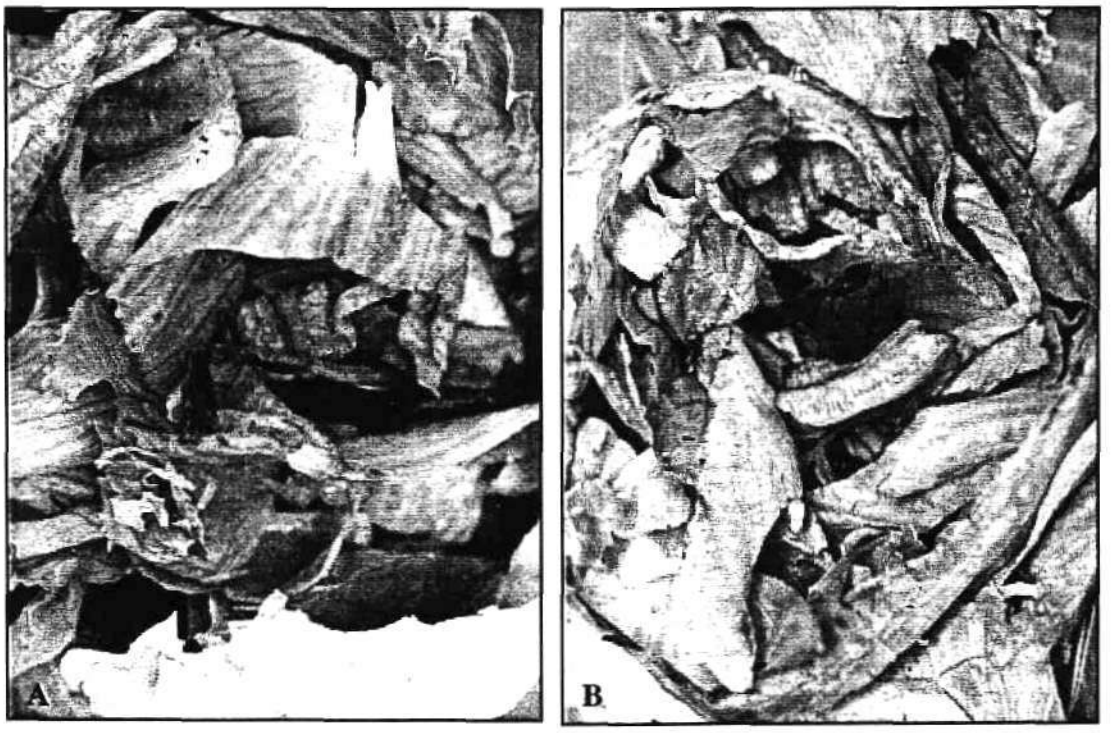


Figure 2.8 Browning on the crisphead cultivars, Ponderosa (A) and Toronto (B) after 7 days storage

2.4 Discussion

Browning of plant tissue was thought to be purely physiological in nature however it has been shown that fluorescent pseudomonads, in part, play a large role in the inducement of browning on lettuce plants. The mechanism by which the deterioration occurs is not fully understood but it has been noted that damage either caused in the field or in processing provides ideal conditions for the multiplication of the bacteria (Brocklehurst & Lund 1981; Nguyen-The & Prunier 1989).

Quality and integrity of the lettuce leaves are the most important factors necessary to maintain during storage. Weakened or over mature plant tissues promote the presence of higher populations of fluorescent pseudomonads due to the breakdown in cellular tissue therefore further browning and subsequent rots could occur (Zagory 1999). Storage conditions have shown to influence the quality of cut lettuces. Temperature and modified atmosphere have been analyzed in order to improve the shelf life and quality of lettuce (Bolin *et al.* 1977; Couture *et al.* 1993). Bolin *et al.* (1977) found that lettuce held at 2°C remained marketable for about 26 days compared to 10 days for the same product at 10°C. The observations in this study showed that quality of lettuce was compromised when held at 12°C for longer than 7 days. Poor quality was detected when lettuce was held at 12°C for up to14 days.

Other conditions that favour the growth of bacteria on the surface of vegetables include high temperatures, overmature plants and injurious levels of CO_2. These not only lead to spoilage of the vegetables but lead to further bacterial proliferation

(Zagory, 1999). Attempts to reduce these bacterial promoting conditions in the field and in storage could also be conducted by maintaining vegetables in a less mature condition so that the growth of most opportunistic microorganisms would be expected to be retarded since they tend to grow most rapidly on aging tissues. (Zagory 1999). As cellular rupturing through mechanical damage to the vegetable's surface promotes bacterial growth, Bolin *et al.* (1977) suggested that the simple action of cutting with a knife could be modified to minimize the acceleration of the browning reaction. By cutting with a very sharp knife or tearing the lettuce leaf into strips could give a better storage life due to the lack of exudation noticed from the cut surface (Bolin *et al.* 1977). Some researchers have actually suggested that the presence of proliferating bacteria, such as fluorescent pseudomonads, on the surface of vegetables may also be seen as a warning system to show that the quality and shelf life of the product has depleted and that the vegetables should be disposed of (Zagory, 1999).

The results from this project indicated that bacteria have an important role in not only rotting the cut surface of lettuce leaves but also in eliciting a browning response. The cut surface of all leaves inoculated with bacteria turned brown whereas the cut surface of leaves that were not inoculated remained free of browning. It has previously been shown that *Pseudomonas* species can induce rapid browning on cut lettuce within 48 hours (Frisina *et al.* 1997). Therefore the results presented in this project support this observation. *Erwinia carotovora*, another bacterial species that causes postharvest soft rots also possesses pectolytic enzymes. Both *Erwinia* species and *Pseudomonas* species are comparable with the degree of damage they cause on vegetables. The difference between the two species is that *Erwinia* species grows slightly at 5°C whereas *Pseudomonas* species are capable of serious rotting even at temperatures close to 0°C (Nguyen-The & Prunier 1989; Snowdon 1991). When compared with other bacteria the browning reaction caused by the fluorescent pseudomonads was more rapid and severe.

As Pseudomonad species have been implicated in exacerbating the browning reaction on cut surfaces of lettuce, initial levels on the plant may play a role in the subsequent browning of the cut surface. Studies by Tomkins *et al.* (2001) have shown variation of postharvest storage quality exists between cos and crisphead cultivars. Couture *et al.* (1993) also noted differences in the visual quality of different cultivars, mostly differences in browning intensity. From the cultivar experiment (2.3.3), observations showed that the different cultivars also showed differences in storage quality and fluorescent pseudomonad levels. Cultivars with lower fluorescent pseudomonad levels showed higher storage quality and cultivars with higher pseudomonad levels showed lower quality. These observations are in accordance with Bolin *et al.* (1977). They noted that samples of fresh-cut lettuce containing higher microorganism loads had shortened storage life and lower quality (Bolin *et al.* 1977). Nguygen-The and Prunier (1989) also noted that the higher frequency of *Pseudomonas marginalis* compared to other Pseudomonas species among the strains present on the lettuce surface lead to a higher deterioration effect of the produce. However, other researchers have rejected observations such as these. Available evidence also suggests that microbial growth and populations depend primarily upon the morphological and physiological condition of the plant tissues. Rather than high microbial populations being responsible for deterioration of fresh produce, deterioration may be responsible for high microbial populations. Those operations that reduce injury and preserve the physiological integrity of fresh produce are

associated with low populations of microorganisms (Zagory 1999). Conflicting stories on whether the storage stability of shredded lettuce can be improved by the application of treatments which reduce the initial microbial load will continue to be debated (Delaquis *et al.* 1999). Previous studies conducted on the effect of chlorinated water on the reduction of other bacteria, such as *E. coli*, on lettuce and broccoli showed no significant effects with reductions of 1.7-2.8 $\log_{10}$ cfu/g, whilst water alone reduced numbers by 1.5-1.8 $\log_{10}$ cfu/g (Behrsing *et al.* 2000). Our studies also showed that chlorinated water did not significantly reduce the levels of fluorescent pseudomonads on the surface of lettuce.

Through the available published research and the work conducted throughout this project it is clear that fluorescent pseudomonads affect the quality and storage longevity of lettuce and other vegetables, by playing a role in the elicitation of the plant's defence mechanisms. The contamination of lettuce and other vegetables by saprophytic and pathogenic bacteria usually occurs before harvest in the field. Monitoring of the levels of fluorescent pseudomonads and their activity in the soil of lettuce growing regions may provide valuable information for further ways to control the presence of pathogenic pseudomonads. This work has been discussed further in the following parts of the report.

PART 3 Analysis of fluorescent Pseudomonads in soil

3.1 Introduction

Fluorescent pseudomonads are widespread in soil. They are distributed unevenly through soil and are largely associated with organic matter at the early stage of decomposition (Rovira & Sands 1971). High numbers of fluorescent pseudomonads, up to 10^4 per gram, are prevalent on recently fallen leaves and in the rhizosphere of living roots (Rovira & Sands 1971; Cuppels & Kelman 1973). The soil populations and types of fluorescent pseudomonads vary widely depending on the state of decomposition of organic matter (Rovira & Sands 1971; Sands & Hankin 1975). Previous studies have found that the fluorescent pseudomonads isolated from soil were usually the saprophytic *Pseudomonas fluorescens* (biovar V) which do not produce pectolytic enzymes (Sands & Hankin 1975; Stanier *et al.* 1966). Soil isolates, which are pectolytic, may represent a residual population incorporated into the soil on plant material, or in water, and are not typical soil inhabitants.

Previous research into the levels of fluorescent pseudomonads in soil has shown that relatively low numbers of the bacteria were found. Sands and Rovira (1971) reported that fluorescent pseudomonads represented <1% of the total bacterial population in the soil and rhizosphere. Similarly Sands *et. al.* (1972) found that the fluorescent pseudomonads comprised no more than 0.27% of the total bacterial population.

The aims were to:
(a) monitor the levels of fluorescent pseudomonads in the soil of lettuce growing regions.
(b) identify the fluorescent pseudomonads present in the soil over the different seasons.
(c) analyze the persistence of fluorescent pseudomonads on the lettuce debris after harvest.

3.2 Method

3.2.1 Analysis of soil for fluorescent pseudomonads from lettuce growing regions

3.2.1.1 Locations of selected lettuce-growing properties

Samples were taken from three lettuce growing regions in Victoria. These included Werribee South, Somerville and Lindenow/Maffra (refer to figure 3.1). Two properties were selected from each region.

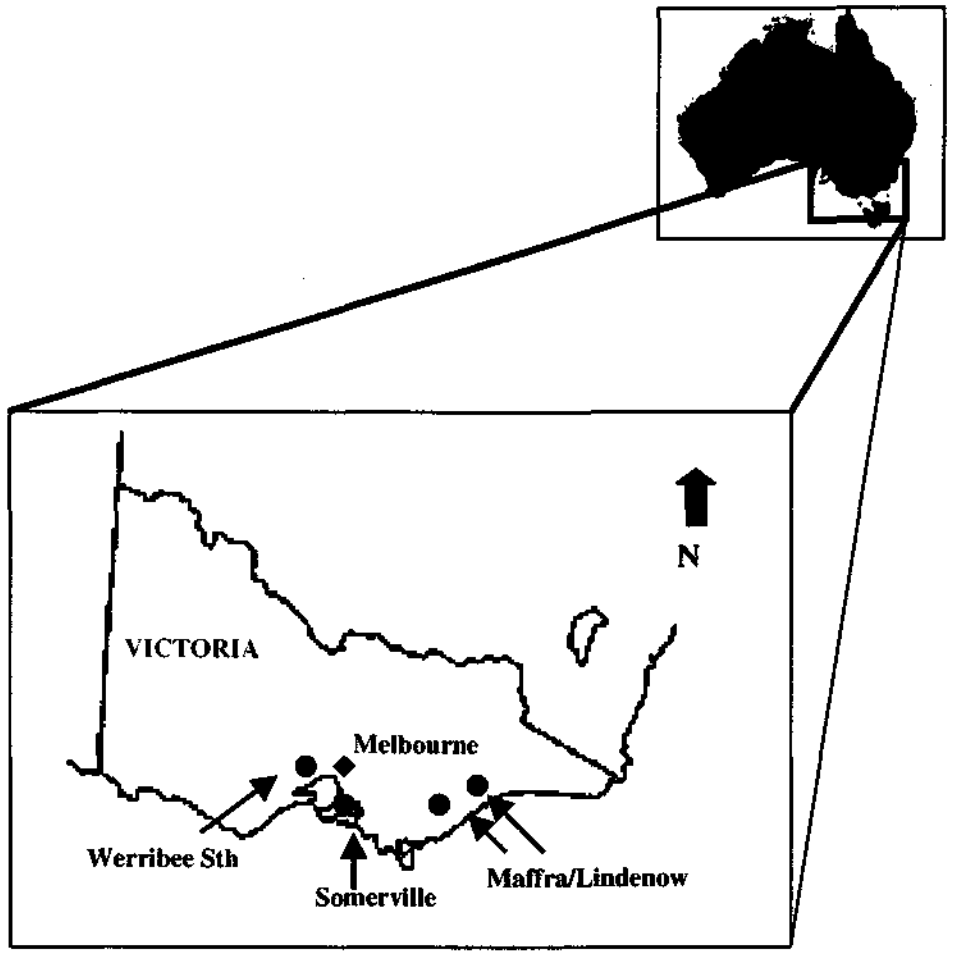


Figure 3.1 Location of lettuce growing regions of Victoria used for soil sampling

3.2.1.2 Soil sampling procedure

Sampling of soil from each property was conducted over 7 to 10 seasons. Within each of the 3 fields within the same property, soil samples were taken from 8 positions reduce variation in results. Each soil sample was analyzed separately for total aerobic bacterial counts and *Pseudomonas* species.

3.2.1.3 Analysis of fluorescent pseudomonads in the soil
From each soil sample 15-20 g was placed into peptone water (0.1%) and agitated for 20 minutes at 150rpm. Serial dilutions of the soil suspension were made in peptone buffer containing 0.1% buffered peptone (Merck, Aust.) in deionised water. These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The quantification of the fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies as an indicator (refer to figure 2.1). Quantification of the total aerobic bacteria was conducted by counting the red bacterial colonies that grew on the petrifilm.

3.2.1.4 Data analysis
REML (Restricted maximum likelihood) analysis (GenStat 5.4.2) was conducted on the log data produced from the soil sampling as some fields were not measured for all 10 times. Least significance differences (LSD) between the region and time means were generated. All tests were at the 5% significance level unless otherwise stated. Bars on graphs are 1x LSD. Where several LSD were generated, the maximum LSD has been shown.

3.2.2 Identification of fluorescent *Pseudomonas* species from soil

Soil was processed according to section 3.2.1.3 and assessed for fluorescent pseudomonad presence under UV light. Fluorescent pseudomonads were identified using the LOPAT system of identification (Lelliott *et al.* 1966). The LOPAT system included Levan production, oxidase activity, potato rot (pectolytic enzyme activity), arginine dihydrolase activity and tobacco sensitivity (Lelliott *et al.* 1966).

3.2.3 Persistence of fluorescent pseudomonads on lettuce debris

3.2.3.1 Experimental development and design
Soil and lettuce were obtained from a property in Werribee South, Victoria. Polystyrene boxes (20-litre capacity) were filled with the soil from the property and transferred to the glasshouse. Overhead water spraying (mist) occurred once daily and air temperatures were similar to daily conditions. Each treatment was randomly assigned to a randomly placed individual block located on the glasshouse benches. Each treatment consisted of 6 replicates.

3.2.3.2 Treatments
The treatments comprised a factorial combination of soil straight from the property or sterilized soil (from the property but autoclaved) mixed with or without lettuce leaves. Treatment combinations were:

1) soil straight from the property
2) sterile soil
3) sterile soil mixed with fresh lettuce leaves
4) soil straight from the property with lots of newly harvested lettuce leaves.

3.2.3.3 Soil sampling and assessment for bacterial levels
Soil samples were collected daily for 3 days then every 2-3 days up to day 24. Six samples were collected from each replicate treatment box and pooled together,

whereby a 25 g sub-sample of soil was removed for analysis. Bacterial analysis of the soil was conducted in accordance to 3.2.1.3.

