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Effect of Calcium Nutrition on Decay of Summer Sown Seed Potatoes

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Project Number: PT98011

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

The research contained in this report was funded by Horticulture Australia Ltd with the financial support of all levy paying industries.

ISBN 0 7341 0610 6

Published and distributed by:
Horticultural Australia Ltd
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Horticulture Australia

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Final Report

PT98011

"Effect of calcium nutrition on decay of summer sown seed potatoes"

Commenced 30/10/98

Completion Date 30/06/01

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Date of Submission: 03/03/03

ISBN: 0 7347 1473 4

HORTICULTURAL RESEARCH AND DEVELOPMENT CORPORATION

FINAL REPORT PROJECT PT98011

"Effect of calcium nutrition on decay of summer sown seed potatoes"

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Commencement Date:	**30/09/98**
Completion Date:	**30/06/02**
Total Funding:	**$160 096**

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1.1 Media Summary

Effect of Calcium Nutrition on Decay Of Summer Sown Seed Potatoes

PT98011, sought to find a solution to the continuing problem of seed tuber loss for Australian potatoes growers. In the field the disease usually manifests itself after summer planting through liquefaction of tuber tissue or black legging of the emerging stems resulting in poor establishment and a consequent reduction of the crop's yield potential. Consumer preference for washed, new, potatoes also favours the decay of the fresh-market product. Development of tuber decay is usually attributed to the soft-rot bacterium *Erwinia carotivora* (syn. *Pectobacterium carotovorum*). Even when infected, tubers only rot in response to specific conditions of bacterial concentration, temperature, oxygen deprivation, free water and the length of time the tubers are exposed to the stress.

Previous investigations have suggested that supplementary applications of calcium to the crop may prevent tuber losses or delay the loss of the tuber long enough to allow the crop to establish. Our study set out to test this proposition and to observe grower practices which may influence rot development. We were unable to confirm an association between applied calcium fertiliser levels and establishment losses, nor could we associate tuber calcium levels with establishment. We did however find that infection history was the most crucial indicator of a tuber's predisposition to rot. Clean, certified seed tubers exhibited the best establishment compared to most grower produced seed. Growers typically grade out and retain smaller tubers for use as seed however we found evidence that this amplified the potential incidence of the disease as infected plants, even if asymptomatic, tend to produce smaller tubers.

More experimental work needs to be undertaken to confirm the potential of selective tuber retention to not only increase the disease potential of a harvested crop but to decrease it as well. Even if a method for decreasing potential is not economically viable for ware producers it might, in combination with emerging PCR detection technology, allow seed growers to certify tubers as free from Ec.

1.2 Industry Summary

Effect of Calcium Nutrition on Decay Of Summer Sown Seed Potatoes

Experimental investigations into the effectiveness of supplementary applications of calcium (Ca) to normally managed crops of cv. Coliban have shown it to be ineffective in providing protection against *Erwinia* soft rot. While previous researchers proposed a correlation between high tuber calcium levels and lower rot levels the results of the present study reported here do not suggest a relationship. This project has shown that while it was possible to elevate average tuber calcium levels that such treatments did not lessen the incidence or severity of rot. Factors such as the prior infection history of the seed pieces combined with exposure to an environment favourable to rot development play a far more important role in the development of the disease. Progress towards managing soft-rot losses within commercially acceptable levels would be better directed away from fertilizers and into seed management.

Key Factors Influencing the Development of Soft Rot in Ware Farm Sourced Seed Tubers

As part of the present study growers were asked about their management practices and to provide seed samples for calcium analysis and for establishment trials. No correlation was found between levels of calcium and the propensity of the seed sample to rot. Given that most Ca is locked into cell wall and is not readily remobilised, a method to separate soluble and insoluble components of total cell calcium was developed and the results compared to establishment levels from the sample. No correlation was found between either fraction and the level of rot. It was concluded that prior exposure to Ec. before planting rather than tuber Ca for the incidence of rot in the sample. The wide variation in establishment success of the various grower-sourced samples suggests that a combination of site specific and cultural practices governed the incidence of seed piece loss during trail work. It was proposed that the process of grading and retaining smaller tubers for seed could amplify the proportion of tubers carrying Ec. and therefore increase their susceptible to soft rot. Experimental investigation confirmed this finding.

Recommendations for the Management of Soft-Rot in Potatoes

Rots will always be present in potatoes unless tubers are grown aseptically since *Erwinia* species are ubiquitous. Growers may try to avoid rots by using non-susceptible varieties or by only cropping at locations or times of the year which are unlikely to favour rots, however this may not be economically viable. Favourable manipulation of the disease's epidemiology (causal organism, host & environment), to eliminate soft rots, can only be achieved in a controlled environment however there is still much that can be done in field operations to reduce the incidence and severity of the disease. Control measures must aim to:

1. Reduce inoculum levels
    - Plant seed with low inoculum levels (eg Certified seed)
    - Rotate potato cropping land with less favourable host species eg cereals or fallow
    - Thoughtfully dispose of crop waste away from drainage lines leading onto crop lands or water sources
2. Decrease transmission rates
    - Horizontal transmission can be minimised by:
        - Minimising tuber injuries – check machinery and avoid cutting seed
        - Keeping equipment clean
        - Eliminate sources of free water (eg condensation, washing, dips, post planting irrigations) or limit exposure time (eg forced air drying) as water assists bacterial motility, opens lenticels &, reduces O_2 diffusion
    - Vertical transmission can be reduced by:
        - Planting Certified seed where ever possible
        - Exploiting any seed size differential caused by loss of mother tubers to select a less infected cohort from the infected population
3. Increase tuber defences
    - Avoid planting physiologically stressed tubers

- Anaerobiosis favours bacterial over plant growth. Do not: water crops until after emergence, let condensation cover tubers in storage or otherwise wet the tubers (eg washing, dipping etc)
- Tubers must be conditioned for at least a week after removal from the cool room
- Store tubers below 35^{o}C since such temperatures induce anaerobic respiration causing leakage of cell contents

- Provide favourable conditions for periderm suberisation
  - Do not lift tubers before skins have set
  - Let tubers rest in well ventilated storage facilities following rough procedures like digging or cutting.
- Poor nutritional status of the parent crop affects cell integrity & defence of the harvested tubers.
  - Use soil and tissue analysis to manage crop nutrition
  - Single episodes of water stress coinciding with critical developmental stages may lead to irreversible nutrient deficiencies which make affected organs susceptible to disease

4. Minimising exposure to environmental conditions that favour the disease both in quantitative and temporal terms.
   - Install appropriate storage facilities (cooling or forced air ventilation)
   - Avoid handling tubers (harvesting, grading, planting) during periods of high temperatures
   - If washing, dipping or cutting become necessary then appropriate disinfection, water filtering and drying protocols should be followed

Promising Lines of Investigation Requiring Further Research

Breeding for less susceptible varieties is possible however it is a long-term goal and can be greatly assisted by advances in molecular biology. Genetic engineering techniques have been used to switch on defence systems that are normally only activated when a pathogen invades. Transformed potatoes have been created to express enzymes normally produced by *Erwinia* and this has triggered mechanisms that have made the transformed potatoes resiste\ant to actual *Erwinia* infection. Other work has shown that it is possible to disrupt the bacteria's quorum sensing capabilities by giving transformed plants the ability to metabolise the chemical signals involved. The non-specific defence system switched on following pathogen attack, commonly called acquired systemic resistance (ASR), may be able activated in some existing varieties with out genetic modification. Emerging research has linked the application of salicylates and methyl jasmonate to ASR and potato glycoalkaloids, so long shunned by the industry, have anti-microbial properties so their induction may yet prove beneficial. Studies of the endophytic bacterial communities in potatoes have shown that at least some are antagonistic to *Erwinia* when grown *in vitro* so there may be a chance that a biological control may be possible.

