



*Know-how for Horticulture™*

## **Postharvest bacterial rots and browning in lettuce**

Sue Pascoe,  
Bruce Tomkins and  
Robert Premier

Department of  
Natural Resources  
and Environment  
Victoria

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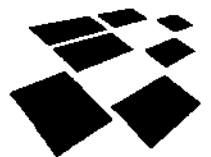
Sydney NSW 2000

Telephone: (02) 8295 2300

Fax: (02) 8295 2399

E-Mail: [horticulture@horticulture.com.au](mailto:horticulture@horticulture.com.au)

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## 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.

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## **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 carotovora* (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:

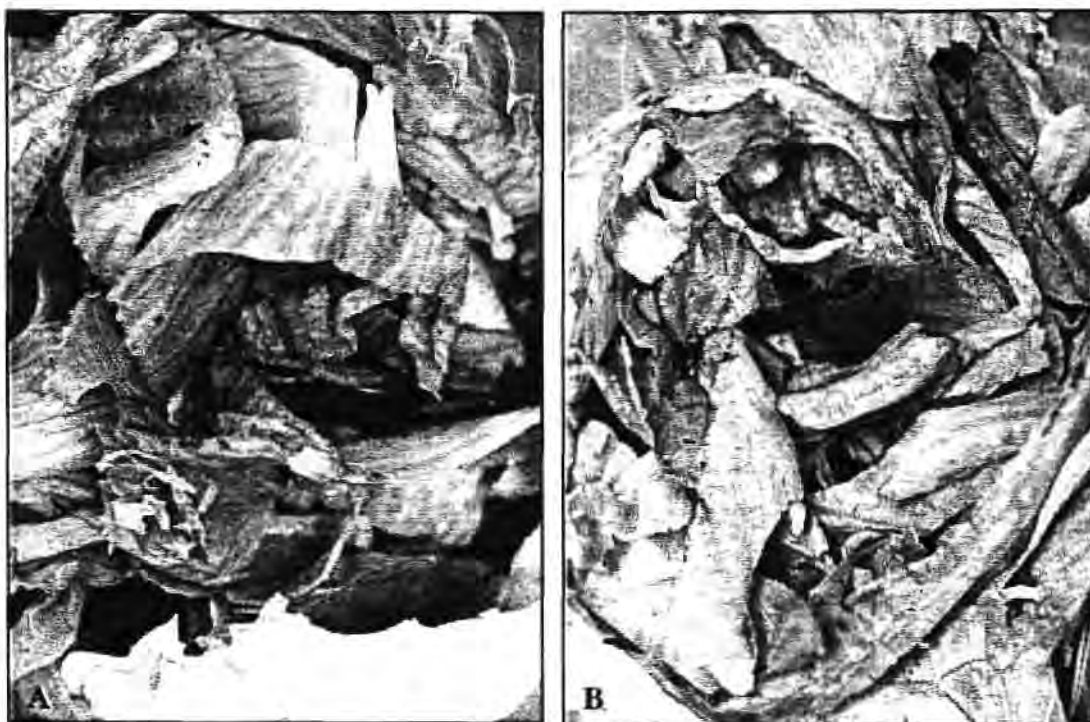
- a) observe the level of fluorescent pseudomonads on cut lettuce in postharvest conditions
- b) evaluate the influence of bacteria on the browning of the cut surface of lettuce
- c) assess the browning reaction and quality of the lettuce caused by fluorescent pseudomonads in postharvest conditions.

### **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.



**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 to 14 days.

Other conditions that favour the growth of bacteria on the surface of vegetables include high temperatures, overmature plants and injurious levels of CO<sub>2</sub>. 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 *Pseudomonas* 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). Nguyen-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<sub>10</sub> cfu/g, whilst water alone reduced numbers by 1.5-1.8 log<sub>10</sub> 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.

### 3.4 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 microorganisms 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 monocytogenes* 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.

**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 28<sup>th</sup> 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). 14<sup>th</sup> November, 2000. (Appendix 1)

S Pascoe, B Tomkins & R Premier (2001) Rapid browning of cut lettuce by Pseudomonas species. 10<sup>th</sup> 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 pseudomonads 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 2. Rapid browning of cut lettuce 48 hours after inoculation with *Pseudomonas* sp.



Figure 1. UV fluorescence of fluorescent pseudomonad

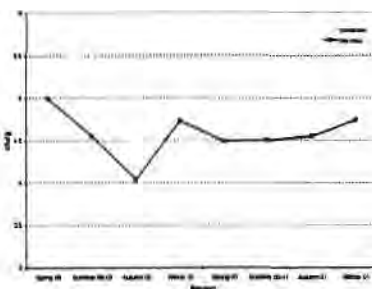


Figure 3. Levels of fluorescent pseudomonads in soils of Somerville & Werribee

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.



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




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



Horticulture Australia

## RAPID BROWNING OF CUT LETTUCE BY *PSEUDOMONAS* SPECIES

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



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**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.

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**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).

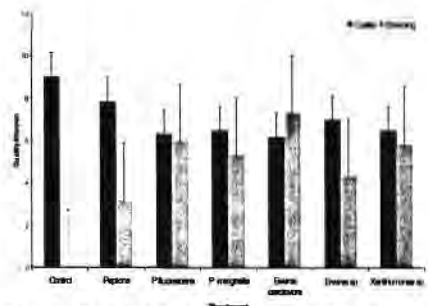


Figure 1 Quality & browning of lettuce 48 hours after bacterial inoculation.

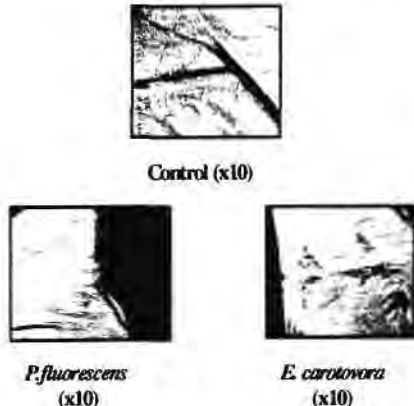


Figure 2 Browning on cut leaf surfaces caused by bacteria (48 hours).

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.



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## **Postharvest bacterial rots and browning in lettuce**

Sue Pascoe,  
Bruce Tomkins  
Robert Premier and  
Bill Washington

Department of  
Natural Resources  
and Environment  
Victoria

Project Number: VG98083