3.2.3.4 Data analysis
Analysis of variance (GenStat 5.4.1) was performed on the data produced from the experiments. Log counts of the bacteria were analyzed to meet the assumption of constant variance. The least significant difference (LSD) was also used to compare treatments. All tests were at the 5% significance level, unless otherwise stated. Bars on graphs are LSD (P=0.05)).

3.3 Results

3.3.1 Analysis of soil for fluorescent pseudomonads from lettuce growing regions

Sampling was conducted over a two-year period to observe the natural levels of fluorescent pseudomonads and total aerobic bacteria in the soil of lettuce growing regions of Victoria. The aim was to observe the fluctuations of the bacterial levels in response to seasonal variations. Figure 3.2 and 3.3 show the results of the samplings. The natural variations in fluorescent pseudomonads and total aerobic bacteria were observed across the three regions and across the different seasons.

Average levels over all sample times of fluorescent pseudomonads were found to be significantly higher in the Somerville region compared with the Werribee and Gippsland region (P=0.001). Average levels in Somerville were 4.71 $\log_{10}$cfu/g of soil compared to Werribee with 3.97 $\log_{10}$ cfu/g of soil and Gippsland with 4.11 $\log_{10}$ cfu/g of soil. In the Somerville region, fluorescent pseudomonad levels were shown to be significantly higher in the later months of 1999 and 2000 and were on the increase in 2001 (Figure 3.2). Samplings conducted in autumn 2000 detected the lowest levels of fluorescent pseudomonads, which were significantly lower than at the other sampling periods.

In the Werribee region, fluorescent pseudomonads were found to be significantly higher in the winter sampling of 2001 compared to all the other samplings (except autumn 2001). The lowest levels were found in summer 2001 which was significantly lower than most of the samplings except those levels detected in spring 1999 and autumn 00 (Figure 3.2).

In the Gippsland region, levels of fluorescent pseudomonads were found to be significantly higher in spring 2001 compared to the other soil samplings (Figure 3.2). In Summer 2001 the level of fluorescent pseudomonads detected was significantly lower than those found in the rest of the sampling period

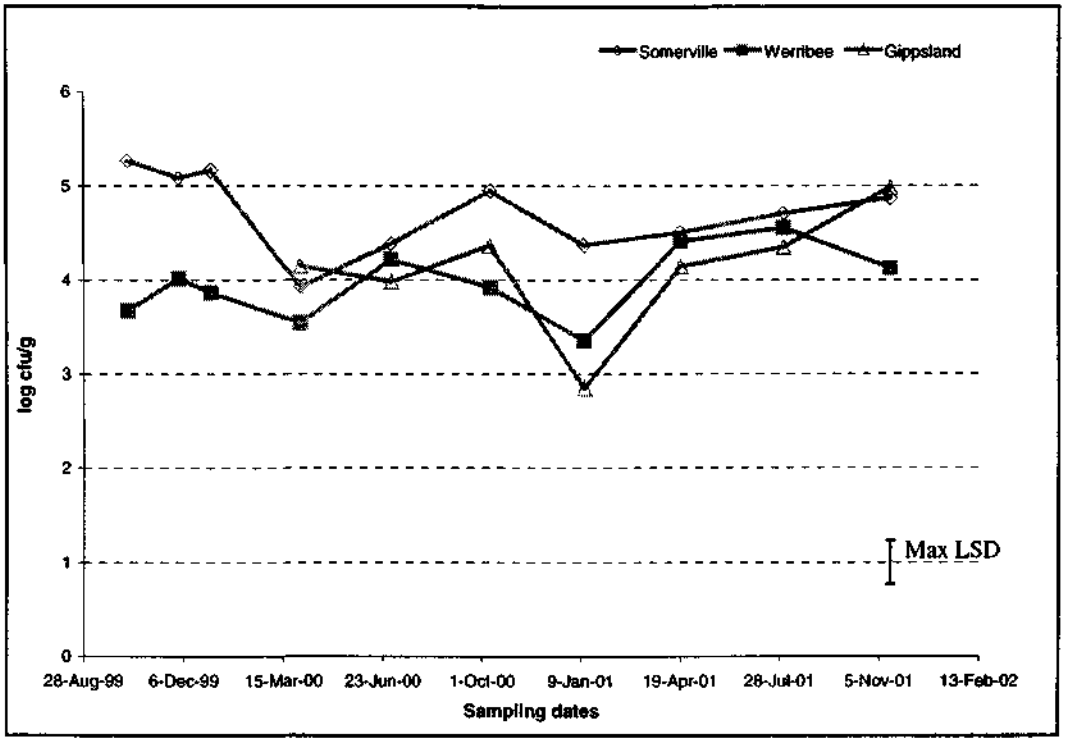


Figure 3.2 Average numbers of fluorescent pseudomonads detected in soils of lettuce growing regions of Victoria [bars are maximum LSD (P=0.001)]

Average levels over all samplings of total aerobic bacteria were found to be significantly higher in the Somerville region compared to the other regions (Figure 3.3). Average levels detected in Somerville were 6.98 $\log_{10}$ cfu/g of soil compared to Werribee with 6.65 $\log_{10}$ cfu/g of soil and Gippsland with 6.64 $\log_{10}$cfu/g of soil.

In the Somerville region average levels of total aerobic bacteria were detected in the range of 6.81$\log_{10}$ cfu/g of soil to 7.17 $\log_{10}$ cfu/g of soil. Levels were significantly higher in spring 2000 compared to most of the other sampling dates except levels detected in summer, autumn and winter 2001. All other levels detected were not significantly different from one another.

In the Werribee region average levels of total aerobic bacteria ranged from 6.2 $\log_{10}$ cfu/g of soil to 7.09 $\log_{10}$ cfu/g of soil. Levels detected in spring 1999 (October) were significantly lower than all the other levels detected. Whilst the highest level detected in winter 2001 (July) was found to be significantly higher than the other levels detected except for the spring 2001 level (Figure 3.3).

In the Gippsland, region average levels of total aerobic bacteria ranged from 6.29 $\log_{10}$ cfu/g of soil to 7.19 $\log_{10}$ cfu/g of soil (Figure 3.3). Significantly higher numbers of total aerobic bacteria were detected in spring 2001, whilst significantly lower numbers were detected in spring 2000 (except the level detected in summer 2001).

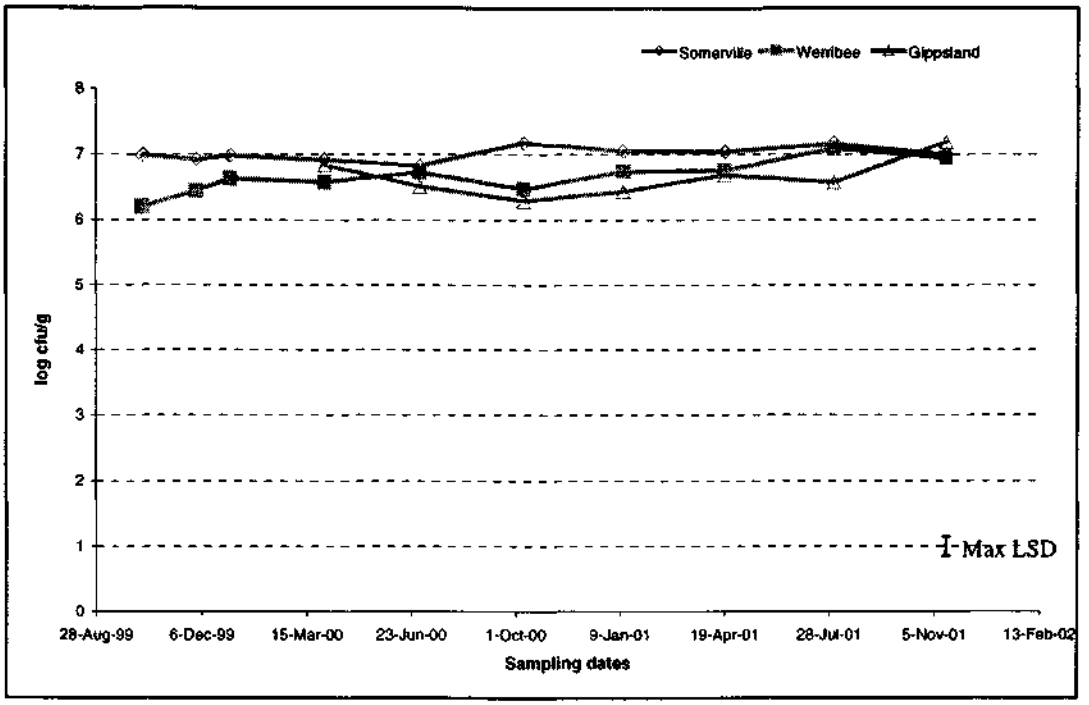


Figure 3.3 Average total aerobic bacterial loads detected in soils of lettuce growing regions of Victoria [bars are maximum LSD (P=0.001)]

Observations of the proportion of fluorescent pseudomonads in the soil varied from each area and each date (Table 3.1). The proportions are not indicative of the actual levels of fluorescent pseudomonads detected over the two-year sampling period (Figure 3.2), but show the average populations present within the soil environment as a proportion of the total aerobic bacterial population. Fluorescent pseudomonad proportions in the Somerville area ranged from 0.39 to 2.8% of the total bacterial population. In Werribee the proportions ranged from 0.29 to 3.6% and in Gippsland the proportions ranged from 0.17 to 3.3% (Table 3.1). No significant differences were detected between the different areas (P=0.553). Variations in fluorescent pseudomonad proportions between dates were significant (P=0.001), however no obvious patterns were detected with the higher proportions

Table 3.1 Observation of the fluorescent pseudomonad population as a proportion of the total aerobic bacteria (%)[Max. LSD 2.37 (when comparing between areas), Max LSD 2.10 (when comparing between dates)]

Date/area	Somerville	Werribee	Gippsland
October 1999	2.5	3.6	*
December 1999	2.6	1.4	*
January 2000	2.8	0.76	*
April 2000	0.39	0.33	0.54
July 2000	0.54	0.45	0.6
October 2000	1.26	0.71	3.3
January 2001	0.29	0.29	0.17
April 2001	0.43	0.49	0.54
August 2001	0.58	0.43	0.88
November 2001	2.8	0.68	2.3

Many different crops of vegetables were planted in the sampling fields over the two-year period. Vegetables planted included broccoli, cauliflower, cabbage, lettuce, celery, sweet corn, parsnips, onions, Asian vegetables and Brussels sprouts. Green manure was also used in sampled fields occasionally. An observation noted throughout the sampling period and across all properties and fields was the elevation in fluorescent pseudomonads in the soil when plant debris from a harvested crop was present. Figure 3.4 shows an example of the increase in pseudomonad levels in the presence of plant debris. All 16 fields observed with plant debris at the time of soil sampling showed an increase in fluorescent pseudomonad levels (Figure 3.4).

The variety of vegetables present during the soil samplings showed no obvious influence on the levels of total aerobic bacteria and fluorescent pseudomonads. For example, levels of fluorescent pseudomonads detected in the soil of a Werribee property during sampling in July 2000 (winter), October 2001 (spring) and January 2001 (summer) were 3.785, 3.105 and 4.706 $\log_{10}$ cfu/g respectively. The crop planted in the area throughout the sample periods was lettuce. Many other factors may have influenced the levels of fluorescent pseudomonads including the maturity of the plant, environmental conditions or farm management practices.

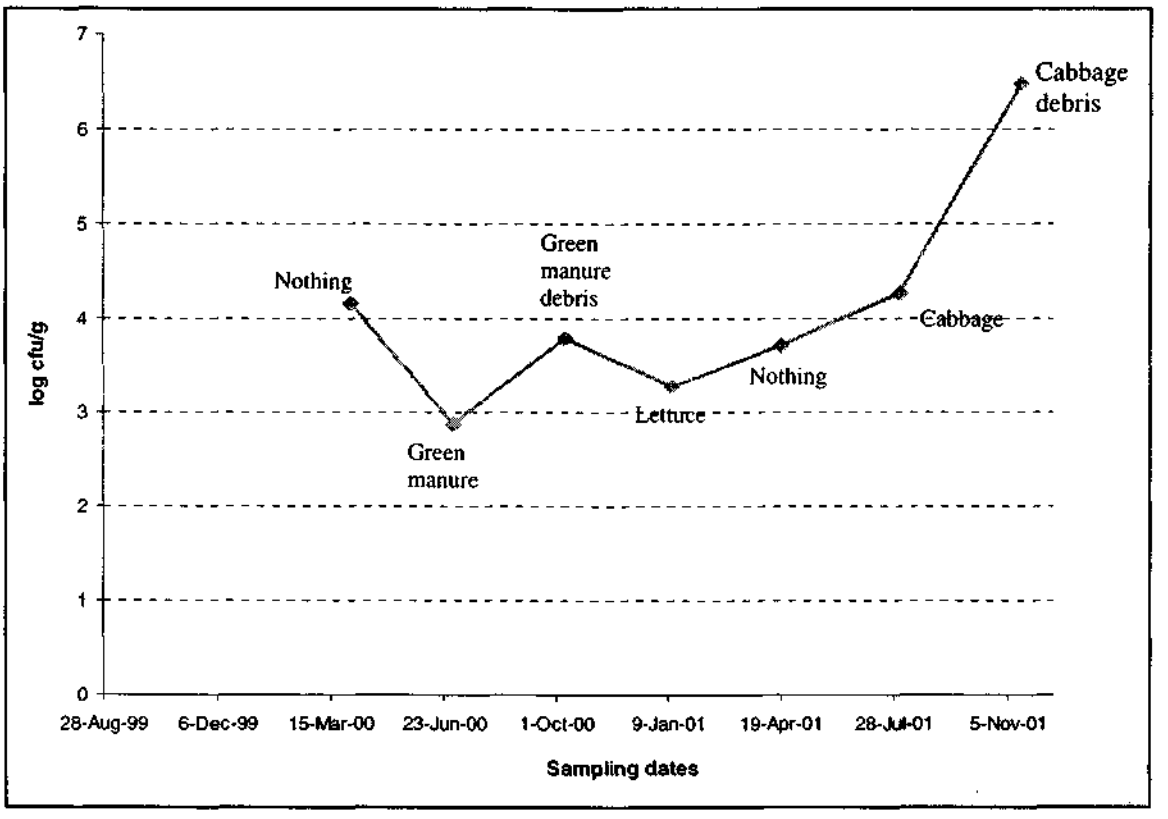


Figure 3.4 Observation of crop or crop debris and average number of fluorescent pseudomonads present in soils of field 2 of Gippsland property 1

3.3.2 Identification of fluorescent *Pseudomonas* species isolated from soil

Fluorescent pseudomonads were isolated from the soil of lettuce-growing regions of Victoria. These species were identified each season according to the LOPAT system. Table 3.2 shows the list of fluorescent pseudomonads identified.

Table 3.2 Number of isolates of fluorescent *Pseudomonas* species identified from soil samples taken from lettuce-growing regions of Victoria

Season	Region	*P. marginalis*	*P.fluorescens* (pectolytic)	*P.fluorescens* (saprophytic)	*P. tolaasii*	*P. cichorii*
Spring 99	Morn. Penn.	3	4	7	2	
	Werribee	1	1	1		
Summer 00	Morn. Penn.	1	1	7	1	2
	Werribee		3	1	2	
Autumn 00	Morn. Penn.	1	2	13	1	2
	Werr. Sth	2	1	8		
	Gippsland			5		
Winter 00	Morn. Penn.			4	7	
	Werr. Sth		3	5	14	
	Gippsland			1	4	
Spring 00	Morn. Penn.				3	
	Werr. Sth		2	4	10	
	Gippsland			1	4	
Summer 01	Morn. Penn.	3	4	7	2	
	Werr. Sth	3	2	7		
	Gippsland		5			
Autumn 01	Morn. Penn.			5	5	
	Werr. Sth			5	6	
	Gippsland			1	6	
Winter 01	Morn. Penn.	1	1	2	6	
	Werr. Sth		1	1	9	
	Gippsland	1	4		6	
	Total	**16 (7.3%)**	**34(15.7%)**	**75(34.6%)**	**88(40.5%)**	**4(1.9%)**

Identification of the bacteria showed similar results with fluorescent pseudomonad species isolated from soil in several growing regions (refer to Table 3.1). Over the two-year period, approximately 217 isolates were identified. Of the fluorescent pseudomonad species isolated 34.6% (75 of 217 isolates) were identified as saprophytic *Pseudomonas fluorescens* (biovar V), whilst 7.3-15.7% (16 & 34 of 217 isolates) were identified as pectolytic *Pseudomonas marginalis* and *Pseudomonas fluorescens* (biovar IV) respectively. The remaining 88 isolates (40.5%) were *Pseudomonas tolaasii*, a saprophytic bacterium (*Pseudomonas fluorescens* biovar V) and 4 (1.9%) identifications of *Pseudomonas cichorii*. At the time of soil sampling plant roots and some lettuce leaf debris were present in some of the samples from which the pectolytic species were isolated. This shows that these bacteria may be associated with organic matter in the soil as opposed to the soil itself.