1.3 Technical Summary

1.3.1 Effect of Calcium Nutrition on Soft Rot Development in Potatoes

A range of calcium levels and application methods (side dress, foliar or combination) were trialled. While it appeared possible to increase tuber Ca levels in some treatments it was not associated with either the total Ca applied nor the resistance of the tubers to Ec. induced rot.

1.3.2 Record of Tuber Calcium and Crop Establishment Levels in Potatoes Sourced From Riverina and MIA Growers

Sample of seed tubers were taken from MIA & Riverina growers just prior to summer sowing. Tubers were analysed for Ca content and planted out in establishment trials. A wide range of Ca and establishment levels was observed. No correlation was found between tuber calcium and establishment. Crop establishment from certified seed was generally higher than grower produced seed regardless of its calcium content.

1.3.3 Filial Transmission of *Erwinia* - Its Consequences and Implications for the Management of Soft Rot in Potatoes

Common biological, cultural and environmental factors were examined to identify the most likely causes of bacterial soft-rot during the potato growth cycle. It was found that daughter tubers from seed tubers infected with Ec. differed in size from those set by healthy seed tubers. If seed tubers rot before plant establishment then no daughter tubers are set, however once established, the later into the season that a seed tuber rots, the smaller the difference becomes between daughters from infected and healthy stocks. Observations taken from affected crops supports the occurrence of the hypothesised tuber size differential.

This finding has profound implications for the profitable management of Coliban crops, which are particularly susceptible to the disease. Modelling indicates selective retention of tubers, in the range 100-150g, will amplify the proportion of infected tubers as it is over represented in the retained population. The model shows an inverse relationship between initial infection rates and the rate of infection in the subsequent generation. Thus an initial 10% infection level may increase to 18% after selective retention, while a 90% rate will only grow to 95%, i.e. an proportional increase of 80% compared to 50%.

1.3.4 *In Vitro* Screening for Microbial Antagonists against *Erwinia*

Two microbes were isolated which were able to inhibit the growth of Ec. *in vitro*, however neither is suited for use as biological control of potato soft rot.

1.4 Extension Summary

1.4.1 Publications and News Letters

Annual reports on the progress of this study were published in the HRDC/HAL supported, nationally distributed potato industry magazine "Potato Australia" during the period 1999-2002. Another four project related articles appeared in the newslettler of the National Vegetable Industry Centres - "Vegiebites". These were:

Howell, G (1999) Potato Research Update. *Vegie Bites* **2**:2
Howell, G (2000) *Erwinia* Amplification. *Vegie Bites* **11**:4.
Howell, G (2001) Potato Soft Rot Management Workshop. *Vegie Bites* **17**:4.
Howell, G (2001) Potato Soft Rot. *Vegie Bites* **18**:1.

A poster titled "Tuber size grading holds the key to amplification of potato soft rots was presented at the Australian Horticulture Society Conference at Sydney University, October 1-4, 2002. Proceedings of this conference are yet to be published.

An article titled, "Avoiding *Erwinia* losses in potatoes" appeared in the February, 2003 issue. of "Good Fruit & Vegetables".

In addition, a scientific manuscript, "Filial transmission of *Erwinia* - its consequences and implications for the management of soft rot in potatoes" was submitted to the CSIRO journal "Australian Experimental Agriculture" in December 2002.

1.4.2 Field Days and Work Shops

Figure 1.4.2.1. Dr Greg Howell (facing centre) discusses soft rot management issues with participants of the soft rot workshop held on January 24, 2002 at Claredale, Berrigan NSW.

A soft rot management field day was held at John Doyle's property, 'Claredale' near Berrigan on January 24, 2002, in conjunction with the Riverina Processing Variety Evaluation Field Day run by Roger Kirkham. The day was well attended by representatives of the major potato processing manufacturers and two Australian and New Zealand seed companies (Figure 1). A poster display and presentation was given by the project leader, Greg Howell, out-lining the results of PT98011, in addition to information from current scientific literature.

The field day was advertised on the NSW Agriculture Website and in the National Vegetable Industry Centre's newsletter "Vegiebites" which was sent directly to over 300 vegetable growers throughout NSW - including all potato growers in the Berrigan-Cobram district. The response of the potato farming community to notification of the work shop was disappointing, generating only one telephone enquiry prior to the workshop. No growers apart from John Doyle, on whose property the work shop was conducted, attended the workshop.

2 INTRODUCTION & BACKGROUND TO THE PROJECT

Under the sand-hill potato (*Solanum tuberosum*) production system two crops a year are produced, a summer crop - grown from certified seed-potatoes and a winter crop grown from seed-potatoes kept from the first crop (Cother & Hocking 1981). Establishment of the summer sown, autumn potato crops in the Riverina ranges from 40-80% (Wade Personal Communication). This figure remains unchanged since Cother (1980) discovered that 85% of planting gaps were attributed to bacterial soft-rot. Soft-rot of summer sown seed-potatoes in the irrigated semi-arid sands of southern New South Wales are primarily caused by the pectolytic activity of *Erwinia carotovora* (Cother 1980). Although may other bacterial genera can cause soft-rots, the range of enzymes produced by *Erwinia carotovora*, allows it to dominate in most cases (Pérombelon 2000).

Cother (1980) found that the rate at which tuber breakdown occurs is positively correlated with temperature but rarely occur at temperatures of 25^{o}C or below. During one of the worst epidemics soft-rot in the Riverina Cother (1981) reported that its incidence was associated with higher than normal temperatures, heavier soils and heavy rain after sowing. Kelman *et al.* (1989) and Pérombelon (1993) note that anerobic conditions favour soft-rot development - this would explain the increased incidence on heavier soils and following rain.

Protecting seed tubers against infection is likely to be the most effective way of controlling soft-rots in the field since *Erwinia* induced soft-rot in Australia, unlike those of the Northern Hemisphere, are due to soil borne as opposed to seed borne inoculum (Cother 1980). Cultural practices or treatments which lessen *Erwinia* development in the summer-sown crop are necessary for ware farmers to reduce establishment costs and increase productivity. The nature of sand-hill potato production, economics and the biology of *Erwinia* and *Solanum tuberosum* interact to narrow the options for soft-rot control.

As previously indicated, lower soil temperatures at the time of planting would reduce the severity of *Erwinia* infections, however if this was achieved through late planting the growing period would be unacceptably short. Mulching could effectively lower soil temperature but is not likely to be economic and would further increase the number of pectolytic organisms. Similarly, crop irrigation at planting would also favour tuber pathogens by increasing humidity and decreasing the oxygen supply. As *Erwinia* can enter through damaged regions of the tuber care must be taken during harvest, transport, storage and planting in order to minimise tuber damage. Immature (new) potatoes are especially susceptible to *Erwinia* infections as their skins are easily damaged. Optimising suberisation conditions following harvest would allow wounds to heal and skins to mature. A formal survey of cultural practices to identify areas of injury minimisation, opportunities for healing (suberisation), hygiene etc. needs to be undertaken in order to identify systematic problems that may promote *Erwinia* development so these can be remedied.

