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

Project Number: VG98083

VG98083

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

Page no.

Media summary............2

Technical summary............3

Acknowledgments............5

PART 1: Background............6

PART 2: Browning of the cut surface of lettuce
- 2.1 Introduction............9
- 2.2 Method............9
- 2.3 Results............14
- 2.4 Discussion............22

PART 3: Evaluation of the presence of fluorescent pseudomonads in soil
- 3.1 Introduction............25
- 3.2 Method............26
- 3.3 Results............28
- 3.4 Discussion............35

PART 4: Reduction measures for bacterial control
- 4.1 Introduction............37
- 4.2 Method............37
- 4.3 Results............41
- 4.4 Discussion............50

Technology transfer............52

Recommendations............54

Appendix 1: Poster shown at NRE Horticultural conference (September, 2000) and Gympie (Qld) field day (November, 2000)............55

Appendix 2: Poster displayed at the Australian Food Microbiology conference (March 2001) Melbourne, Victoria............56

Appendix 3: Detection methods of phytopathogenic bacteria (Milestone 3)..57

References for Appendix 3............62

References for final report............66

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.~~

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.

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.~~

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.