3.3.3 Persistence of fluorescent pseudomonads on lettuce debris

The level of fluorescent pseudomonads showed variations in response to the different treatments over the three week period (refer to Figure 3.5). Farm soil only, showed fluorescent pseudomonads consistent over the entire duration with a slow reduction in numbers towards the end of the trial. Treatment 2 with the sterile soil only, showed that the autoclaving process did not entirely kill off the bacteria in the soil (levels found in 2 of the 6 blocks). Therefore the small population present (avg. 2350 cfu/g) was able to take advantage of the available nutrients and water and grow. As figure 3.5 shows, levels significantly grew from day 2 up to day 6 and then reached a plateau of population growth between day 6 and day 10. From this time the fluorescent pseudomonad levels started to decline to levels comparable with the other treatments.

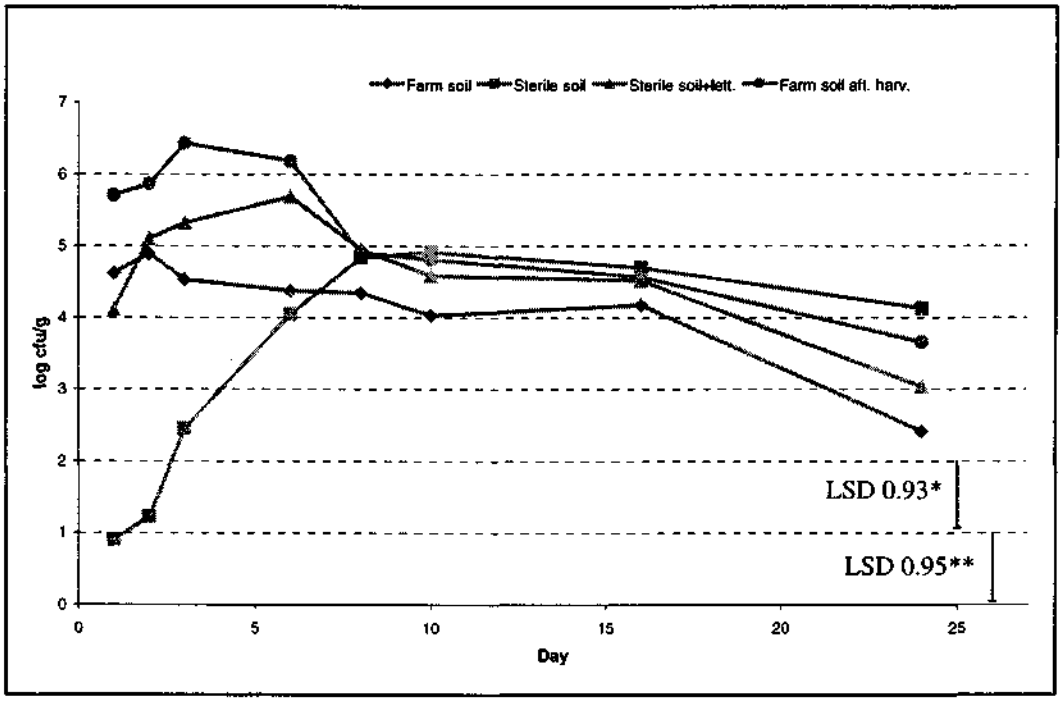


Figure 3.5 Average level of fluorescent pseudomonads in soil with and without lettuce [* LSD (P=0.05) for treatment/day, ** LSD (P=0.05) for same level of treatment]

Treatment 3 (sterile soil and lettuce) showed the influence of the fresh lettuce in the environment. Fluorescent pseudomonads remained low for 2 days then the bacteria made use of the available nutrients and reached higher levels over 5.5 x 10^5 cfu/g by day 6. Population levels dropped between day 6 and day 8 but then evened off to become comparable with the other treatments from day 16 to 24 (Figure 3.5).

Treatment 4 with the soil from the property mixed up with the lettuce waste after harvest simulated what was to be expected in a natural field situation. Figure 3.6 shows the appearance of an area shortly after lettuce harvest. Note the large number of lettuce leaves left on the ground. Populations of fluorescent pseudomonads followed a similar trend to treatment 3 with an increase in the population for the first 6 days, and a reduction towards the end of day 24. The initial levels of fluorescent

pseudomonads were significantly higher with this treatment than the other treatments due to the combination of the soil's natural fluorescent pseudomonads and the population on the lettuce leaves. At the peak of population growth at day 3, the average levels were 3.4 x 10^6 cfu/g (significantly higher than for the other treatments on the same day).

Figure 3.6 Appearance of field after lettuce harvest

The lettuce debris was apparent in the soil for up to 14 days. Initially the leaves were green (for the first 3 days) then started drying out and turning brown (days 6-8). By day 10 the leaves were starting to decompose so by day 14 to 16 there was only a small amount present. By day 21 to 24 the leaves had totally broken down.

Overall the fluorescent pseudomonad levels showed significance between treatments until day 6. By day 8 and onwards, the levels were not significantly different. Levels remained quite high 10^4 to 10^6 cfu/g for up to 16 days after which time reductions in populations became apparent. Levels reduced to between 10^2 and 10^4 cfu/g by day 24. The gradual decrease in the fluorescent pseudomonad population levels in the glasshouse was probably due to the gradual reduction of available nutrients in the boxes of soil for the fluorescent pseudomonads to thrive on. In a field situation this would be a similar case, however the decomposition of the lettuce debris would probably be accelerated by natural conditions, such as solarization.

3.4 Discussion

Many factors effect the presence of saprophytic and pathogenic bacterial populations within soils. These include environmental conditions such as temperature and water availability, farm management practices, such as fertilizer or compost application, soil type and the state of the plant material within the soil (eg. whole plant or debris) (Rovira & Sands, 1971; Cuppels & Kelman, 1980; Schober & Zadoks, 1999).

Detection of the total bacteria and fluorescent pseudomonads has shown to be widespread throughout the soil of all the growing areas sampled. Great variability was observed in the natural fluorescent pseudomonad levels detected across the different seasons. High numbers of fluorescent pseudomonads, up to 10^4 per gram, are prevalent on recently fallen leaves and in the rhizosphere of living roots (Rovira & Sands 1971; Cuppels & Kelman 1973). In this study, levels of fluorescent pseudomonads were often observed at these high numbers across the different seasons and areas. Cuppels and Kelman (1980) noted that fluorescent pseudomonads could withstand winter conditions in aqueous environments, therefore surviving from one growing season to the next not only on seeds or plant debris but also in soil and water.

Higher concentrations of fluorescent pseudomonads have been noted in sandy soils compared to clay based soils (Cuppels & Kelman, 1980) though in another study of fluorescent pseudomonads in soils in South Australia, the opposite was found. This may have been due to the higher temperatures and soil desiccation before sampling occurred from the sandy soils (Rovira & Sands, 1971). Levels of fluorescent pseudomonads were also noted to be in higher concentrations in sandy soils compared to clay based soils in this study. Average levels in Somerville soils were often found to be higher than those found in the Werribee region.

The presence of fluorescent pseudomonads has been linked with the presence in the soil of organic material at the early stages of decomposition (Rovira & Sands, 1971). This was confirmed here with an increase in fluorescent pseudomonad numbers from the previous sampling date noted in the presence of plant debris within the soil (Figure 3.4). Adding plant debris to the soil showed that fluorescent pseudomonad levels increased 1000 fold compared to natural levels (Figure 3.5). An association with organic matter would improve the chances of the bacteria surviving the high summer temperatures and desiccation. It has been noted that as the organic matter in the soil is further decomposed by other organisms the fluorescent pseudomonads remain as residual populations and either die or reside on pockets of fresh material (Rovira & Sands, 1971).

The soil populations and types of fluorescent pseudomonads vary widely depending on the state of decomposition of organic matter (Rovira & Sands 1971; Sands & Hankin 1975). Previous studies have found that the fluorescent pseudomonads isolated from soil were usually the saprophytic *Pseudomonas fluorescens* (biovar V) which do not produce pectolytic enzymes (Sands & Hankin 1975; Stanier *et al.* 1966). Soil isolates, which are pectolytic, may represent a residual population incorporated into the soil on plant material, or in water and are not typical soil inhabitants. The species of fluorescent pseudomonads varied from each area and each sampling period (Table 3.2). The dominant species isolated and identified was *Pseudomonas tolasii* (biovar Va), a saprophytic bacteria (40.5%). Saprophytic bacteria composed the

greater numbers identified from the total numbers isolated. Including the *Pseudomonas fluorescens* (biovar Vb), saprophytic bacteria made up 75% of the total isolates identified (Table 3.2). Pathogenic fluorescent pseudomonads including *Pseudomonas marginalis* (biovar IVa) and *Pseudomonas fluorescens* (biovar IVb) were identified in 23% of the isolates. Based on these observations, pathogenic fluorescent pseudomonads are most probably a residual population in the soil environment. The presence of the bacteria as a residual population may be due to the water availability in the soil. According to Schober and Zadoks (1999) water activity below the level suitable for bacteria growth but still allowing for survival, occurs frequently in the soil environment.

Previous research into the levels of fluorescent pseudomonads in soil has shown that relatively low numbers of the bacteria were found. Sands and Rovira (1971) reported that fluorescent pseudomonads represented <1% of the total bacterial population in the soil and rhizosphere. Similarly Sands *et. al.* (1972) found that the fluorescent pseudomonads comprised no more than 0.27% of the total bacterial population. In this study levels of fluorescent pseudomonads in the soils of the Victorian growing regions were often higher than this. Although the level of the fluorescent pseudomonads was often found less than 1% in 9 out of 30 sampling periods (10 sampling dates over 3 areas), proportions ranged from 1.27% to 3.6% (Table 3.1). Lower proportions of fluorescent pseudomonads may be due in part to high temperatures and desiccation of the soil and partly to the more advanced stage of decomposition of organic material. These results show that the high variability in total numbers and the widespread distribution of fluorescent pseudomonads in the soil.

Identification of fluorescent pseudomonads and other soil-borne bacteria is often difficult due to the complex nature of the soil environment and the multitude of bacteria within it. Better detection methods need to be developed in order to provide more accurate results of fluorescent pseudomonad levels, not only in the field but in postharvest processing. A review examining the different detection methods available for pathogenic bacteria is included in Appendix 3. As mentioned in the review current methods or combinations of methods, for bacterial identification are often time consuming, cumbersome and costly. For detection systems to be readily accepted and used by the vegetable industry and more specifically the grower, quantitative and identification tests need to be rapid, accurate, accessible for field use and inexpensive.

PART 4: Reduction measures for bacterial control

4.1 Introduction

Essential oils, such as tea tree or eucalyptus and many others have demonstrated their potential use against fungal pathogens and human bacterial pathogens (Ouattara *et al.* 1997; Smith-Palmer *et al.* 1998; Harkenthal *et al.* 1999; Washington *et al.* 1999). The means by which microoorganisms are inhibited by essential oils seems to involve different modes of action. The most frequent mode involves the phenolic components of oils that sensitize the phospholipid bi-layer of the cell membrane, causing an increase of permeability and leakage of vital intracellular constituents or impairment of bacterial enzyme systems (Ouattara *et al.* 1997). Much of the research on the antimicrobial effect of the essential oils has concentrated on human pathogens such as *Listeria monocytongenes* and *Staphylococcus aureus*.

Soil additives such as mulches and composts have shown success with weed and fungal disease suppression. Composts made from organic green waste have also demonstrated success in the horticultural industry with benefits to vegetable yields and quality (Anon, 2000b). Environmental benefits have also resulted from the use of composts and mulches. These have included increasing the level and quality of soil organic matter, pesticide and fertilizer reductions and reduction of water usage during irrigation. The effect of composts on the soil microflora has shown an increase in total aerobic counts due to the nutrient and moisture availability. The bacterial species present is determined by the rate of decomposition of the organic material and the temperature of the compost (Anon 1998).

In order to test the antimicrobial potential of essential oils and determine the benefits of the mulches with respect to product quality, a combination of tea tree and eucalyptus mulches, were tried. No studies have been conducted to evaluate the effect of these types of soil additives on bacteria in the field.

The aims were to:
(a) observe the direct effect of an antimicrobial oil against the browning of cut lettuce.
(b) evaluate the use of mulches to control the level of fluorescent pseudomonads in the field.

4.2 Method

4.2.1 Observation of bacterial reduction on cut lettuce using eucalyptus oil

4.2.1.1 Preparation of bacterial suspensions
The bacteria used in this study was *Pseudomonas fluorescens* (isolated from lettuce, 31/5/99). The bacteria were cultured each week on king's B medium (KB). One loopful of culture was inoculated into a flask of nutrient broth (Difco) and incubated with shaking at 27°C for 48 hours. The concentration of this stock suspension was confirmed by making serial dilutions in peptone buffer containing 0.1% buffered peptone (Merck, Aust.) in deionised water. These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and incubated for 48 hours at 27°C.

4.2.1.2 Eucalyptus oil sachet preparation
Thin tissue paper was immersed in a concentrated eucalyptus oil. Time was given to allow the oil to absorb into the paper then fastened to the inside top of the sterile bag.

4.2.1.3 Lettuce preparation
Crisphead lettuce (*Lactuca sativa* L.) was obtained from a local market (unknown cultivar). Lettuce wrapper and core leaves were discarded and the rest of the leaves were trimmed and cut. Cut leaves were rinsed twice in double deionised water and centrifuged with a manual salad spinner to remove excess water. Pieces were randomly mixed and 50 g lots were placed in sterile bags.

4.2.1.4 Treatment combinations
The cut lettuce was subjected to 4 treatment combinations (Table 4.1)

Table 4.1 Treatment combinations of inoculations and sachet addition

Title	Treatment
Control (-)	Cut lettuce only
Control (+)	Cut lettuce + euc. oil sachet
Infected (-)	Cut lettuce + inoculation
Infected (+)	Cut lettuce + inoculation + euc. oil sachet

4.2.1.5 Bacterial inoculation
An inoculation solution of the bacteria was prepared by dilution of the stock suspension in peptone buffer (0.1%) to make a concentration of 10^4. Approximately 5 mL of the inoculation suspension was aliquoted into the bags of lettuce (+/- eucalyptus oil sachet) and mixed thoroughly for 3-5 minutes. The bag was drained of excess inoculation suspension and stored at 8°C for 48 hours.

4.2.1.6 Assessment for fluorescent pseudomonads and quality
Cut lettuce samples of 50 g were placed into sterile bags and stomached for 2 min in 225mL of sterile peptone buffer containing 0.1% buffered peptone. Serial dilutions were made in peptone buffer (0.1%). These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The detection and quantification of the fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies as an indicator (refer to figure 2.1). Visual quality of the cut lettuce was assessed after 7 days. The rating was conducted according to a 1-9 rating scale described by Kader *et al.* (1973) where 9= excellent, 5 =limit of saleability and 1 =extremely poor.

4.2.2 Use of mulches to reduce bacterial levels in soils of lettuce fields

4.2.2.1 Mulch preparation

Eucalyptus chipbark mulch (Foster mulch, c/- Banksia nursery) and Tea tree mulch (*Melaleuca alternofolia*) (waste material from the oil distillation process, from Port Macquarie, NSW) were used in this study.