Given sound seed and ideal planting conditions establishment losses from soft-rot still occur It is hypothesised that the ability of potato tubers to resist the action of *Erwinia* depends on the integrity of the middle lamella. The middle lamella is a region of polymerised carbohydrate which sticks adjacent cells together. Resistance to the pectolytic action of *Erwinia* should be evident in tubers with thickened middle lamellae, those with a modified chemical structure or those expressing inhibitory substances (Stow 1989, 1993; Pérombelon

1993). Modifying the genotypic expression of the middle lamella to ameliorate soft-rot development is beyond the scope of the present proposal, but such research is being done in the United Kingdom. Pérombelon (1993) notes that "In the long term, only resistance to infection will achieve consistent blackleg (*Erwinia*) control, but previous attempts to breed for resistance have failed because this character is absent in conventional potato breeding lines…"

Seed-piece breakdown continues to be a chronic problem for Australian sand-hill potato production area such as the MIA, Riverina and Riverlands and is endemic to most sand production systems throughout the world. This project has sought to find a correlation between calcium nutrition of potato seed tubers and their susceptibility breakdown in the field since *in vitro* studies have shown a positive correlation between the calcium content of tubers and their ability to resist *Erwina* induced soft rots.

The methodology of this study was to experimentally manipulate tuber calcium levels by altering fertiliser protocols (form, rate, timing), to measure the differences in calcium levels and to correlate these with susceptibility to *Erwinia* inoculation and to decay in the field. Understanding these correlations and having the ability to manipulate tuber calcium levels will allow growers to combat the financial losses associated with anticipated or actual depressed yields from soft-rot.

2.0 REVIEW – FACTORS AFFECTING SOFT ROT DEVELOPMENT IN POTATOES

2.1 Introduction

Many potential pathogens are able to produce enzymes capable of liquefying tuber tissue however the range of enzymes and the speed with which *Erwinia carortivora* is able to grow clearly places this organism ahead of rest (Pérombelon & Kelman 1980, Pérombelon 2000). This summary will review *Erwina* soft rot and the conditions under which it proliferates but for a more detailed discussion readers should refer to Pérombelon (2000) or Toth *et al.* (2003).

2.2 Presence of Soft Rot Bacteria

Erwinia species are responsible for the majority of soft-rot outbreaks both in Australia and overseas (Cother and Cullis 1992, Pérombelon 2000, Pérombelon & Kelman 1980). These bacteria are frequently imported in commercial seed grade material (Pérombelon 2000). Ec. infections are passed asymptomatically from infected mother plant to daughter tubers (De Boer 2002, Helias *et al* 2000. Under Australian field conditions however Cother (1980 and present – pers com.) believes that most cases are acquired in the field *de novo*. The source of inoculum is not usually speculated however in the USA, research has shown that Ec. is present in the irrigation water of most production regions in Colorado regardless of its source (Maddox & Harrison 1988).

2.2.1 Erwinia Taxonomy and Pathology

Ewrinia carotovora (Ec.) is now formally known as *Pectobacterium carotovorum* (Jones 1901) Waldee 1945 (Anonymous 1999). Ec. belongs to a group of flagellated free-living or pathogenic gram positive bacilli which are facultative aerobes (Pérombelon & Kelman 1980). The most comprehensive overview of the pathology of soft rot causing erwiniæ is given by Pérombelon (2000). In his review Pérombelon (2000) notes differences between taxa responsible for black legging (*Ewrinia carotovora ssp atroseptica*) and those primarily responsible for tuber loss *E. carotovora ssp carotovora* or *E. chrysamthemi.* In each case the mode of action of the bacteria is similar: intercellular invasion, followed by bacterial proliferation and maceration of affected tissue. As discussed by Toth *et al*. (2003), the various forms (Ech, Ecc, Eca) each produce a signature profile of enzymes and their isomers which are adapted to the host.

2.2.2 Quorum Sensitivity and Pathology

While *Erwinia* may survive as commensal rhizobacteria, saprophytes or endophytes it is their ability to produce a wide range of wall degrading enzymes that makes them pathogenic (Pérombelon 2000, Pérombelon & Kelman 1980, Toth *et al*. 2003). A distinct change in the profile of the enzymes produced during colony growth, characterises the shift from commensal to pathogenic status and is triggered by the concentration of a pheromone (N-acyl homoserine lactone) in the surrounding medium in a process termed quorum-sensing (Swift *et al* 2001, Welch *et al* 2000, Williams *et al* 2000). Pérombelon (2000) interprets quorum sensing as an aid to efficient regulation of enzyme production, so that a colony consumes a food source through serial exploitation of each carbon source within the host environment.

In terms of pathogenesis the threshold level for the change from latent to blatant is around 10^7 CFU/g tuber skin (Pérombelon 2000). Subject to the right environment, this level can be quickly reached after the introduction of as few as 10^2 CFU (Pérombelon & Kelman 1980)

2.3 Environment favourable to *Erwinia*

2.3.1 High Temperatures

Researchers have consistently reported associations between soft rot incidence and development and the tuber pulp or soil temperature (Cother 1980, 1981; Kleinhenz & Palta 2002; Morgan & Wicks 2000; Pérombelon & Kelman 1980). Pérombelon (2000) contends that tubers are at higher risk of *Erwinia* rots at higher temperatures because increased metabolic activity leads to anaerobiosis, which results in leakage of cell contents, nourishing the bacteria. The optimal temperatures for growth of pathogenic *Erwinia* species are: Eca < 37 ^{o}C, Ecc 37-39^{o}C, Ech >39 ^{o}C with both Ecc & Eca having similar growth at 22^{o}C (Pérombelon & Kelman 1980).

Elevated temperature indirectly effects the incidence and severity of soft rot. Kleinhenz & Palta (2002) found calcium requirements for a potato plant's growth rose during periods of heat stress as more calcium was required for axillary shoot growth and cell expansion compared to control material. The higher growth rates of potato plants under hot conditions has been linked to calcium deficiencies which may then depress the ability of tubers to resist *Erwinia* (Tzeng *et al* 1986). Jiang and Huang (2001) believe that calcium (Ca^{2+}) may be involved in plant tolerance to heat stress by regulating antioxidant metabolism or/and water relations.

In the absence of a host, *Erwinia* numbers decline rapidly in soil, the rate of decline being related to both the species in question and to the soil temperature (Pérombelon & Kelman 1980).

2.3.2 Free Water

The presence of free water is able to directly or indirectly advantage *Erwinia* in four ways:

1. As a medium for the mass transfer of inoculum

   Infection of the crop may come about through the use of contaminated irrigation water or may be transferred between tubers during post harvest washing (Morgan & Wicks 2000, Pérombelon 2002).

2. As a medium for cellular motility

   Erwinia and similar coliform bacteria are flagellated and so are motile over short distances in water films (Pérombelon & Kelman 1980, Pérombelon 2000). While perhaps not directly related Shih *et al* (1999) was able to demonstrate increased pathogenicity and motility in a hyper-flagellated mutant of Eca.

3. Protection from desiccation

Erwina are a non-spore forming bacteria and so will not tolerate desiccation and can only survive short periods in hot dry soils (Pérombelon & Kelman 1980, Pérombelon 2000).