4.2.2.2 Treatment combinations

Application of the mulch was conducted at the start of the trial period. The mulch was applied to the top of the soil a few days prior to the lettuce seedling being planted. The grower carried out their usual fertilizer regime for the production of lettuce. Two application rates of 30t/ha and 50t/ha were applied. Table 4.2 shows the treatments used for the trial.

Table 4.2 Treatment combinations of the mulch trial

Treatment No.	Treatment
A	Eucalyptus mulch – 50t/ha
B	Eucalyptus mulch – 30t/ha
C	Tea tree mulch – 50t/ha
D	Control – no mulch
E	Tea tree mulch – 30t/ha

4.2.2.3 Assessment of bacterial levels in soil

Soil was analyzed for bacterial levels prior to the application of the mulch. Weekly soil samples were taken from each treatment plot up to and including the harvest week. Soil samples (six) from each treatment plot were pooled together. From each weekly soil sample 15-20 g was placed into peptone water (0.1%) and agitated for 20 minutes at 150rpm. Serial dilutions of the soil suspension were made in peptone buffer containing 0.1% buffered peptone (Merck, Aust.) in deionised water. These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The quantification of the fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies as an indicator (refer to figure 2.1). Total aerobic bacterial counts were conducted by counting the red bacterial colonies produced on the petrifilm plates.

4.2.2.4 Assessment of lettuce quality

During week 4, week 7 and at harvest (week 8) of the trial, lettuce heads were removed and transported back to the laboratory. Upon arrival the lettuce heads were weighed. Classification of firmness as an indication of maturity for the crisphead lettuce was conducted after harvest (week 8) in accordance to Kader *et al.* 1973 (Table 4.3).

Table 4.3 Rating scale for lettuce head firmness based on hand pressure

Score	Firmness description
1	*Soft*, easily compressed or spongy
2	*Fairly firm*, neither soft nor firm, good head formation
3	*Firm*, compact but yields slightly to moderate pressure
4	*Hard*, compact and solid, does not yield under slight pressure
5	*Extra-hard*, over-mature, may have cracked mid ribs

Assessment of the disorders of the lettuce within the harvest area was conducted during the harvest week. The disorders that were assessed included tipburn, *Sclerotinia* rots, othe rots, damage to the head (i.e. insect) and incomplete formation of the head.

4.2.2.5 Postharvest assessment of fluorescent pseudomonads on lettuce
Leaves were shredded, mixed thoroughly and 50 g was placed into a sterile bag and stomached for 2 min in 225mL of sterile peptone buffer containing 0.1% buffered peptone. Serial dilutions were made in peptone buffer (0.1%). These dilutions were plated onto Petrifilm total aerobic count plates (3M Australia, NSW) and KB medium (Merck, Aust.) and incubated for 48 hours at 27°C. The quantification of the fluorescent pseudomonad species was conducted under ultraviolet light (λ465nm) using the fluorescence of the pigment produced from the bacterial colonies as an indicator (refer to figure 2.1). Total aerobic bacterial counts were conducted by counting the red bacterial colonies produced on the petrifilm plates.

Visual quality rating was according to a 1-9 scale described by Kader *et al.* (1973), where 9= excellent, 5 =limit of salability and 1 =extremely poor.

4.2.2.6 Data analysis
The analysis of the data was conducted using Analysis of covariance (GenStat 5.4.2). Log counts of bacteria were analyzed to meet the assumption of constant variance. The covariate used was a measurement of the $\log_{10}$ cfu/g of fluorescent pseudomonads in the soil prior to planting. Weight, firmness and quality components measured were analyzed using Analysis of variance (GenStat 5.4.2). The least significant difference (LSD) was determined to compare treatments. All tests were at the 5% significance level, unless otherwise stated. Bars on graphs show 1x LSD. (P=0.05). Where data has more than one LSD, the maximum LSD has been shown on the graph for simplicity.

Six replicates of each treatment were set up in a row-column design (6x5). Each treatment appeared in each row and column. Treatment plots were 4 metres long, with the centre 2 metres of lettuce reserved for the harvest (Figures 4.1 & 4.2). Space (50cm) at either end of the plot was left as a buffer region between treatments. Remaining space (50cm) on either side of the harvest region was reserved for lettuce removal during the trial period (week 4).

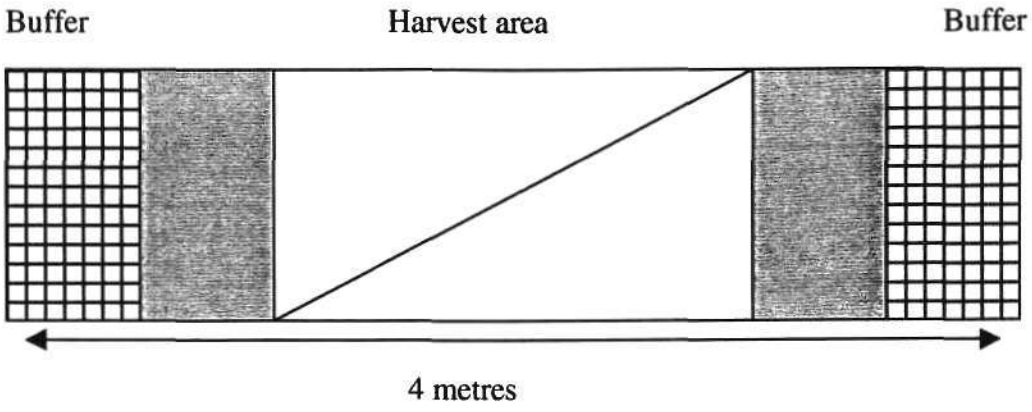


Figure 4.1 Treatment plot arrangement

Figure 4.2 Demonstration of treatment plot

4.3 Results

4.3.1 Observation of bacterial reduction on cut lettuce using eucalyptus oil sachets

The results from this experiment were mainly observational. No statistical analysis was conducted on the quality data due to the lack of the appropriate number of replicates. Observations of the bacterial loads of the cut lettuce showed lower levels of fluorescent pseudomonads on the treatments exposed to the eucalyptus oil sachet (Table 4.4). Cut lettuce exposed to the eucalyptus oil vapour showed a reduction in total aerobic bacteria and fluorescent pseudomonads compared to the non-exposed lettuce. The pseudomonad inoculated lettuce treatments also showed a reduction in

bacterial levels (Table 4.4). However as no statistical analysis was conducted on the data, significant differences cannot be identified.

Quality of the cut lettuce was also noted to be improved by exposure to the oil vapour. No differences were noted between the control treatments, however differences in the quality rating was noted between the inoculated treatments (Table 4.4). Observations of the overall quality noted browning on most of the cut edges of the inoculated lettuce not exposed to the oil vapour, whilst little browning was observed on the exposed inoculated lettuce.

Table 4.4 Observation of the total aerobic bacteria (TAC) (cfu/g) and the fluorescent pseudomonads (Pseudos.) (cfu/g) on cut lettuce exposed to eucalyptus oil vapour after 48 hours (Quality scores: 1=very poor to 9=very good)

Treatment	TAC	Pseudos.	Quality
Control (-)	3.4×10^5	1.95×10^5	9
Control (+)	3.0×10^5	7.0×10^4	9
Infected (-)	1.45×10^8	1.35×10^8	3
Infected (+)	8.8×10^7	7.5×10^7	7

*No statistical analysis conducted
(-) no oil sachet in bag, (+) oil sachet in bag

Another observation was noted when the lettuce was stomached prior to bacterial analysis. The diluent appeared clearer and 'greener' in colour with the treatments exposed to the eucalyptus oil vapour. Whilst the diluent in the non-exposed bags was turbid and light brown in colour. These differences were indicative of the higher bacterial growth and browning exhibited in the non-exposed lettuce treatments.

Observations from this experiment suggest that the use of antimicrobial oil sachets such as eucalyptus may play a role in reducing bacterial levels within the environment of bags during postharvest storage. Further work is needed to ascertain the usefulness of antimicrobial oil sachets aiding product quality, however regulations for the use of these products with food need to be examined.

4.3.2 Use of mulches to reduce bacterial levels in soils of lettuce fields

Before harvest

Tea tree and eucalyptus mulches were applied to soils on a property in the Werribee South region. Mulches were applied prior to planting the lettuce seedlings. The effects of the different mulch treatments on the total bacterial and fluorescent pseudomonad loads were analyzed weekly. Figures 4.3 and 4.4 show the results of the bacterial analysis.

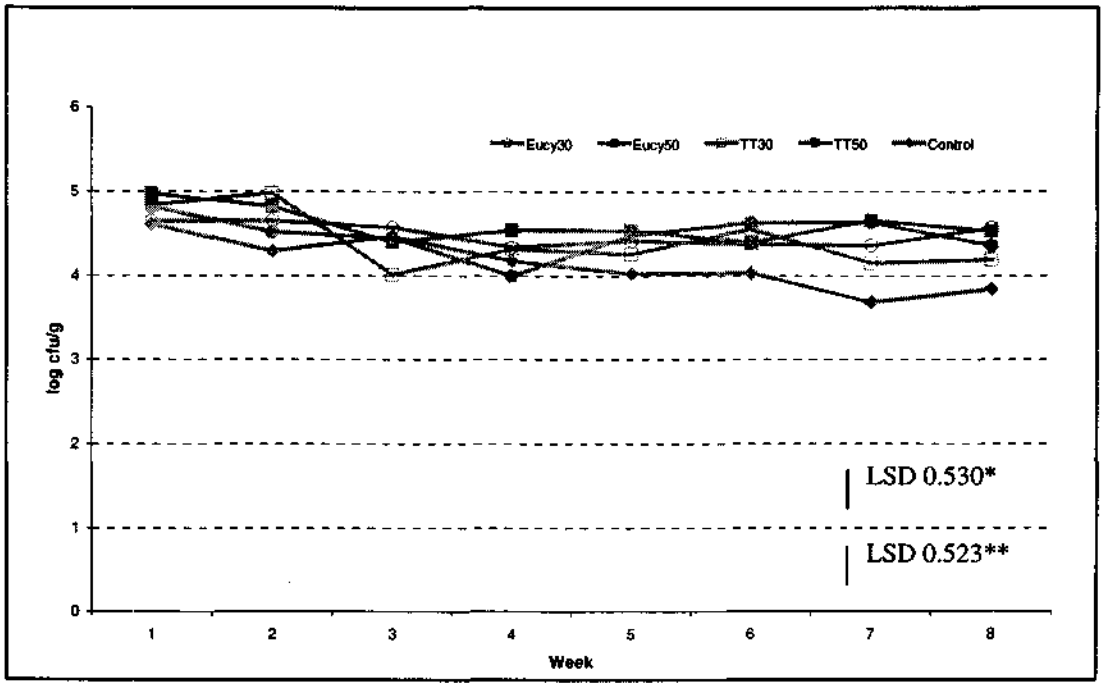


Figure 4.3 Fluorescent pseudomonad levels in soil treated or untreated with mulch over an 8 week lettuce production period [* LSD (P=0.05) for week/mulch/rate/type, ** LSD (P=0.05) when comparing same level of mulch/rate/type]

The average level of fluorescent pseudomonads varied in all treatments over the 8 week period of the trial. The application of the mulch influenced the level of fluorescent pseudomonads with significantly higher levels observed in mulched treatments compared to un-mulched treatments (Figure 4.3). Levels of fluorescent pseudomonads within the treaments remained reasonably constant during the 8 weeks with counts ranging between 1000s cfu/g of soil and 10,000s cfu/g soil. The background level of natural fluorescent pseudomonads had no influence on the results obtained during this trial (P=0.234).

Table 4.5 shows the influence of the mulch and no mulch treatments. Levels of fluorescent pseudomonads were not significantly different between treatments for the first 4 weeks, however levels were significantly different for the last 4 weeks of the trial. This may have been due to the reducing levels of nutrients over time in the unmulched areas compared to the level of nutrients available in the mulched areas. No differences were noted between the mulch application rates of 30t/ha and 50t/ha (P=0.170). There was also no differences noted between the types of the mulches used during the trial (P=0.779).

Table 4.5 Influence of the mulch/no mulch treatments on the average fluorescent pseudomonad levels (log_{10} cfu/g) over 8 weeks (P= 0.033)

Week	Mulch	No mulch
1	4.82	4.58
2	4.74	4.30
3	4.35	4.47
4	4.28	4.22
5	4.43	3.97
6	4.48	4.03
7	4.46	3.62
8	4.39	3.93

The average levels of total aerobic bacteria also varied over the 8 week trial period. Figure 4.4 shows the results of the mulch and no mulch treatments on the bacterial counts. The bacterial loads in the soil for the mulch treatments were similar to each other and the control at the start of the trial. With the application of mulch, the levels slowly rose for the first 3 weeks, then remained fairly constant after this. As can be seen in figure 4.4, no significant difference between treatments during the later weeks was observed. Levels of aerobic bacteria ranged from high 10^7 and low 10^8cfu/g of soil in the early weeks of the trial to high 10^6 and low 10^7 cfu/g soil during the later weeks of the trial, most likely due to the decomposition of the mulch.

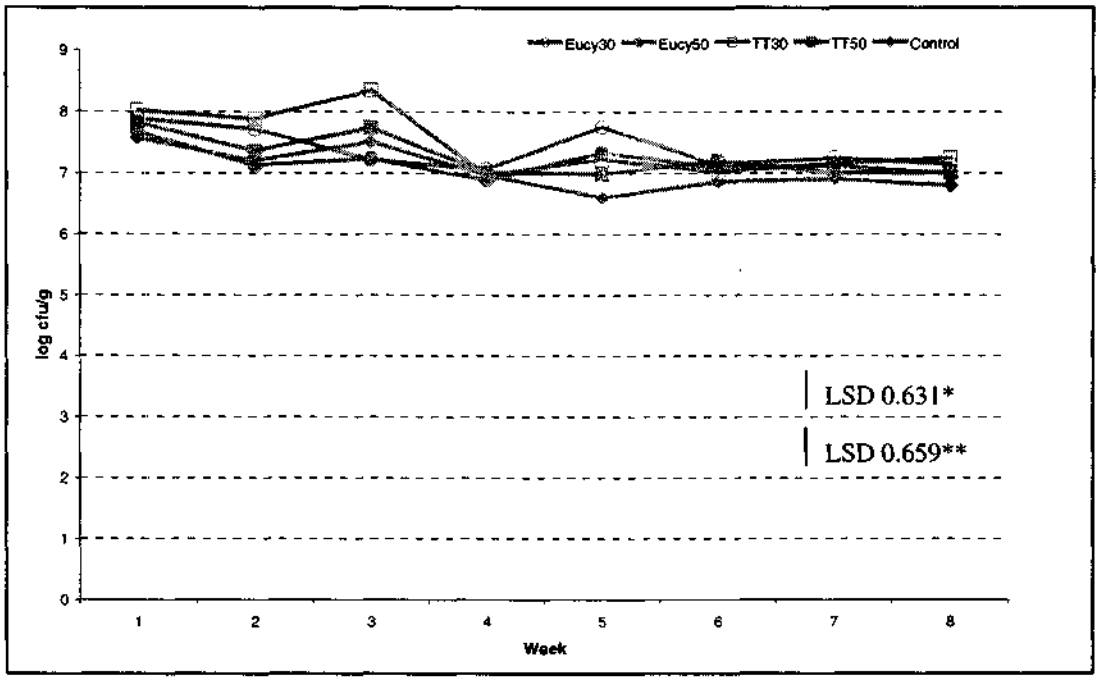


Figure 4.4 Total aerobic bacterial levels in soils treated and untreated with mulches over an 8 week lettuce production period [* LSD (P=0.05) for week/mulch/rate/type, ** LSD (P=0.05) when comparing same level of mulch/rate/type]

As found with the average fluorescent pseudomonad counts, the mulch treatment showed significantly higher aerobic bacterial levels compared to the no mulch treatment during several weeks of the trial (P=0.001). The rate of application of the mulch had an influence on the bacterial counts, as table 4.6 shows, 50t/ha produced lower levels of aerobic bacteria compared to the 30t/ha rate. Though the control treatment showed significantly lower levels than both rates. The effect of the type of mulch used on the aerobic bacteria showed no significance between treatments (P=0.113).

Table 4.6 Effect of application rate of mulch on the total aerobic bacteria (P=0.001) averaged over the 8 week lettuce production period

Treatment	Aerobic bacteria (log cfu/g)
Control	7.05
30t/ha	7.44
50t/ha	7.23

Quality of lettuce before and at harvest

The effect of the treatments on the average weight of lettuce at week 4 is shown in figure 4.5. The average weight of the lettuce head was not significantly affected by the type of the mulch or the application rate (P=0.847; P=0.957). However, the presence of the mulch compared to no mulch was significantly different (P=0.001). The average weight of the lettuce heads grown on mulched soil at week 4 was 34.5 grams, whilst on un-mulched soil it was 24.3 grams.

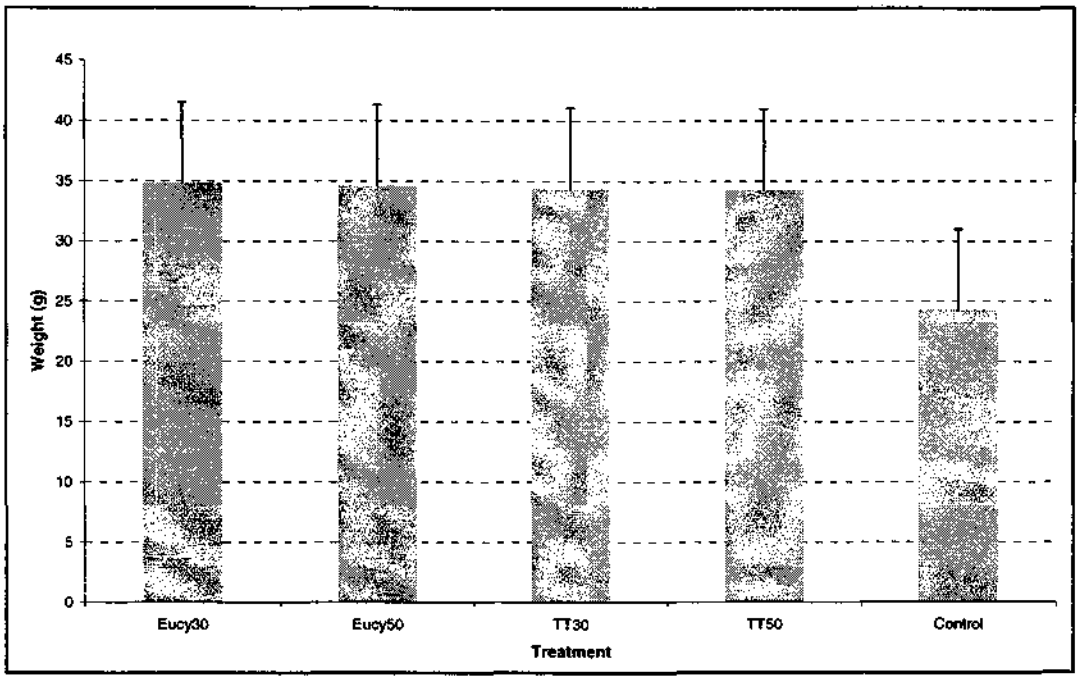


Figure 4.5 Average weight of lettuce head at the 4th week of the production period [bars are 1x maximum LSD (P=0.05)]

The average weight of the lettuce heads was also assessed at week 8 of the trial at harvest. As figure 4.6 shows, the presence or absence of the mulch affected the average weight of the lettuce head at harvest. As was observed at week 4, lettuce grown on mulched soils was significantly heavier than lettuce grown on un-mulched soils (P=0.014). The average weight of the lettuce at harvest, grown on mulched soils was 605 grams whilst lettuce from un-mulched soils was 513 grams. This difference in average lettuce head weight between the mulched and un-mulched treatments showed an average increase of 15% due to the presence of the mulch. No significance between lettuce head weights was noted between the un-mulched control treatment and the 50t/ha of both tea tree and eucalyptus mulch. Significant differences were noted between the other 2 treatments of 30t/ha-tea tree and eucalyptus mulch and the control treatment (Figure 4.6).

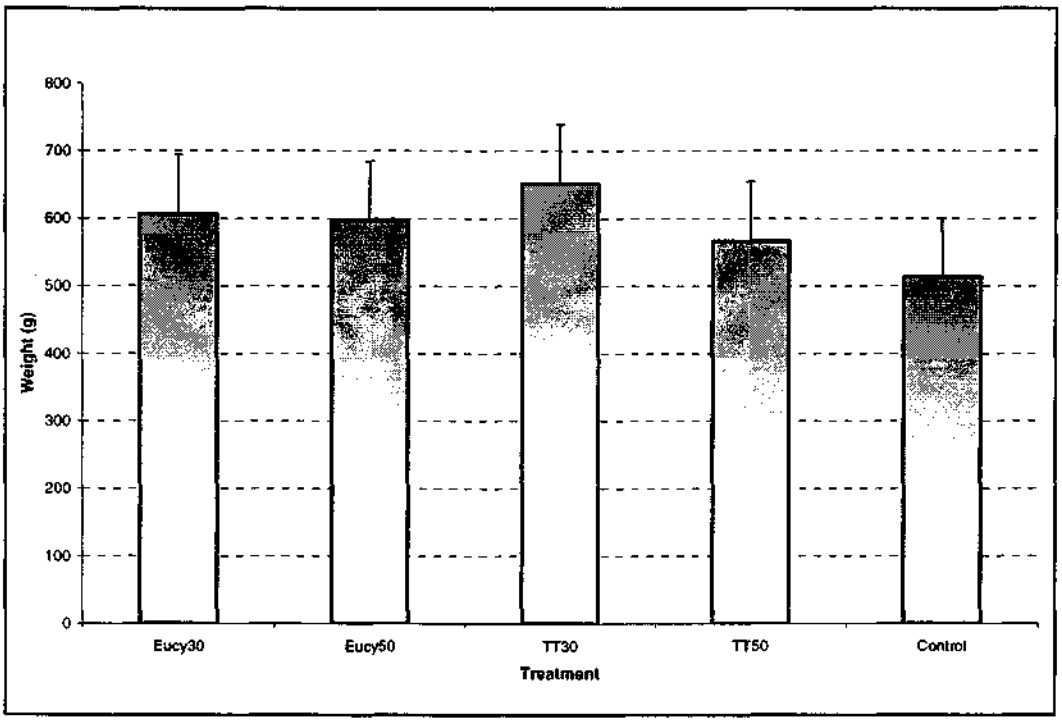


Figure 4.6 Average weight of lettuce heads at harvest (8th week)
[bars are 1x maximum LSD (P= 0.05)]

An assessment of the quality of the lettuce heads at the time of harvest was undertaken as an observation. Table 4.7 shows the different disorders that were noted on the lettuces grown in the soil of the trial area. A large mixture of disorders were noted across the range of treatments including incomplete or abnormal lettuce head formation, holes in the lettuce leaves (usually caused by insects or birds), the presence of soft rots or tissue discolouration under the outer leaves (Varnish spot), tipburn and *Sclerotinia*. No statistical analysis was conducted on the data.

Table 4.7 Number of lettuce heads affected by disorders in the planting area

Disorder	Treatment				
	Control	Eucy 30t/ha	Eucy 50t/ha	Tea tree 30t/ha	Tea tree 50t/ha
Incomplete head	11	6	5	3	2
Damaged –holes, tears	5	12	2	11	14
Soft rot/ Varnish spot	3	6	4	4	6
Tipburn	5	4	2	1	3
Sclerotinia	0	0	8	2	0
Total # unaffected	74	64	55	76	71

Further assessment of the harvested lettuce heads was conducted to observe any differences between treatments. The rating scale used is described in table 4.3 in section 4.2.2.4. The firmness rating between the lettuce grown in the mulched soil shows no significant differences between the different rates and types of mulch applied. However, when compared to the un-mulched treatment, the lettuces from mulched treatments were firmer (P=0.001). The firmness of lettuce grown in mulched soils produced heads which were firm and compact, whilst the average firmness of lettuce grown in un-mulched soils was rated between fairly firm and firm, compact lettuce heads (Table 4.3 & Figure 4.7).

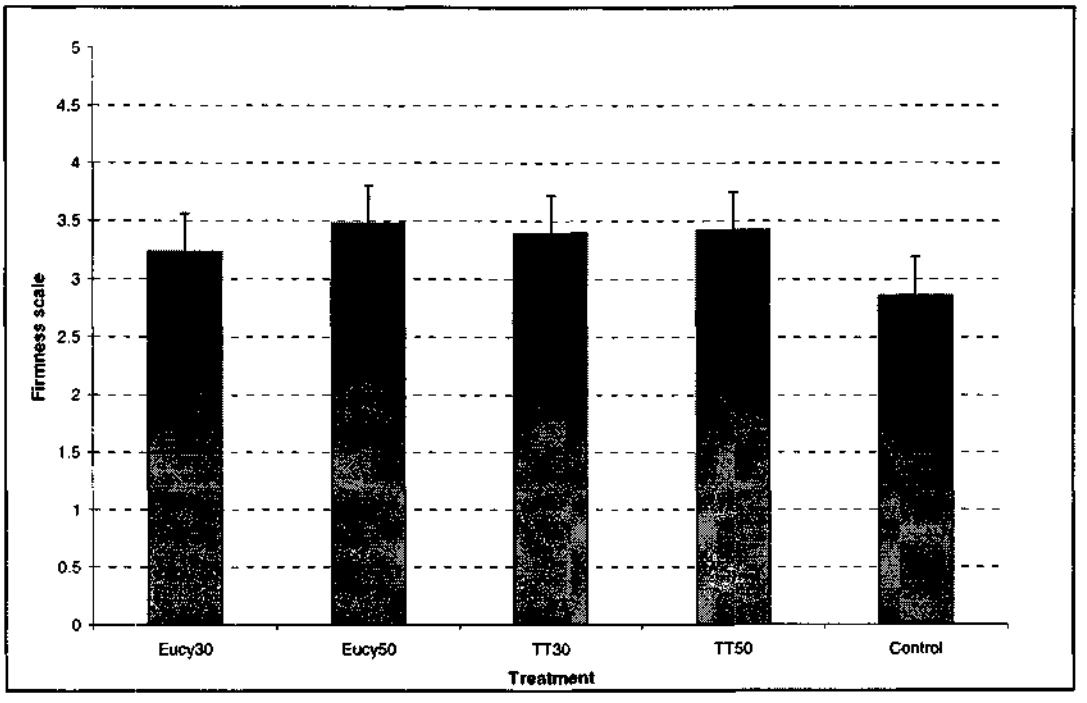


Figure 4.7 Average firmness quality of harvested lettuce heads grown in mulch treated and untreated soil [bars are 1x maximum LSD (P= 0.05)] (Firmness scale – 1 Soft to 5 Extra hard)

Bacterial levels on lettuce at harvest

Levels of fluorescent pseudomonads and total aerobic bacteria were analyzed on the lettuce leaves at harvest to observe any effects of the treatments (Figure 4.8). No significant differences of fluorescent pseudomonads were found between lettuce leaves taken from un-mulched and mulched soils (P=0.420). Fluorescent pseudomonad levels on leaves taken from lettuce grown on tea tree mulch applied at 30t/ha were significantly lower than leaves taken from lettuce grown on the mulch applied at 50t/ha. This may have influenced the overall levels of fluorescent pseudomonads observed between the two rates of applied mulch. Significant differences were noted between the rates of 30t/ha and 50t/ha, with lower levels detected at 30t/ha (P= 0.034).

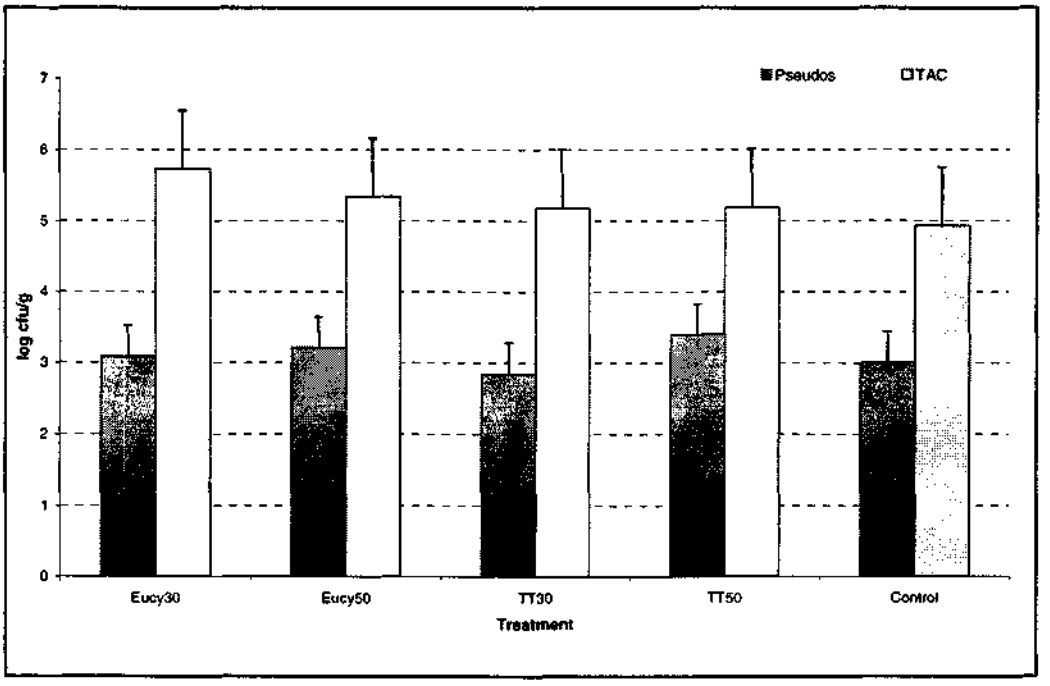


Figure 4.8 Average fluorescent pseudomonad and total aerobic bacteria on lettuce leaves at harvest [bars are 1x maximum LSD (P=0.05)]

Levels of total aerobic bacteria detected on lettuce leaves at harvest showed no significant differences between any of the treatments or for any of the effects (Figure 4.8).

Postharvest storage

Cut-lettuce was stored for 7 days after which time the levels of fluorescent pseudomonads and total aerobic bacteria were counted and the quality of the lettuce were assessed (Figure 4.9). The average levels of fluorescent pseudomonads and total aerobic bacteria are a reflection of the amounts observed before postharvest storage. No significant differences were noted in fluorescent pseudomonad levels on leaves taken from plants grown in either mulched or un-mulched areas (P=0.541). No differences were noted between the types of mulch used (P=0.121). However, as

previously shown in the levels detected at harvest, significant differences were noted between fluorescent pseudomonads on leaves taken from lettuce grown in soil mulched with the two different rates. Again, fluorescent pseudomonads were lower on leaves mulched with 30t/ha compared to 50t/ha (P=0.043). Another observation in Figure 4.9 shows that the levels of fluorescent pseudomonads on leaves taken from lettuce grown in soil mulched with 30t/ha of tea tree mulch were significantly lower than levels detected from 50t/ha tea tree mulch and 30t/ha eucalyptus mulch.