4. Increasing oxygen tensions

   Even at maximum saturation liquid water contains far less available oxygen than does air and its presence, even as a thin film greatly increases resistance to diffusion. Since *Erwinia* are facultative anaerobes they are able to proliferate under conditions which cause severe stress or death to affected potato tissue – the associated leakage of cell contents then nourishes the bacteria (Pérombelon & Kelman 1980, Pérombelon 2000). Tubers planted into wet soils are at risk as above field capacity air is excluded and in certain situations the soil may become anoxic due to crusting or from microbial respiration.

2.3.3 Substrate Availability

Erwinia bacteria do not synthesise efficient energy reserve compounds and so must be continually supplied with a suitable carbon source in order to multiply (Pérombelon & Kelman 1980). Pérombelon (2000) considers Ecc, Ecc & Ech to be opportunistic pathogens that produce a diverse range of enzymes in order to gain access and utilise tuber cell wall carbohydrates and more importantly the starch reserves within. Pérombelon & Kelman (1980) note that Eca can live harmlessly in the rhizosphere of a range of plants or saprophytically for over 100 days. Pérombelon (2000) emphasises that initial multiplication of bacteria is aided by events that provide them with extra carbon such as tuber injury or stresses, which cause the leakage of cell contents - this is particularly so during periods of anaerobiosis. In damp anaerobic conditions lenticels open up in the periderm, giving any resident or applied bacteria access to the thin-walled storage parenchyma (Scott *et al* 1996).

As previously mentioned, the ability of *Erwinia* to macerate tissue is quorum dependant and without the associated metabolic shift the infection will remain latent, as the tuber's starch reserves will be unavailable to the bacteria (Swift *et al* 2001, Welch *et al* 2000, Williams *et al* 2000).

2.3.4 Susceptible Cultivars

Potato cultivars vary in their susceptibility to Ec infection however screening does not appear to be consistently undertaken. In 1993, Pérombelon reported that attempts to breed for resistance have failed because the character is absent in conventional potato breeding lines. More recently work has being carried out to introduce resistance into cultivated potatoes from wild relatives (Bain *et.al*. 1999, Berrocal-Lobo *et. al* 2002, Cappo *et. al*. 2002, McGrath *et. al*. 2002). Work by Bain *et al.* (1999) concluded that the transferred *Erwinia* resistance genes from *S. brevi,* are simply inherited. During the course of this investigation it was observed that cv. Coliban was more susceptible to soft rot than cv. Desiree both in field following deliberate inoculation (see Fig. 1.3.4.1)

Genetic transformation of existing cultivars to express some form of resistance is also being looked at. The expression of antibiotics within tubers has been demonstrated to reduced susceptibility to Ec. (Arce *et. al.* 1999). Priming endogenous defence systems is yet another approach; tubers transformed to endogenously express, the pectin destroying enzymes from erwiniæ were shown to have enhanced resistance when challenged with the bacteria itself

(Wegener 2001, 2002). Another approach has been to modify ATP/ADP transporter proteins (Linke *et. al*. 2002), however this system severely inhibits energy transport resulting in yield loss.

Fig. 1.3.4.1 Comparison of leision size in cv. Coliban and cv. Desiree tubers, developed after 48h incubation following deliberate inoculation of a puncture wound with *Erwinia carotivora* ssp. *carotivora*. Note that all macerated tissue has been washed from the lesions.

2.3.5 Diminished microbial antagonists

The constant renewal of potatoes from sterile cultured material may come at a price as potato tubers are know to host around 20 endophytic bacteria some of which show potential to retard the growth of Ec. (Reiter *et. al* 2002, Sturtz *et. al*. 1999). Sharga & Lyon (1998) identified a strain of *Bacillus subtilis* that was able to retard the growth of Eca. and Ecc. both in *vitro* and in *vivo*. The idea that ecological disequilibrium or vacuums are inherently unstable is widely held so it is not surprising that Ec. or other pathogens can take hold under such conditions.

2.4 Compromised Tuber Defences

2.4.1 Lack of Oxygen

All potato cells require oxygen, which in tubers may have to diffuse though centimetres of tissue as well as the periderm. The periderm itself forms an impervious layer around the living tissue and is punctuated by lenticels that aid in respiration (Esau 1965). As could be expected the diminished surface area to volume ratio of large tubers, compared to smaller tubers, increases the risk that deep tissues will suffer from oxygen deprivation. To counter this effect Scott *et al* (1996) found that that tuber size was proportional to lenticel density. This study by Scott *et al* (1996) also demonstrated that when tubers were deliberately inoculated with *Erwinia*, rots would only progress in anaerobic conditions and that lenticels tended to remain open in mature tubers only when the soil was wet. This observation fits in with the contention that tubers are at extreme risk during wet, anaerobic episodes as it is during these periods that any bacteria inhabiting the lenticels gain access to the living tissue and additionally the cell contents can leak thus nourishing and bacteria present (Pérombelon 2000). The instability of cellular membranes has been extensively investigated by Rawler *et al.* (2002). Anoxic conditions set in place a cascade of events, starting the release of ionic

calcium in the mitochondria, which eventually lead to programmed cell death in both plant and animal cells (Virolainen *et al*. 2002). In this manner both cell death and loss of membrane integrity contribute to the ease with which *Erwinia* can access the starch reserves of affected tubers.

Pérombelon (2000) notes that anaerobiosis inhibits the ability of tubers to resist infection by diminishing their ability to produce defence chemicals and their ability to strengthen cell walls through lignification or suberisation.

2.4.2 Immaturity

The suberised periderm of potato tubers provides an effective barrier to the entry of Ec. as it does not produce enzymes capable of digesting suberin (Lulai & Corsini 1998). Immature periderm however is easily damaged exposing susceptible tissue to Ec present in the environment, for this reason alone tuber skins should be allowed to set before digging and handling. Not only is immature periderm unable to provide adequate mechanically defence but it is low in calcium rich pectin (Sabba & Lulai 2002) – a factor associated with susceptibility to Ec.

2.4.3 Poor Nutritional Status

Calcium plays an important role in the structural integrity of plant cell walls (Bain *et al* 1996, Bush *et al* 2001, Parker *et al* 2000), tolerance to heat stress (Jiang & Huang 2001), intra-cellular signalling (Reddy *et al* 2002) and the regulation of defence mechanisms (Kim *et al* 2002). Low calcium level are often cited as a reason for the susceptibility of potato tubers to decay, however the effect is not consistently correlated (Bartz *et al* 1992, Cother & Cullis 1992, Kelman *et al* 1989). Given the function that calcium plays within the plant it is not surprising that deficiencies have been linked to physiological disorders (Tzeng *et al* 1986) which might then predispose affected tubers to pathological conditions. In fruit, calcium availability not only affects a plants perception and resistance to diseases but has also been shown to directly depress fungal pathogenicity in *vitro* but not in *vivo* (Tian *et al* 2002).

The softening evident in *Erwinia* infected tubers occurs because pectolytic enzymes cause cells to separate and the tissue to become macerated (Kelman *et a* 1989, Pérombelon.1993). In plants, pectin forms an intercellular matrix which literally glues adjacent cell walls together and its degradation is associated with tissue breakdown (Stow 1989; Glenn, & Poovaiah 1990). Pectin itself is a carbohydrate polymer of rhamnose-galacturonic acid, held together by various chemical and electrostatic bonds. The most important of these linkages are formed by methyl ester and ionic calcium bonds (Kelman *et al*. 1989).