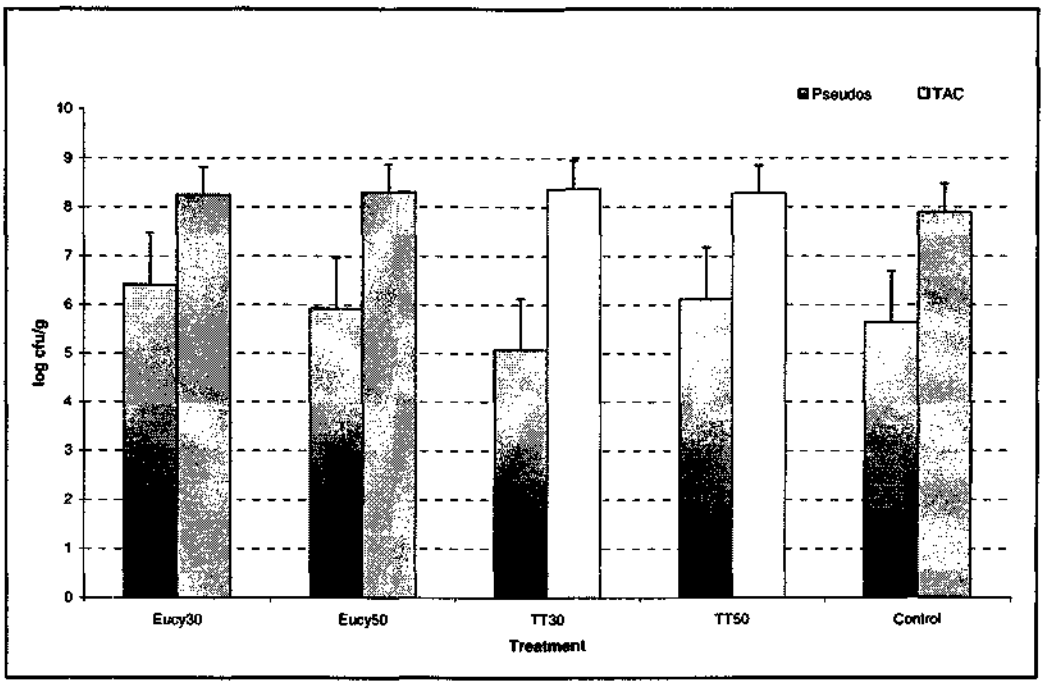


Figure 4.9 Average fluorescent pseudomonads and total aerobic bacteria on lettuce leaves after 7 days storage at 8°C [bars are 1x maximum LSD (P=0.05)]

No significant differences were noted between the mulched and un-mulched treatments of the levels of aerobic bacteria (P=0.077), type of mulch used (P=0.810) and the rates of mulch applied (P=0.917).

The average postharvest quality of the cut-lettuce for all treatments was reasonably poor after 7 days storage at 8°C (Table 4.8). Based on the quality ratings in Table 4.2 all the treatments produced cut-lettuce that was between the fair and the poor scale of quality. No significant differences were noted between the lettuce from the mulched and un-mulched treatments (P=0.052), the types of mulch used (P=0.776) and the rates of mulch application (P=0.772). Overall average quality of the lettuce was considered unsaleable.

Table 4.8 Average postharvest quality of cut lettuce stored at 8°C for 7 days (Quality scores: 1=very poor to 9=very good)

Treatment	Quality
Eucy 30	2.9
Eucy 50	3.1
TT 30	3.1
TT 50	3.2
Control	4.4

In this trial there was no significant treatment effect on the level of fluorescent pseudomonads or on the quality of the lettuce.

4.4 Discussion

As bacterial contamination usually occurs before harvest in the field, reduction methods need to be tailored around monitoring bacterial levels in the field and during postharvest handling. Many methods have been developed to aid the inhibition of browning during postharvest processing including chemical inhibitors such as citric acid, ascorbic acid, modified atmosphere packaging, and hot water dips (Laurila *et al.* 1998; Zagory 1999). The reduction of levels of fluorescent pseudomonads, particularly the pathogenic pseudomonad species in the field may potentially have an impact on postharvest storage life and quality of the whole lettuce heads and fresh-cut products. However, many debates exist on these views.

The use of essential plant oils for control of microbes has been understood for a long time. However, most research conducted has evaluated their effect on fungal species and medical-related bacteria (Ouattara *et al.* 1997; Smith-Palmer *et al.* 1998; Harkenthal *et al.* 1999; Washington *et al.* 1999). The use of essential oils has a potential role in the reduction of plant spoilage bacteria, such as fluorescent pseudomonads in postharvest processing and storage. The exposure of lettuce to eucalyptus oil vapour reduced the quantity of bacteria on the lettuce surface and had a positive effect on the quality of the lettuce. Similar results have been observed with the effect of eucalyptus oil on mushroom quality. The oil vapour was observed to reduce the growth of the bacteria that cause browning of mushrooms, notably fluorescent pseudomonads, therefore increasing the quality of the mushrooms (Jobling 2000). The potential use of plant essential oils could be incorporated into postharvest processing in a number of ways. These include a natural alternative for the washing of selected fresh salad produce to replace or reduce the chlorination of water, possible delivery of the essential oils to the product as a post wash application incorporated into an edible coating, or the application of the oil as a vapour to the product during handling and storage (Wan *et al.* 1998; Jobling 2000).

The benefits of mulches and composts in the landscaping industry have been widely acknowledged. However, it is becoming clear that they also have a large role to play in the horticultural industries (Anon 2000b). With the increasing volumes of composted products not being able to be solely consumed by the home and landscape markets, new areas, such as vegetable production industries are being evaluated (Anon 2000b; Anon 2001).

Mulches have been shown to increase the levels of bacteria in the areas they have been applied in. This is largely due to the availability of water, oxygen, nutrients and soil temperature (Anon 1998; You & Sivasithamparam 1995). The study conducted in this project has shown similar results even though the mulches were made up of components that possess antimicrobial properties when used in concentrated oil forms. The increases in total aerobic bacteria and fluorescent pseudomonad populations in the presence of the tea tree and eucalyptus mulches were most likely due to the organic nature of the plant material and an increase in the availability of nutrients and water. The bacterial population increases would however probably provide some other benefits to the soil environment. You and Sivasithamparam (1995) suggested that the high microbial populations in a plant growth medium could result in the medium becoming disease suppressive. This is due to the competitive nature of the existing populations of bacteria and fungal species for the new niches developed with the mulch application. The mulch may have influenced the pathogenic and saprophytic fluorescent pseudomonad species present at the time of application and during the decomposition over time. This has been observed with fungal species such as *Phytophthora cinnamomi* (You & Sivasithamparam 1995). This suggests that disease severity could be limited by the monitoring and manipulating of the temperature and moisture in the mulch. Our study did not identify the individual species of fluorescent pseudomonads present in the soils of mulched regions. The evaluation of the infectivity of pathogenic bacteria, especially pseudomonad species such as *Pseudomonas marginalis* and *Pseudomonas cichorii* could be analyzed to see if they react in the environment due to the mulches in a similar way.

The application of the mulches provided other benefits for the lettuce plants. Lettuce heads at harvest were on average heavier when grown in mulched areas compared to un-mulched areas. Other studies have shown that the addition of compost onto cauliflower crops resulted in an increase yield of 18% when applied at 50t/ha (Anon, 2000c). Similar increases in lettuce yields were also observed in our studies in both the application rates of the different mulches. These increases in yield of lettuces grown in mulched areas is most likely due to the increase in soil water retention through reducing evaporation and improved water infiltration.
Other benefits of mulch application include the increased organic matter in the soil over time and the potential reduction of fertilizer and pesticide requirements. Both would reduce the potential water and soil damage that is experienced with vegetable production. Furthermore, savings in water usage and the prevention of moisture stress to plants would also provide large savings in costs to the industry (Anon 2001).

To provide a more comprehensive study into the effect of these products and other antimicrobial products such as using essential oil based fungicides/bactericides, further work would be necessary. Oils or soil additives with potential antimicrobial properties, such as eucalyptus and tea tree, could be used to aid the reduction of the pathogenic bacteria either in the field or postharvest. This would not only aid the environment and vegetable industry but would enable the oil distillation industry the opportunity to utilize their waste products into a potentially new market.

Technology transfer

Articles

'A study of bacterial rots and browning in lettuce and the development of control methods' Lettuce Leaflet, October 1999

'Browning of cut lettuce leaves more than a quality issue' Good Food and Vegetables, February 2000

'Browning discovery' The Weekly Times, September 28th 2000

'Rots and browning in lettuce' Lettuce Leaflet, November 2000

'Lettuce gets new life' National Marketplace News, December 2000

'Bacteria Link' Southern Farmer, December 2000

'When can I re-plant?' Vegetable Matters, June 2001

'Soft rots and browning in lettuce' Lettuce leaflet, November 2001

'Controlling Pseudomonas on lettuce before the rot sets in' Good Food and Vegetables, January 2002

Rots and Browning in Lettuce. IHD Annual Review. 1999-2000, 2000-2001

S Pascoe & R Premier (2000) Fluorescent Pseudomonads – contributors to rots and browning in lettuce. Practical Hydroponics and Greenhouses. Nov/Dec 2000. pp.36-39

Conferences

S Pascoe & R Premier (2000) Fluorescent Pseudomonads – contributors to rots and browning in lettuce. Australian Lettuce Industry Conference. 6-8 June 2000. Hay, NSW. (paper & invited speaker)

S Pascoe, B Tomkins & R Premier (2000) Fluorescent Pseudomonads: Contributors to rots and browning in lettuce. NRE horticulture conference. 6-7 September, 2000. Melbourne, Vic. (abstract & poster) (Appendix 1)

S Pascoe, B Tomkins & R Premier (2000) Fluorescent Pseudomonads: Contributors to rots and browning in lettuce. Gympie field day (Qld). 14th November, 2000. (Appendix 1)

S Pascoe, B Tomkins & R Premier (2001) Rapid browning of cut lettuce by Pseudomonas species. 10th Australian Food Microbiology Conference. 28-30 March, 2001. Melbourne, Vic. (abstract & poster) (Appendix 2)

S Pascoe, B Tomkins & R Premier (2001) Assessment of *Pseudomonas* populations on different lettuce cultivars after harvest. Australasian Postharvest Conference. 23-27 September, 2001. Adelaide, SA. (paper & poster) pp 9

Meetings

GSF lettuce growers meeting, Warrigal (Vic.) September, 1999.
Werribee Growers meeting, May 2000.
Scientific seminar, IHD Knoxfield, May, 2000.
JIFSAN (Joint Institute for Food safety and applied Nutrition) (US) meeting

RECOMMENDATIONS

- Testing for fluorescent pseudomonads on the lettuce surface before processing to use as a warning system for lettuce that is likely to go brown.

- Testing for fluorescent pseudomonads on lettuce as an indicator of the effectiveness of browning inhibition treatments, such as heat treatments, antimicrobial washes and chlorination.

- Persistence of fluorescent pseudomonads on lettuce waste indicates that re-planting should be conducted 10-14 days after the last harvest to reduce the chances of bacterial re-infection.

- The use of plant based essential oils pre or post-harvest may be beneficial for the reduction of bacteria on the surface of fresh-cut lettuce (needs further research and development).

- The use of mulches (of any type) are beneficial to lettuce production and increasing yields due to the soil water retention and the improved water infiltration in the field. They could provide large savings on water usage.

- Maintenance of good postharvest conditions such as proper temperature management (<4°C) to improve the overall quality of lettuce and to retard growth of fluorescent pseudomonads.

- Further research
    - efficient, rapid and more applied use of a diagnostic test kit for the detection of fluorescent pseudomonads in the field and after harvest.

    - extensive research needed into the use of antimicrobial oils to reduce the overall bacterial levels in fresh-cut products.

    - further evaluation of the use of mulches to observe the effects on pathogenic bacteria in lettuce and other produce in the field.

    - evaluating the infectivity of particular pathogenic bacteria in the presence of different types of mulches.

    - Further examination of the role of fluorescent pseudomonads in browning by repeating the experiments and analyzing the phenolics component on the cut surface of the lettuce.

Appendix 1: Poster from NRE Horticulture conference (2000) and Gympie field day (2000)

FLUORESCENT PSEUDOMONADS: CONTRIBUTORS TO ROTS AND BROWNING IN LETTUCE

SUSAN PASCOE, BRUCE TOMKINS & ROBERT PREMIER
Institute for Horticultural Development
Private Bag 15, Scoresby Business Centre, Vic 3176

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is an economically important food crop grown worldwide. Demand for quality lettuce in the Australian domestic and export markets is high. Visual quality of lettuce can be threatened by rots and browning, often leading to reduced shelf life and product quality issues with consumers. We believe that the presence of plant pathogenic bacteria may play a role in the elicitation of the plant's defence mechanisms and be a major cause of browning at the cut surface.

FLUORESCENT PSEUDOMONADS

The genus *Pseudomonas* is the predominant bacteria on the surface of the lettuce leaf. *Pseudomonas* species notably the fluorescent pseudomonads species comprise both saprophytic and pathogenic bacteria that produce pectolytic enzymes capable of degrading plant tissue. They are also the main contributors to some field and postharvest disease. As figure 1 shows fluorescent pseudomnads fluoresce when exposed to UV light on particular media, therefore making detection quite easy.

PRESENCE OF FLUORESCENT PSEUDOMONADS IN SOIL

Fluorescent *Pseudomonas* species are distributed widely but unevenly through soil and are largely associated with organic matter. Analyses of soil samples from Werribee and Somerville have shown varying levels of these bacteria. Levels of fluorescent pseudomonads in the soil samples showed a peak around late winter-spring (Figure 3). Declining proportions were observed during summer and autumn.

Figure 1. UV fluorescence of fluorescent pseudomonad

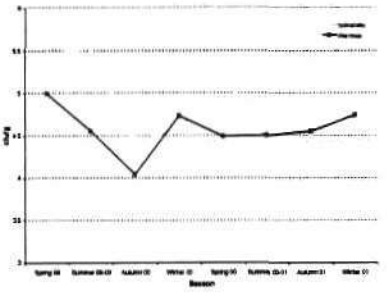


Figure 3. Levels of fluorescent pseudomonads in soils of Somerville & Werribee

Figure 2. Rapid browning of cut lettuce 48 hours after inoculation with *Pseudomonas* sp.

Experiments conducted at Knoxfield have shown that different *Pseudomonas* species can induce browning of cut lettuce within 48 hours (Figure 2). Browning is quite severe and rapid compared with other bacteria such as *Erwinia* and *Xanthomonas* species.

FUTURE WORK

Future work aims to fully understand the ecology of fluorescent pseudomonads in order to develop measures to reduce the levels in the field. The control of fluorescent pseudomonads will result in fewer problems with postharvest quality, thus increasing returns to growers.

Horticulture Australia

This project is funded by the leafy vegetable industry, the HAL and NRE (Victoria). Project VG 98083.

Victoria The Place To Be

Appendix 2: Poster from Australian Food Microbiology conference (2001)

Horticulture Australia

RAPID BROWNING OF CUT LETTUCE BY *PSEUDOMONAS* SPECIES

Victoria The Place To Be

Susan Pascoe, Bruce Tomkins, Peter Franz & Robert Premier
Institute for Horticultural Development, Private Bag 15, Scoresby Business Centre, Victoria, 3176.

INTRODUCTION

Consumers use visual quality as a criterion for selection of lettuce. Rots and browning reduces the visual quality of the produce especially in fresh cut products. Minor discolouration leads to loss of consumer appeal whilst major problems lead to rejection of the product. An investigation into the role of bacteria on rots and browning of cut lettuce is reported here.

Browning by bacteria

A group of bacteria seen to play a role in the elicitation of the browning reaction of cut lettuce surfaces is the fluorescent Pseudomonads. This group makes up to 90% of the total bacterial population on the leaf surface. The population contains saprophytic and pathogenic species that produce pectolytic enzymes capable of breaking down plant tissue and they can be the main contributors to some field and postharvest diseases.

Aim

To determine the role of bacteria in the browning of cut leaf surfaces of lettuce.

METHOD

Cos lettuce plants were germinated and grown in tissue culture conditions for 4 weeks. Plants were removed and the leaves cut and placed in sterile bags. Suspensions of 10^5 bacteria were used for inoculations of the leaf cut surface. Plants were stored at 8°C for 48 hours. Analysis of the quality of the leaves and the number of cut surfaces exhibiting browning were conducted after 48 hours.

RESULTS & DISCUSSION

Figure 1 shows the results obtained for lettuce quality and the number of cut surfaces exhibiting browning. Control lettuce was of good quality with no browning at the leaf cut surface. Lettuce inoculated with *P. fluorescens* and *E. carotovora* showed poor quality and a high number of cut surfaces exhibiting browning (Figure 2).

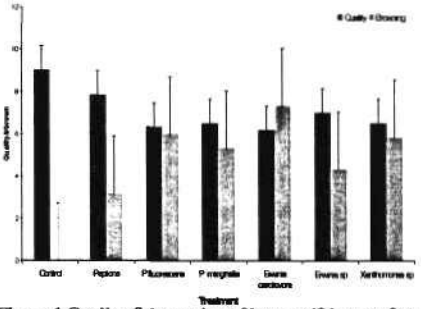


Figure 1 Quality & browning of lettuce 48 hours after bacterial inoculation.

Control (x10)

P.fluorescens (x10)

E. carotovora (x10)

Figure 2 Browning on cut leaf surfaces caused by bacteria (48 hours).

The results indicate that bacteria have an important role in not only rotting the cut surface of lettuce leaves but also in eliciting a browning response. The cut surface of all leaves inoculated with bacteria turned brown whereas the cut surface of leaves that were not inoculated remained free of browning.

Appendix 3

Detection methods for phytopathogenic bacteria

The bacterial agents responsible for diseases in lettuce belong to several different species including *Pseudomonas*, *Erwinia* and *Xanthomonas*. Detection of these phytopathogenic species is undertaken by a variety of methods. Classical diagnosis was carried out using morphological characteristics of the bacteria along with the use of selective media and biochemical tests. These methods are still used however, due to their time consuming preparation and long wait for results, quicker and less cumbersome techniques have been developed. Technological advancements such as serological and molecular techniques have made it easier to routinely identify differences between species and even differences between pathovars.

Biochemical tests and selective media

Nutritional and physiological characteristics have been used in the identification of bacteria. Some of these such as gram reaction, ability to use oxygen as a terminal electron acceptor, presence of cytochrome c oxidase, accumulation of poly-β-hydroxybutyrate, arginine dihydrolase activity and pectate lyase activity are based upon important taxonomic characteristics and have been shown to be quite reliable (Lelliott & Stead 1987). Different species generally react in a similar way whilst individual isolates of a species may often show different nutritional features which can be further identified by other biochemical tests including ability to utilize or produce acid from particular sugars, amino acids etc. (Lelliott & Stead 1987). Lelliott, Billing & Hayward (1966) devised a determinative scheme for the fluorescent pseudomonads. The biochemical tests of levan production, oxidase activity potato rot ability, arginine dihydrolase activity and the tobacco hypersensitivity test (LOPAT system) were found to be adequate enough to differentiate between the species. These tests are routinely used in the identification of these species, including *Pseudomonas fluorescens* and *Pseudomonas marginalis*. Inhibition of growth by 0.1% (and usually 0.02%) tetraphenyltetrazolium chloride (TTC) is usually a good distinctive biochemical test for *Xanthomonas* species (Kreig & Holt 1984).

Preparation of the biochemical tests and incubation times can be laborious and time consuming. Miniaturized identification systems are also used. Such tools are designed to perform several biochemical tests simultaneously and can identify bacteria quite rapidly (Tortora *et al.* 1995). Manufacturers of these mini systems provide charts, tables, coding systems and computer databases for particular test systems and different multi tests are often directed to different groups of bacteria (Holt *et al.* 1994). For example, the API 20E system from bioMerieux has an identification system for Enterobacteriaceae and other gram-negative rods as well as a separate test for non-enteric gram-negative rods. Experiments conducted by Lacroix *et al.* (1995) using the miniaturized test systems found that the BIOLOG system accurately recognized *P. marginalis* and *P. viridiflava*. The API NPT system was efficient for the identification of *P. marginalis*, *P. viridiflava* and *P. syringae* whilst the API 20E system worked reliably with the *Erwinia* species.

The use of selective media has been used routinely in the isolation and classification of plant pathogenic bacteria. Selective media aids the growth of the desired bacteria whilst suppressing the growth of competing species (Tortora *et al.* 1995). The isolation of some *Erwinia* species can be facilitated by the use of selective media. For pectolytic erwinias such are *Erwinia carotovora*, CVP medium containing crystal violet and sodium polypectate is commonly used (Cuppels & Kelman 1973). Soft rot Erwinias were easily recognized by the deep cup-like depressions that were formed in the medium. Other soft rot bacteria such as some fluorescent pseudomonads usually produced shallow and wide depressions distinctively different from the *Erwinia* species. A medium frequently used for direct isolation of fluorescent pseudomonads is medium B of King *et al.* (1954). This medium enhances the fluorescent diffusible pigment (or pyoverdin) which is produced abundantly in media of low iron content and readily fluoresces a green-yellow to blue under UV light (Krieg & Holt 1984).

Further modifications of King's medium B have been developed since the 1970s. Sands & Rovira (1970) exploited the fluorescent pseudomonads resistance to compounds such as penicillin G, novobiocin and cycloheximide to create a more selective media. Modifications of these media are also being developed such is the case with semi selective media development by Gitiatis *et al.* (1997) for the isolation of *P. viridiflava* in onions and selective media for *P. cichorii* by Jones *et al.* (1990). These media use D (-) tartaric acid and L (+) tartrate as a carbon source. For *Xanthomonas* species such as *X. campestris*, a suitable selective media has not been common for identification. Hayward (1979) comments on a medium known as SX agar that has been developed which is based upon the ability of *X. campestris* (from black rot of crucifers) to produce wide zones of starch hydrolysis. In Bergey's manual of Systematic Bacteriology (1984) it is suggested Dye's GYCA medium (glucose, yeast extract, $CaCO_3$ and agar) is useful for the isolation of *Xanthomonas* species because the characteristic yellow pigment (xanthomonadin) is greatly enhanced.

Serology & Fatty acid analysis (FAA)

Serology has become an important tool in diagnostics. This area deals with the response of the bacteria to particular antibodies. Some serological techniques, which are used, include slide agglutination tests, Ouchterlony immunodiffusion precipitation, fluorescent antibody techniques, enzyme linked immunosorbent assays (ELISA) and the use of monoclonal and polyclonal antibodies. A major limitation with this area is the fact that the outer surface of bacterial cells are antigenically heterogenous. Therefore some of the bacterial antigens are quite common between species and pathovars, therefore cross-reactions may frequently be obtained when using polyclonal antisera or non-specific monoclonal antibodies (Lelliott & Stead 1987). Antisera can be made more specific by dilution, in the hope of diluting out cross-reacting antibodies, or with more extensive knowledge of the desired bacterial species targeting more specific bacterial antigens. Monoclonal antibodies can also increase the specificity by being selective to only one type of antigen. In *Pseudomonas* species, *P. aeruginosa* has been studied more thoroughly than any other *Pseudomonas* species. However studies of plant pathogenic pseudomonads have utilized some of these findings to further typing methods (Holt *et al.* 1994). Festl *et al.* (1986) noted that a monoclonal antibody against an outer membrane protein from

P. aeruginosa had been used that was specific for the *P. fluorescens* group and the *Aztobacter* species. However, the antibody described exhibited broad specificity and a moderate cross-reaction with another species. Serotyping methods using the heat stable O antigen represented by the lipopolysaccharide (LPS) have been used in research conducted by Saunier *et al.* (1996). Twenty-three O serogroups were defined for *P. syringae* and *P. viridiflava* strains where various levels of specificity and cross-reactions were compared. Detection of plant pathogenic pseudomonads was also conducted using immunofluorescent antibodies. Hikichi *et al.* (1996) used this serological method to study the infection route of *P. cichorii* into lettuces. The investigation looked at the invasion sites by using fluorescein isothiocyanate (FITC)-conjugated antibody against *P. cichorii* (Hikichi *et al.* 1996).

Serological techniques have also been used in the differentiation and identification of *Erwinia* species. De Boer *et al.* (1979) determined various serogroups for *E. carotovora* whilst serological characterization of potato isolates of *E. carotovora* subsp. *atroseptica* and subsp. *carotovora* were done using polyclonal and monoclonal antibodies (Alarcon *et al.* 1995). The serogroups from each *E. carotovora* subsp. were observed by ELISA and indirect immunofluorescence (IIF) (Alarcon *et al.* 1995). The use of serology in *Xanthomonas* according to Bergey's manual of Systematic Bacteriology (Kreig & Holt 1984) hasn't been too successful. Work with the large numbers of pathovars of *X. campestris* has resulted in multiple groupings and many cross-reactions. Since then serological differences among strains of *X. campestris pv campestris* have been detected with monoclonal antibodies, which divided 200 strains into 6 groups (Alvarez *et al.* 1985). Further research by Alvarez *et al.* (1994) has evaluated more monoclonal antibodies and combined it with the increased specificity of DNA based techniques. The references observed in literature searches largely involved the serological analysis of *X. campestris pv. campestris* and related species. Unfortunately information relating to *X. campestris pv. vitians* was not found. Possibly indicating little to no research conducted on this strain.

Another technique that has become quite valuable in identifying plant pathogenic bacteria is Fatty Acid analysis (FAA) or cellular fatty acid composition. The fatty acid composition of microorganisms has been used extensively to aid microbial characterization. It is thought that FAA is specific enough to differentiate genera, species and in some cases subspecies and can serve as a primary tool or a confirmatory technique for previously identified bacteria (Wells *et al.* 1993). Information about the fatty acid composition of *Erwinia* species, *Pseudomonas* species and *Xanthomonas* species has been published (Wells *et al.* 1993). The fatty acid composition of plant pathogenic bacteria is analysed by gas-liquid chromatography (GLC) to yield fatty acid profiles. These profiles (chromatograms) are compared with other species and with known standards to identify. As Wells *et al.* (1993) explains fatty acid profiles can be affected by culture conditions, physiological age of cells and experimental factors in the laboratory, such as media. Khan *et al.* (1999) showed that by using FAA in combination with other techniques rapid identification of phytopathogens of pear and radish leaves were obtained. Differences in the fluorescent pseudomonads that were studied showed similarities between *P. syringae* pathovars and *P. viridiflava* and similarities in fatty acid composition between *P. marginalis* and *P. fluorescens* (Khan *et al.* 1999). Fatty acid analysis has also examined the similarities and differences in fatty acid composition of *Xanthomonas* species (Yang *et al.* 1993).

Molecular biology techniques

Molecular biology techniques represent the latest technology for definitive identification of phytopathogenic bacteria. Techniques including nucleic acid hybridization, Polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD) techniques and amplified fragment length polymorphic (AFLP) DNA techniques are being used to provide more specific and rapid ways of identifying species and sub species, as well as expanding the phylogenic studies of the bacteria.

Nucleic acid hybridization is based on the ability of nucleic acids to hybridize to specific sites on the DNA or RNA. Because the reactions are highly specific probes will bind to their complementary sequence even if the sequence accounts for a small portion of the target nucleic acid (Schleifer *et al.* 1992). The two approaches to hybridization that have been applied in investigations are dot blot hybridization and *in situ* colony hybridizations. Similar results are viewed from each method but the dot blot method can be quite laborious because nucleic acids have to be extracted prior to hybridization. *In situ* colony hybridization provides a more rapid screening of mixed populations of culturable bacteria because the bacterial colonies are transferred to a membrane filter, cells are lysed and the nucleic acids are bound *in situ* (Festl *et al.* 1986; Schleifer *et al.* 1992). Festl *et al.* (1986) and Schleifer *et al.* (1992) used probes derived from 16s and 23s rRNA of *P. aeruginosa* to detect the *P. fluorescens* group, through hybridization methods. Lübeck *et al.* (2000) used the fluorescence *in situ* hybridization (FISH) techniques using 16s rRNA target oligonucleotide probes to detect *Pseudomonas* species in soil and plant roots.

RNA and DNA probes have also been used in various hybridization experiments to study bacterial release and identification in the environment (Braun-Howland *et al.* 1993; Boye *et al.* 1995) and to observe the DNA relatedness between different *Pseudomonas* pathovars (Gardan *et al.* 1999). Detection of *Xanthomonas* species using DNA and RNA hybridization techniques has also been applied. Hartung and Pruvost (1996) used a cloned DNA fragment as a biotin labeled hybridization probe for detection of a *Xanthomonas campestris* pathovar. Palleroni *et al.* (1997), Jones *et al.* (2000) and Rademaker *et al.* (2000) investigated the taxonomic relationship and relatedness between *Xanthomonas* species and pathovars. Also Kwon *et al.* (1997) investigated the phylogenetic relationships of some *Erwinia* species using sequences of the 16s rRNA genes in hybridization studies.

Another molecular technique that is increasingly common in the identification of phytopathogenic bacteria is the Polymerase Chain Reaction (PCR). This procedure involves the amplification of a specific portion of DNA/RNA where the borders of the region are known (primers). PCR is carried out through a series of cycles which denature the DNA/RNA, anneals the primers, synthesizes a new strand then proceeds to the next stage of denaturing, annealing and synthesizing (Brown 1990). PCR is highly specific, rapid and can detect minute levels of nucleotide sequences, where some techniques require large numbers of bacteria for an accurate identification (Audy *et al.* 1996; Llop *et al.* 1999). PCR based assays have been used in the detection of *X. campestris* pathovars and *P. syringae* pathovars in diseases of beans (Audy *et al.* 1996). Also genes encoding unusual characteristics in bacteria have used PCR detection systems to identify bacteria. For example, Verhagen *et al.* (1995)

identified various *Pseudomonas* species such as *P. cichorii* and *P. fluorescens* through PCR amplification of a gene encoding the ability to degrade dichloropropene (a nematocide) in soils.

In *Xanthomonas* species, PCR has been used to identify and detect the diversity between *X. campestris* pathovars (Alvarez *et al.* 1994) and to amplify rRNA genes in studies researching ribosome-binding sites (Lin & Tseng 1997). In the identification of *Erwinia* species PCR has been used to amplify genes specific to *Erwinia carotovora* pathovars. Using a sequenced pectate lyase gene (pel gene) primers were designed to amplify a 434 bp fragment in different strains (Darrasse *et al.* 1994) The PCR method was also used to detect the *E. carotovora* subsp. *atroseptica* (potato pathogen) (Hyman *et al.* 2000) and to aid in the study of differential gene expression in *E. carotovora* subspecies *atroseptica* and *carotovora* (Dellagi *et al.* 2000).

Using the PCR system random amplified polymorphic DNA (RAPD) techniques have been developed to aid in the genetic analysis of bacterial DNA. This technique is based on the enzymatic amplification of non-selected DNA fragments, initiated by arbitrarily chosen DNA primers. Even though this technique is rapid, simple and inexpensive complex profiles may be generated by RAPD primers making their analysis difficult (Parent *et al.* 1996). Selection of primers is often critical to produce reproducible, clear and useful RAPD patterns. Parent *et al.* (1996) used RAPD analysis to identify *E. carotovora* species and to look for differences between *Erwinia* and *Pseudomonas* species (including *P. fluorescens, P. marginalis and P. viridiflava*). RAPD fingerprinting procedures have also been used to assess genetic diversity within subspecies of *P. syringae* (Clerc *et al.* 1998) and have been used as DNA probes in hybridization experiments to detect *P. putida* in the rhizosphere (Hansen & Winding 1997).

A recent addition to the PCR based detection systems which is becoming widely used in bacterial taxonomy is AFLP or the amplified fragment length polymorphic technique. AFLP has major advantages over other PCR based fingerprinting techniques. It is fast, highly sensitive, requires no prior sequence knowledge and gives access to a very large range of polymorphisms due to access to the complete genome. AFLP is based on the selective amplification of genomic restriction fragments by PCR to differentiate bacterial strains even to the biovar level (Janssen *et al.* 1996). In an evaluation of this method total genomic DNA from the bacteria being analysed was digested with particular restriction enzymes and the resulting fragments were ligated to specific adaptors which served as primer binding sites. These sites allowed the fragments to be amplified by selective PCR primers that extended beyond the restriction site sequence. The resulting amplified products could be visualized on an autoradiograph due to the radioactive labeling of one of the primers (Janssen *et al.* 1996). *Pseudomonas* and *Xanthomonas* species were subjected to AFLP fingerprinting techniques to differentiate highly related strains (Janssen *et al.* 1996). AFLP fingerprinting techniques have been used to assess genetic diversity and relatedness in *P. syringae* subspecies (Clerc *et al.* 1998) and in *Xanthomonas* species (Rademaker *et al.* 2000). Finally AFLP has been used in studies of differential gene expression of *Erwinia carotovora* subspecies (Dellagi *et al.* 2000).

Conclusion

A great array of techniques have been used to isolate, identify and study phytopathogenic bacteria. Early techniques focused on the differences of nutritional and physiological characteristics between bacteria in order to separate them into different groups. New methods have concentrated on the molecular and immunological differences and similarities between bacteria. PCR based techniques such as RAPD and AFLP have exposed the genetic variations that exist between genera, species and closely related species, subspecies, biovars and pathovars. All of the different detection systems from the use of selective media and biochemical testing through to the molecular based techniques are currently being used in laboratories all over the world. They are often used as confirmatory tools in conjunction with 1 or 2 of the other methods. Even though detection methods are more specific and rapid, problems still arise with cost, ease of preparation and clarity of analysis, however as our knowledge and technology develops inconveniences such as these will be minimized and possibly eliminated.