Since potato tubers naturally have low calcium levels compared to emergent parts and much of it is fixed into the cell walls of the cortex (Cother & Cullis 1992, Kelman *et al*. 1989) it may be that they are more at risk of experiencing critical deficiencies as there is little mobile calcium reserve to call upon. The distribution of calcium within the tuber shows that it is most abundant in or near the skin and lowest inside the vascular ring (Bretzloff & McMenamin 1971). In a sense nutritional deficiency might be viewed as another stress which predisposes affected tubers to soft-rot however the relationship may not be causal but consequential of the conditions under which the tuber formed.

In hot conditions the requirement for calcium rises in response to additional plant growth and possibly to overcome difficulties in cell expansion (Kleinhenz & Palta 2002). Tuber rots are strongly related to temperatures as the warmth not only favours the growth of *Erwinia* but it induces anaerobiosis leading to leakage of cell contents that actually feed the *Erwinia* (Pérombelon 2000). The anoxic and frequently wet conditions under which *Erwinia* bacteria proliferate (Pérombelon 2000) are similar to those which can shut down the transpiration stream of plants (Sailisbury & Ross 1991). Since calcium is transported exclusively via the xylem (Kelman *et al* 1989, White *et al* 2001) its movement is regulated by the transpiration stream and correlates closely to environmental conditions (Tzeng *et al* 1986). Under this scenario daughter tubers set on plants experiencing elevated temperatures and wetness would not only have depressed calcium levels but they may carry elevated inoculum loads from their proximity to a rot affected mother tuber.

Conditions for optimising middle lamella development in potato tubers, through phenotypic manipulation, to resist *Erwinia* soft-rot have not been thoroughly investigated. Poor cation exchange capacity (CEC) in sandy soils is thought to contribute to inadequate levels of calcium assimilation (McGuire & Kelman 1984, Tzeng *et. al* 1986, Bartz *et. al* 1992). Inadequate calcium nutrition is associated with tissue necrosis and inadequate middle lamella formation (Salisbury & Ross 1985; Davies & Ross 1985; Tzeng *et. al* 1986). *In vitro* studies (McGuire & Kelman 1984; Kelman *et al*. 1989) concluded that calcium nutrition can be used to increase soft-rot resistance in potato, however conferring soft-rot resistance in the field, via fertilizer, applications has not been demonstrated. Bartz *et. al* (1992) and Cother & Cullis (1992) failed to prove consistent association between applied calcium and soft-rot development. They concluded that unknown interactions of environmental (edaphic and climatic) and genetic (cultivar) influences are more important to soft-rot development than any conferred resistance associated with fertilizer applications.

Poor CEC in sandy soils may explain observed inconsistencies in tuber resistance to soft-rot in crops treated with a calcium fertiliser. Like all mineral nutrients, aqueous ionic calcium is the only form of this element which can be absorbed and in contrast to anions its intra-cellular transport is comparatively slow (Salisbury & Ross 1985). As ionic calcium is quickly lost from sand due to poor CEC, deficits may occur in tubers which are rapidly filling in the heat of summer.

2.4 Cultural Practices

2.4.1 Seed Selection – Certified V. Non-Certified Seed

The use of certified seed is one of the most reliable methods of reducing disease incidence in the subsequent crop (Janse & Wenneker 2002), however not all diseases are seed or soil borne and the certification scheme only guarantees that the tubers are no more than three field generations out of tissue culture and are virus free (Steven Wade pers. comm.). While the use of certified seed is recommended it is not widely acknowledged that the causal agent for soft rot is frequently imported in certified seed (DeBoer 2002, Pérombelon & Kelman 1980).

2.4.2 Rough Handling and Washing

Rough handling can result in mechanical injuries which allow the entry of Ec. into tubers (Perombelon *et. al*. 1976; Pérombelon & Kelman 1980, Pérombelon 2000). Once infected, a tuber of a susceptible cultivar is unlikely to survive if subsequently exposed to conditions which allow the bacteria to proliferate (Pérombelon & Kelman 1980).

Recycled wash water is of great concern as it can carry, transmit and amplify disease if filtration and disinfection procedures are inadequate. Work on rot reduction, undertaken for Horticulture Australia (Morgan & Wicks 2000, 2002), has shown that sanitizing agents added to wash water can reduce levels of Ec. however without effective drying treatment is ineffective. Since even surface sterilised tubers will go onto develop rot if left wet, it appears that either sanitisers can not penetrate lenticels or that the seat of infection lies in deeper tissue.

2.4.3 Inadequate Storage

As previously discussed, the use of strict hygiene in conjunction with controlled temperature and atmosphere within a storage facility can reduce the incidence severity of soft rot or limit its spread. Even under relatively ideal storage conditions (95% relative humidity @ 10 ^{o}C) it has been found that *E. carotivora* ssp *atroseptica* can proliferate (Costello *et. al*. 1999). The movement of air is essential in order to dry tubers, thereby minimising rots. From the present study it is apparent that very few fresh-market growers own or have access to bulk cool-store facilities - this is a major factor determining the deterioration of seed tubers over summer. While most growers still use ½ tonne bins to store potatoes the move to 1 tonne polypropylene, bulk bags must be detrimental to tuber health since very little air exchange is possible.

Bulk storage of tubers poses another threat to the continued integrity of non-infected tubers – that of direct contact with a rotting tuber, which by itself provides a source of inoculum and moisture. Under such circumstances the decay of a single tuber may catalyse the destruction of the tubers around it and may eventually affect the entire store. The ability to detect and remove the first signs of rot is therefore a major advantage and is the subject of ongoing research (Costello *et. al*. 1999, 2000).

3 TECHNICAL REPORT

3.1 Effect of Calcium Nutrition on Soft Rot Development in Potatoes

Introduction

Decreases and delays in *Erwinia* disease development (tuber rots and black leg) have been observed when deliberately infected tubers have been sown into soil augmented with gypsum (Bain *et al* 1996, Cother & Cullis 1992) but why this should be so is not completely understood. There is a general belief that high levels of tuber calcium in potato tubers are associated with resistance to the action of *Erwinia carotivora* (Ec.) soft rots (Pérombelon 2002), however experimental investigations have proven inconsistent (Bartz *et al* 1992, Cother & Cullis 1992, Kelman *et al* 1989). There is however a strong foundation for this belief as calcium plays an important role in the structural integrity of plant cell walls (Bain *et al* 1996, Bush *et al* 2001, Parker *et al* 2000), tolerance to heat stress (Jiang & Huang 2001), intra cellular signalling (Reddy *et al* 2002) and the regulation of defence mechanisms (Kim *et al* 2002).

Previous studies investigating the affect of calcium nutrition on soft-rot resistance have found calcium deficits in susceptible tubers but not in the emergent stem and leaf system (Tzeng *et. al* 1986; Kelman *et al*. 1989). Clearly the potato plant is able to differentially allocate calcium to its various parts and as was demonstrated by Cother & Cullis (1992). Bain *et. al.* (1996) found that the plant can so some extent alter the amount of calcium allocated to individual tubers. The control mechanism for calcium allocation is not completely understood (White 2001) but it may be that localised and episodic interruptions to calcium availability during growth may have lasting effects. If just a small region of a tuber is susceptible this area may catalyse the destruction of the entire tuber if it becomes infected before the shoot system is established. Because of inter-and intra-tuber variation in calcium deposition Cother & Cullis (1992) recommend that investigations of calcium allocation should be based on individual rather than bulked samples.

If calcium can be delivered to nutrient sink areas at rates that match their needs then the true potential of calcium to ameliorate soft-rot incidence would be gained. Previous field-based studies investigating the effect of calcium on soft-rot development have examined soil applied fertilisers (Bartz *et. al* 1992, Bain *et. al* 1996, Cother & Cullis 1992; Kelman *et al* 1989). In the study by Bain *et. al* (1996), the effect of such fertilisers was shown only to provide protection against black-leg early in the growth period. Earlier, Cother & Cullis (1992) supplemented a pre-sowing gypsum application with a more soluble fertiliser (calcium nitrate) later in the season, and they still found inconsistent protection against soft-rot and allocation of calcium to daughter tubers. Why this should be has not been adequately explained, however the literature is replete with assertions that in the subsequent crop a positive correlation exists between applied calcium, usually as gypsum and tuber resistance to rot (see review by Pérombelon 2002).

Given that gypsum is almost insoluble (saturation at 0.012M $CaSO_4 \cdot 2H_20$), it was decided to test the claim using a more available form of calcium – calcium nitrate (saturation at 5.843 M $Ca(NO_3)_2 \cdot 4H_20$ - saturation based on solubility as stated by Aylward & Findlay 1971) as both a soil amendment and in combination as a foliar spray. The present proposal seeks to

circumvent the apparent differential and undesirable allocation of calcium by regular foliar applications of ionic calcium to satisfy the demand near the site of deposition so that calcium absorbed by the roots might stay in the subterranean parts- specifically the tubers.

Materials and Methods

An experimental site was established at tubers at the Yanco Agricultural Institute, in the Riverina district of New South Wales Australia. The soil at the site was a red sandy loam and had not been used previously to grow potatoes. Soil testing (0-20cm) was undertaken (Sydney Environmental and Soil Laboratory, PO Box 357 Pennant Hills NSW 2120) to determine the suitability of the experimental site and to calculate fertiliser levels. Top-soil at the field site was slightly acid (pH (1:2 in water) 6.8) and had fairly low salinity (EC (1:2) =0.14 $mScm^{-1}$). Calcium levels were good, making up 65.9% of the exchangeable cations and the calcium to magnesium ratio was 3.8, which was also considered to be good. Adjustment to soil's cation pool was deemed unnecessary and a fertiliser recommendation was set at N:P:K, 275:80:200 $kgHa^{-1}$.

Plot	Base Fertilizer				Side Dressing				Foliar				Total			
	N	P	K	(Ca)	N	P	K	Ca	N	P	K	Ca	N	P	K	Ca
AD	137.5	80.0	100.0	181.8	120.4	0.0	100.0	55.1	17	0.0	0.0	0.0	274.9	80.0	200.0	236.9
AE	137.5	80.0	100.0	181.8	115	0.0	100.0	27.6	22.5	0.0	0.0	27.6	275.0	80.0	200.0	237.0
AF	137.5	80.0	100.0	181.8	103.4	0.0	100.0	55.1	34	0.0	0.0	0	274.9	80.0	200.0	236.9
AG	137.5	80.0	100.0	181.8	92.5	0.0	100.0	0	45	0.0	0.0	55.1	275.0	80.0	200.0	236.9
AD	137.5	80.0	100.0	181.8	120.5	0.0	100.0	110.2	17	0.0	0.0	4.94	275.0	80.0	200.0	296.9
AE	137.5	80.0	100.0	181.8	115	0.0	100.0	83.6	22.5	0.0	0.0	27.6	275.0	80.0	200.0	293.0
AF	137.5	80.0	100.0	181.8	103.5	0.0	100.0	110.2	34	0.0	0.0	4.94	275.0	80.0	200.0	296.9
AG	137.5	80.0	100.0	181.8	92.6	0.0	100.0	55.1	45	0.0	0.0	55.1	275.1	80.0	200.0	292.0
CD	137.5	80.0	100.0	181.8	120.4	0.0	100.0	147.3	17	0.0	0.0	10.1	274.9	80.0	200.0	339.2
CE	137.5	80.0	100.0	181.8	115	0.0	100.0	141	22.5	0.0	0.0	27.6	275.0	80.0	200.0	350.4
CF	137.5	80.0	100.0	181.8	120.1	0.0	100.0	147.3	34	0.0	0.0	10.1	274.8	80.0	200.0	339.2
CG	137.5	80.0	100.0	181.8	92.5	0.0	100.0	113.4	45	0.0	0.0	55.1	275.0	80.0	200.0	350.3

Table 3.1.1. Fertiliser rates used for experimental manipulation of a potato crop cv. Colliban. Rates are in $kgHa^{-1}$. Base and Side Dressing : N as Ammonium Nitrate, P as single super phosphate, K as potassium chloride, (Ca) as inclusion in single super phosphate. Side & Foliar applied fertilisers: N as ammonium nitrate, Ca as calcium nitrate.

Whole 60mm diameter certified Coliban seed tubers were used to a spring (September) crop in 2000 using the twelve fertiliser regimes. Four randomised replicate blocks of each combination of pre-plant, side and foliar applied fertiliser were established at the experimental site. Rates of fertiliser application are given in Table **3.1.1.**

Individual plots measured 5m long by 5 rows wide. Rows were 900mm apart and tuber spacing was set at 400mm. Pre-planting fertiliser treatments were broadcast over the plot and later dragged up into the rows in a mechanised hilling operation. Side dressings were applied midseason as were foliar sprays. Daughter tubers from the middle two rows of each treated plot, from the crop sown in September were machine harvested in late December and assessed for: a) tuber calcium content; b) field establishment rate and; c) resistance to soft-rot following inoculation. The results of these observations were then subject to analysis of

variance to determine significant shifts from control treatments. Protocols for the analysis procedures are set out here:

Tuber calcium content

Ten tubers from each treatment plot were randomly chosen, taking care to exclude injured and excessively large or small tubers. The tubers were then washed and rinsed in distilled water. The tubers were then individually identified and their weights recorded. The tubers were then peeled, using a domestic vegetable peeler and the peel weight recorded. Peels were then oven tried at 60^{o}C for 48h and again the weight recorded to determine the moisture content. Dried peels were then ground, and a 0.5g sample digested in 5 mL of 70% nitric acid (Sigma Chemicals) at 140^{o}C for 1.5h. Each sample was than diluted to 25 mL with water and the calcium content determined by a commercial laboratory using ICP-OES (Zarcinas *et al.* 1987). The results were then expressed as mg Ca/g FW of tuber peel, as that has been the standard in previous studies and reflects the calcium concentration in a form that is biologically meaningful.

Resistance to soft-rot following inoculation

Ten tubers from each treatment plot were randomly chosen, taking care to exclude injured and excessively large or small tubers. Tubers were then soaked in diluted domestic bleach (10% available chlorine) for 30minutes and then rinsed in distilled water and allowed to air dry. Each tuber was then weighed and inoculated with 5µL of an Ec. culture, wrapped in polythene film and incubated at 25-27^{o}C for 48h. Following incubation the tubers were cut in two and the macerated tissue washed away under running water. After washing, excess water was blotted off and the tuber reweighed to determine the amount of rotted tissue. Control inoculations with killed cultures were not deemed necessary as preliminary work showed that such controls did not rot.