References for the Appendix

Alarcon B, Gorris MT, Cambra M, Lopex MM (1995) Serological characterization of potato isolates of *Erwinia carotovora ssp atroseptica* and *carotovora* using polyclonal and monoclonal antibodies. *Journal of Applied Bacteriology* **79**(6), 592-602.

Alvarez AM, Benedict AA, Mizumoto CY (1985) Identification of Xanthomonads and grouping of strains of *Xanthomonas campestris* pv *campestris* with monoclonal antibodies. *Phytopathology* **75**, 722-728.

Alvarez AM, Benedict AA, Mizumoto CY, Hunter Gabriel DW (1994) Serological, pathological and genetic diversity among strains of *Xanthomonas campestris* infecting crucifers. *Phytopathology* **84**, 1449-1457.

Audy P, Braat CE, Saindon G, Huang HC, Laroche A (1996) A rapid and sensitive PCR based assay for concurrent detection of bacteria causing common and halo blights in bean seed. *Phytopathology* **86**(4), 361-366.

Boye M, Ahl T, Molin S (1995) Application of a strain specific rRNA oligonucleotide probe targeting Pseudomonas fluorescens AG1 in a Mesocosm study of bacterial release into the environment. *Applied and Environmental Microbiology* 61(4), 1384-1390.

Braun-Howland EB, Vescio PA, Nierzwicki-Bauer SA (1993) Use of a simplified cell blot technique and 16s rRNA directed probes for identification of common environmental isolates. *Applied and Environmental Microbiology* **59**(10), 3219-3224.

Brown TA (1990) Gene cloning: An introduction. 2nd Edition. Chapman and Hall. London.

Clerc A, Manceau C, Nesme X (1998) Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology* **64**(4), 1180-1187.

Cuppels D, Kelman A (1973) Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. *Phytopathology* **64**, 468-475.

Darrasse A, Priou S, Kotorjansky A, Bertheau Y (1994) PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato disease. *Applied and Environmental Microbiology* **60**(5), 1437-1443.

De Boer SH, Copeman RJ, Vruggink H (1979) Serogroups of *Erwinia carotovora* potato strains determined with diffusible somatic antigens. *Phytopathology* **69**, 316-319.

Dellagi A, Birch PRJ, Hielbronn J, Lyon GD, Toth IK (2000) cDNA-AFLP analysis of differential gene expression in the prokaryotic plant pathogen *Erwinia carotovora*. *Microbiology* **146**(1), 165-171.

Festl H, Ludwig W, Schleifer KH (1986) DNA hybridization probe for the *Pseudomonas fluorescens* groups. Applied and Environmental *Microbiology* **52**(5), 1190-1194.

Gardan L, Shafik H, Belouin S, Broch R, Grimont F, Grimont PAD (1999) DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *International Journal of Systematic Bacteriology* **49**(2), 469-478.

Gitaitis R, Sumner D, Gay D, Smittle D, McDonald G, Maw B, Johnson III WC, Tollner B, Hung Y (1997) Bacterial streak and bulb rot of Onion: I. A Diagnostic medium for the semiselective isolation and enumeration of *Pseudomonas viridiflava*. *Plant Disease* **81**(8), 897-900.

Hansen BM, Winding A (1997) Detection of *Pseudomonas putida* B in the rhizosphere by RAPD. *Letters in Applied Microbiology* **24**(4), 249-252.

Hartung JS, Pruvost O (1996) Detection of *Xanthomonas campestris pv. citri* by hybridization and polymerase chain reaction assays. *Biotechnological Advances* **14**(3), 296.

Hayward AC (1979) Isolation and characterization of *Xanthomonas*. In: Identification methods for Microbiologists (2nd Ed) (Eds. Skinner FA, Lovelock DW) Academic Press, London. pp 15-30.

Hikichi Y, Saito A, Suzuki K (1996) Infection sites of *Pseudomonas cichorii* into head leaf of lettuce. *Annual Phytopathological Society of Japan* **62**, 125-129.

Hyman LJ, Birch PRJ, Dellagi A, Avrova AO, Toth IK (2000) A competitive PCR-based method for the detection and quantification of *Erwinia carotovora subsp. atroseptica* on potato tubers. *Letters in Applied Microbiology* **30**(4), 330-335.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (eds) (1994) Bergey's manual of Determinative Bacteriology. 9th edition. Williams and Wilkins. Baltimore.

Janssen P, Coopman R, Huys G, Swings J, Bleeker M, Vos P, Zabeau M, Kersters K (1996) Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* **142**(7), 1881-1893.

Jones JB, Randhawa PS, Sasser M (1990) Selective isolation of *Pseudomonas cichorii* from soil and from leaves and buds of *Dendranthema grandiflora*. *Plant Disease* **74** (4), 300-303.

Jones JB, Bouzar H, Stall RE, Almira EC, Roberts PD, Bowen BW, Sudberry J, Strickler PM, Chun J (2000) Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. International *Journal of Systematic and Evolutionary Microbiology* **50**(3), 1211-1219.

Khan AA, Furuya N, Matsumoto M, Matsuyama N (1999) Trial for rapid identification of pathogens from blasted pear blossoms and rotted radish leaves by the direct colony TLC and whole cellular fatty acid analysis. Journal of the Faculty of Agriculture, Kyushu University **43**(3-4), 327-335.

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

Krieg NR, Holt JG (eds) (1984) Bergey's manual of Systematic Bacteriology Volume 1. Williams and Wilkins, Baltimore.

Kwon SW, Go SJ, Kang HW, Ryu JC, Jo JK (1997) Phylogenetic analysis of *Erwinia* species based on 16s rRNA gene sequences. *International Journal of Systematic Bacteriology* **47**(4), 1061-1067.

Lacroix M, Vezina L, Desjardins S, Beaulieu C (1995) Comparison of identification methods for *Erwinia* and *Pseudomonas* species causing soft rot diseases. *Phytoprotection* **76**(1), 27-37.

Lelliott RA, Billing E, Hayward AC (1966) A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of applied Bacteriology* **29**(3), 470-489.

Lelliott RA, Stead DE (1987) Methods in Plant Pathology Volume 2. Methods for the diagnosis of bacterial diseases of plants. Blackwell Scientific Publications, Oxford.

Lin NT, Tseng YH (1997) Sequence and copy number of the *X. campestris* gene encoding 16s rRNA . *Biochemical and Biophysical research communications.* **235**(2), 276-280.

Llop P, Caruso P, Cubero J, Morente C, Lopez CC (1999) A simple procedure for efficient routine detection of pathogenic bacteria in plant materials by Polymerase Chain Reaction. *Journal of Microbiological methods* **37**, 23-31.

Palleroni NJ, Hildebrand DC, Schroth MN, Hendson M (1993) Deoxyribonucleic acid relatedness of 21 strains of *Xanthomonas* species and pathovars. *Journal of Applied Bacteriology* **75**(5), 441-446.

Parent JG, Lacroix M, Page D, Vezina L (1996) Identification of *Erwinia carotovora* from soft rot diseased plants by Random amplified polymorphic DNA (RAPD) analysis. *Plant Disease* **80**, 494-499.

Rademaker JLW, Hoste B, Louws FJ, Kersters K, Swings J, Vauterin L, Vauterin P, De Bruijn FJ (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology* **50**(2), 665-677.

Sands CC, Rovira AD (1970) Isolation of fluorescent Pseudomonads with a selective media. *Applied Microbiology* **20**(3), 513-514.

Saunier M, Malandrin L, Samson R (1996) Distribution of *Pseudomonas syringae* pathovars into twenty-three O serogroups. *Applied and Environmental Microbiology* **62**(7), 2360-2374.

Schleifer KH, Amann R, Ludwig W, Rothemund C, Springer N, Dorn S (1992) Nucleic acid probes for the identification and in situ detection of Pseudomonads. In: Pseudomonas: Molecular biology and bitoechnology. (Eds. Galli E, Sliver S, Witholt B) American Society for Microbiology, Washington DC. pp127-134.

Tortora G.J, Funke GR, Case CL (1995) Microbiology: An Introduction (5th edition) The Benjamin/Cummings Publishing Company, Inc. California.

Verhagen C, Smit E, Janssen DB, Van Elsas JD (1995) Bacterial dichloropropene degradation in soil: screening of soils and involvement of plasmids carrying the dhlA gene. *Soil Biology and Biochemistry* **27**(12), 1547-1557.

Wells JM, Butterfield JE, Revear LG (1993) Identification of bacteria associated with postharvest disease of fruit and vegetables by cellular fatty acid composition: An expert system for personal computers. *Phytopathology* **83**, 445-455.

Yang P, Vauterin L, Vancanneyt M, Swings J, Kersters K (1993) Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology* **16**(1), 47-71.

References for the Final report

Anon. (1998) Guide to Best Practice: Composting green organics. A guide for achieving best practice in Victoria. Natural Resources and Environment and EcoRecycle Victoria (Ed. L Chaplin).

Anon. (2000a) ExpHORT Publication No. 87 Seven vegetables (7 veg) Final report. (Eds. J Faragher, W Morgan, S Barry, R Dimsey, B Ashcroft). Department of Natural Resources and Environment, Victoria. High value horticulture initiative.

Anon (2000b) Green organics: risks, best practice and use in horticulture. A report on the IHD green organics research program, 1995-99. (Eds. K Wilkinson, S Tymms, V Hood, E Tee, I Porter).

Anon (2000c) Green waste benefits cauliflower growers. In: Good fruit and Vegetables, July 2000

Anon (2001) Looking at compost as a boost to horticulture production. In: Good Fruit and Vegetables, May 2001 p 6.

Barriga MI, Trachy G, Willemot C, Simard RE (1991) Microbial changes in shredded iceberg lettuce stored under controlled atmospheres. *Journal of Food Science* **56**(6), 1586-1599.

Behrsing J, Winkler S, Franz P, Premier R (2000) Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharvest Biology and Technology* **19**, 187-192.

Bolin HR, Stafford AE, King Jr AD, Huxsoll CC (1977) Factors affecting the storage stability of shredded lettuce. *Journal of Food Science* **42**(5), 1319-1321.

Brocklehurst TF, Lund B M (1981) Properties of pseudomonads causing spoilage of vegetables stored at low temperatures. *Journal of Applied Bacteriology* **50**, 259-266.

Castañer M, Gil MI, Artes F, Thomas-Barberan FA (1996) Inhibition of browning of harvested head lettuce. *Journal of Food Science* **61**(2), 314-316.

Couture R, Cantwell MI, Ke D, Saltveit Jr. ME (1993) Physiological attributes related to quality attributes and storage life of minimally processed lettuce. *Hortscience* **28**(7), 723-725.

Cuppels D, Kelman A (1973) Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. *Phytopathology* **64**, 468-475.

Cuppels D, Kelman A (1980) Isolation of pectolytic fluorescent pseudomonads from soil and potatoes. *Phytopathology* **70**, 1110-1115.

Delaquis PJ, Stewart S, Toivonen PMA, Moyls LA(1999) Effect of warm, chlorinated water on the microbial flora of shredded lettuce. *Food Resources International* **32**, 7-14.

Frisina C, Hutchins J, Morris C, Tomkins B, Premier R (1997) The isolation of *Pseudomonas fluorescens* and its effect on the shelf life of minimally processed lettuce. Proceedings of the 1997 Australian Postharvest Horticulture Conference. Hawkesbury, NSW. pp 106.

Harkenthal M, Reichling J, Geiss H-K, Saller R (1999) Comparative study on the in vitro antibacterial activity of Australian tea tree oil, cajuput oil, niaouli oil, manuka oil, kanuka oil, and eucalyptus oil. *Pharmazie* **54**(6), 460-463.

Hikichi Y, Saito A, Suzuki K (1996) Infection sites of *Pseudomonas cichorii* into head leaf of lettuce. *Annual Phytopathology Society of Japan* **62**, 125-129.

Jay JM (1996) Fresh and fermented fruit and vegetable products. In: Modern Food Microbiology (Ed: JM Jay) Chapman & Hall, New York. pp 149-176.

Jobling J (2000) Oils: the essence of pest control? Good Fruit and Vegetables. August 2000. pp 50.

Kader AA, Lipton WJ, Morris LL (1973) System for scoring quality of harvested lettuce. *Hortscience* **8**(5), 408-409.

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

Laurila E, Kervinen R, Ahvenainen R (1998) The inhibition of enzymatic browning in minimally processed vegetable and fruits. *Postharvest News and Information* **9**(4), 53N-63N.

Lelliott RA, Billing E, Hayward AC (1966) A determinative scheme for the fluorescent plant pathogenic pseudomonads. *Journal of Applied Bacteriology* **29**,470-489.

Loaiza-Velarde JG Saltveit ME (2001) Heat shocks applied either before or after wounding reduce browning of lettuce leaf tissue. *Journal of American Society of Horticultural Science* **126**(2), 227-234.

Michelmore RW (1997) Applications of biotechnology to disease resistance in lettuce. In: Compendium of lettuce diseases. (eds. Davis RM, Subbarao KV, Raid R N, Kurtz EA) pages 11-13.

Morris CE, Nguyen-The C (1996) The role of plant surface bacteria in the hygienic and market quality of minimally processed vegetables. In: Aerial Plant Surface Microbiology. (eds. Morris CE, Nicot PC, Nguyen-The C) Pages 191-208.

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

Nguyen-The C, Prunier JP (1989) Involvement of pseudomonads in deterioration of 'ready-to-use' salads. *International Journal of Food Science and Technology* **24**, 47-58.

Ouattara B, Simard RE, Holley RA, Piette GJ-P, Bégin A (1997) Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. *International Journal of Food Microbiology* **37**, 155-162.

Patterson CL, Grogan RG, Campbell RN (1986) Economically important diseases of lettuce. *Plant Disease* **70**(10), 982-987.

Rovira AD, Sands DC (1971) Fluorescent pseudomonads – a residual component in the soil microflora? *Journal of Applied Bacteriology* **34**(1), 253-259.

Sands DC, Hankin L (1975) Ecology and physiology of fluorescent pectolytic pseudomonads. *Phytopathology* **65**, 921-924.

Sands DC, Hankin L, Zucker M (1972) A selective medium for pectolytic fluorescent pseudomonads. *Phytopathology* **62**, 998-1000.

Sands CC, Rovira AD (1970) Isolation of fluorescent pseudomonads with a selective medium. *Applied Microbiology* **20**(3), 513-514.

Sands DC, Rovira AD (1971) *Pseudomonas fluorescens* Biotype G, the dominant fluorescent pseudomonad in South Australian soils and wheat rhizospheres. *Journal of Applied Bacteriology* **34**(1), 261-275

Schober BM, Zadoks JC (1999) Water and temperature relations of soft rot bacteria: growth and disease development. *Annal of applied Biology* **134**, 59-64.

Smith-Palmer A, Stewart J, Fyfe L (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology* **26**(2), 118-122.

Stanier RY, Palleroni NJ, Doudoroff M (1966) The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* **43**,159-292

Tomkins B, Frisina C, Murdoch C, Hopkins F, Wilkinson I (2001) Effect of cultivar on the storage life of whole and fresh-cut cos and crisphead lettuce. Australasian postharvest conference 23-27 September, 2001. Adelaide, South Australia. pp 69

Wan J, Wilcock A, Coventry MJ (1998) The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *Journal of Applied Microbiology* **84**, 152-158.

Washington WS, Engleitner S, Boontjes G, Shanmuganathan N (1999) Effect of fungicides, seaweed extracts, tea tree oil, and fungal agents on fruit rot and yield in strawberry. *Australian Journal of Experimental Agriculture* **39**, 487-94.

You MP, Sivasithamparam K (1995) Changes in microbial populations of an avocado plantation mulch suppressive of *Phytophthora cinnamomi. Applied Soil Ecology* **2**, 33-43.

Zagory D (1999) Effects of post-processing handling and packaging on microbial populations. *Postharvest Biology and Technology* **15**, 313-321.