Field establishment rates

Thirty tubers from each treatment plot were randomly chosen, taking care to exclude injured and excessively large or small tubers. These were replanted approximately 6-8 weeks after harvest in late February – early March, into conventionally prepared hills. The rows were then irrigated after planting to encourage rot and emergence and establishment figures recorded

Results and Discussion

In contrast to previous studies where gypsum was used to augment soil calcium (Bain *et al* 1996, Bartz *et al* 1992, Cother & Cullis 1992, Kelman *et al* 1989) the calcium source in the present trial occurred as part of single superphosphate and calcium nitrate. The dominance of calcium makes it unlikely that the soil's Mg pool could competitively inhibit Ca uptake as may sometimes occur (Tzeng *et al* 1986). Bartz *et al* (1992) found that rots were more closely correlated to rainfall than to calcium (as gypsum) application. Their study concluded that the "efficiency of Ca applications to potatoes grown in low Ca soils for enhancement of tuber resistance to bacterial soft rot may be limited by factors associated with the environment or cultivar". While the importance of optimal calcium nutrition in plant defense

can not be dismissed, in the absence of any soil analysis it may be that the action of the gypsum on the soil may be responsible for decreasing crusting and waterlogging in amended soils. Given that the low solubility of gypsum and that soils supporting rot-affected crops may not be considered calcium deficient (Bain *et al* 1996, Kelman *et al* 1989), perhaps the nutrient effect of calcium is secondary to the gypsum's physical and chemical modification to the soil. Reduction in soil crusting and an increased hydraulic conductivity is a recognized consequence of gypsum application (Loveday 1976). This explanation, while mainly applicable to sodic clay soils, needs to be more thoroughly investigated since even relatively sandy and marginally saline soils used in this study contains some clays which may be gypsum responsive.

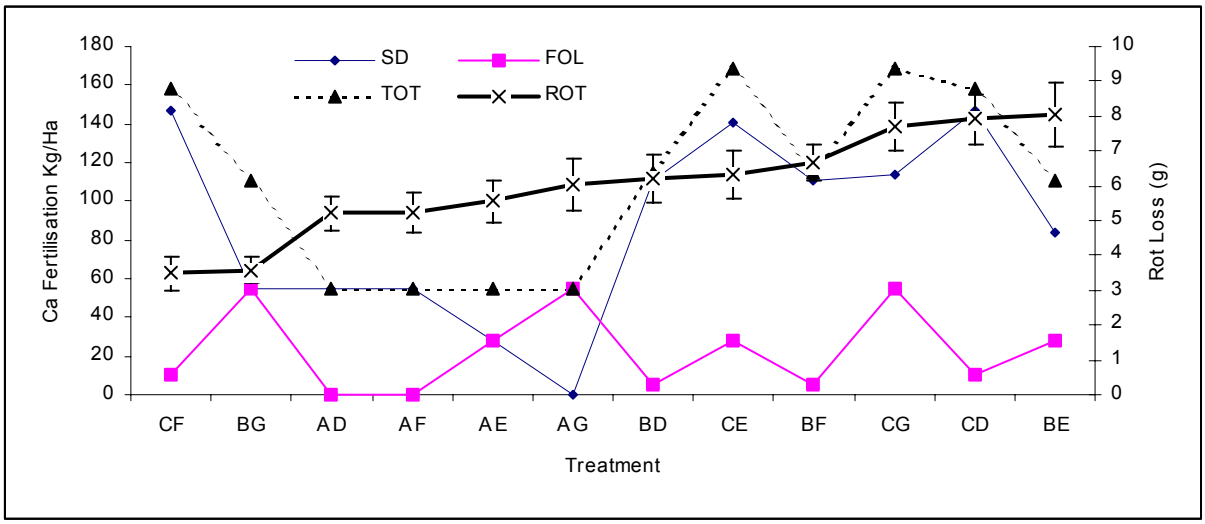


Figure 3.1.1 Decreasing rot resistance (L-R) of Coliban tubers grown under differential calcium fertiliser regimes and then inoculated with *Erwinia*. Applications of foliar (FOL), side dressed (SD) and total (TOT) calcium fertilisation are shown on the "Y" axis and rot ± standard errors are given on the secondary "Y" axis.

Ordering the rot resistance results for tubers grown under differential calcium fertiliser regimes and then inoculated with *Erwinia* (Figure 3.1.1) show only two distinct groups. Statistically, treatments BG and CF were significantly different to from all other treatments and were the most resistance to rot. The highest rates of rot were exhibited by treatments BE, CD, CG but the difference to AF, AE, AG, BD, CE and CF was not significant. Treatment CG represented one of the highest rates of both soil and foliar applied calcium and could have been expected to have the lowest rot loss if calcium availability to the growing tuber is a dominant factor influencing its susceptibility to rot.

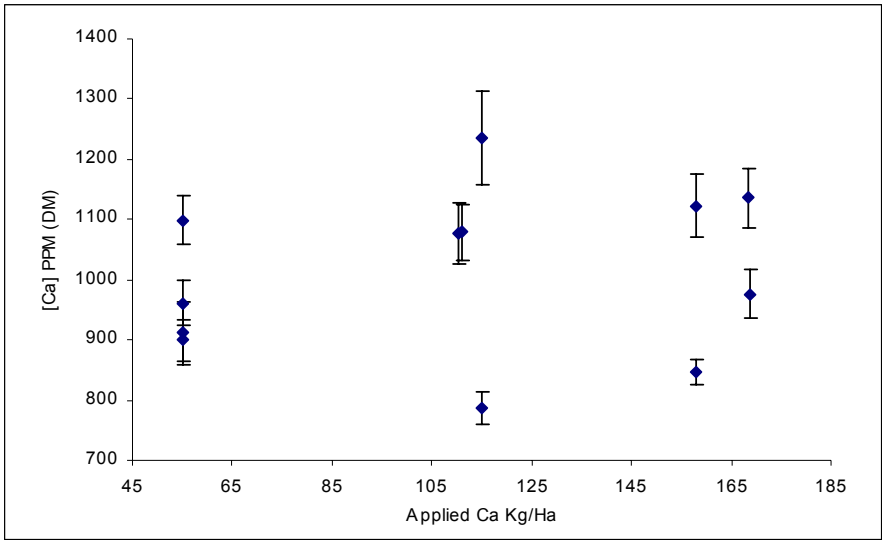


Figure 3.1.2 Relationship between recorded tuber calcium levels (DM) and applied calcium in a Coliban crop receiving various levels of calcium fertilisation. Standard errors shown for observed tissue Ca levels.

Both "resistant" treatments (ie BG and CF) had very different soil and foliar amendments, and their placement in a matrix of increasing soil and foliar amendments did not correlate well with increasing resistance to *Erwinia*. Without further experimentation, under the conditions experienced during the trial, there is little evidence to show that that rot resistance was correlated with increasing availability of calcium. As shown in Figure 3.1.2, the belief that the level of applied calcium will be directly related to bulked tuber calcium levels was shown to be at best unreliable.

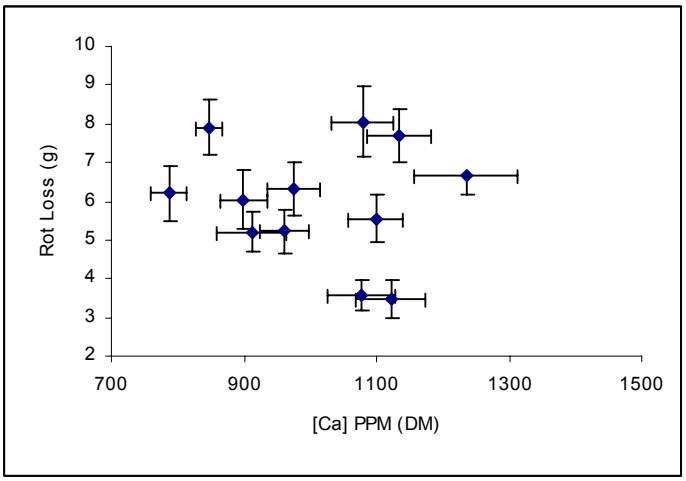


Figure 3.1.3. Rot loss in Coliban tubers plotted against tuber perderm calcium levels following infection with *Erwinia carotivora*. Standard errors shown on both axes.

Rot resistance when plotted against tuber calcium levels (Figure 3.1.3) is likewise poorly correlated. The expected negative correlation (increascing Ca levels associated with decreased rot) is barely measureable (r^2=0.01) but is in the right direction. Overall there is no convincing evidence that Ca levels in range observed in Coliban tuber periderm had any effect on the severity of Ec mediated tissue loss.

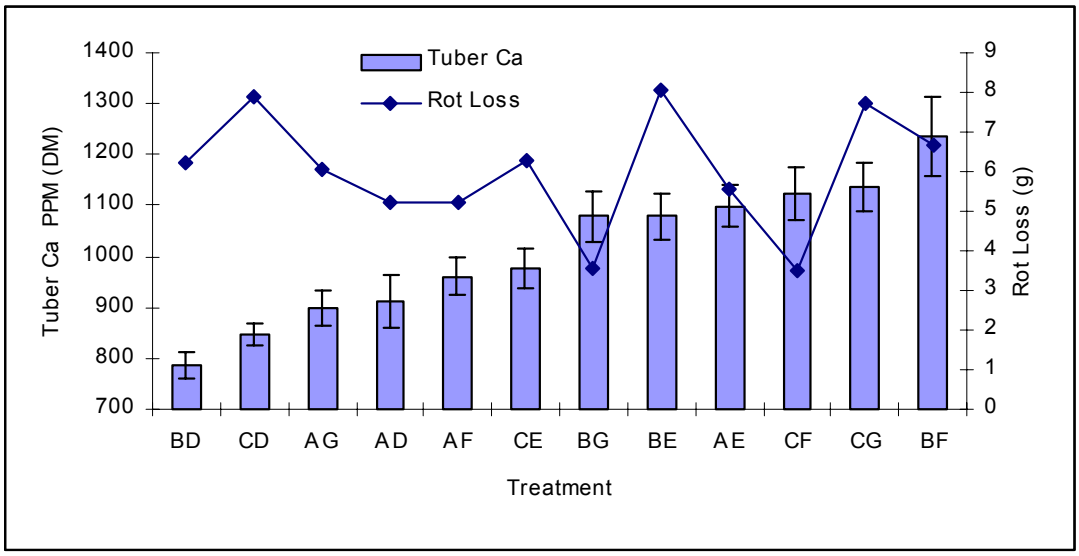


Figure 3.1.4. Associated rot loss in Coliban tubers plotted against tuber perderm calcium levels following infection with *Erwinia carotivora* for the various treatments. Standard errors for Ca content of tuber periderm shown.

It appears from this study that tuber periderm calcium levels are not that important in providing protection against Ec. under the conditions of the experiment. As shown in Figure 3.1.1 and reproduced in Figure 3.1.4 to show increasing order of tuber periderm calcium level, the average amount of rot experienced by tubers exposed to Ec does not correlate with the average calcium level. This may represent a limitation of the method as very little Ca is associated with medullary parenchyma in tubers (Locascio *et al*. 1992) and this where Ec would have been introduced during the inoculation. It might be argued that the severe wounding of the tissue during the assay is artificial; however it should provide an indication of useful resistance since Ec are naturally transported beyond the periderm via vascular tissue (Helias *et al* 2000).

The range of calcium application levels used and calcium levels in tuber periderm observed in the present study are within that reported by other researchers (Bartz *et al.* 1992, Locascio *et al*. 1992). As was found by Bartz *et al*. (1992) and in the present study, application of Ca fertilizer is not consistently associated with increased tuber calcium levels or with rot resistance. While their and other studies have used higher rates of applied calcium and sometimes found higher tissue calcium; Bain *et al*. 1996 notes that even in soils not considered to be low in calcium that the effect of calcium supplementation was inconsistent.

Rot resistance may be confounded by the pattern of calcium assimilation in potatoes. Work by Bush *et al.* (2001) found that aside from being restricted to the corners of cells, calcium deposition is restricted to the initial tuberisation period; i.e. the cell division rather the cell expansion phase. The implication of this work is that the timing of application and the availability of the calcium are critical to the calcium content of the mature tuber. The pattern of calcium assimilation is likely to be cultivar specific and modified by factors which affect the tuberisation and calcium absorption by the plant. Such factors would include: temperature, planting density, water availability, the physiological age of the seed tuber and

fragmentation of the plant (see Chapter 3.1.4). Management of the crop to ensure optimal Ca levels should avoid creating episodes where large numbers of tubers per plant are initiated early in the growth cycle, during hot periods and more or less simultaneously on all stolons. In this way, manipulating tuber composition to enhance the nutrient content would offer benefits to consumers and growers.

Calcium is transported exclusively in the xylem (White 2001). There may however be problems with its assimilation, particularly interference from other divalent cations, like Mg^{2+} (Allison *et al*. 2001). During periods of low transpiration (eg water stress or at night), the amount of nutrient moving in the apoplast and loading into the xylem decreases dramatically; in relative terms more nutrients are moved through the symplast where their admission is regulated by element specific trans-membrane gateways (proteins) and it is at these sites that divalent elements can saturate and competitively inhibit the entry of calcium (White 2001). Thus even in crops receiving adequate calcium fertilisation, periods of water stress can induce calcium deficiency especially when requirements are high, such as at tuber initiation.

Kleinhenz & Palta (2002) found that supplementary calcium restored normal cellular expansion in heat-stressed potato plants. Ionic calcium, is required for the normal functioning of calmodulin in non-specific plant defence reactions (Kim *et al*. 2002) and is also an important trigger for tuberisation (Reddy *et al*. 2002). Since heat is one of the critical factors in the development of soft-rot it may be that higher levels of calcium may restore normal defence functions to heat affected tubers.

Conclusion

Calcium is essential for potatoes to maintain normal cellular structure and function. Inadequate calcium nutrition has been implicated in potato tuber resistance to the action of Ec. The provision of supplementary calcium fertilizer to reduce the incidence and severity of *Erwinia* diseases in potatoes has not provided consistent protection. When calcium is applied, either as sulphate (gypsum) or as calcium nitrate, neither the resulting calcium levels nor resistance to Ec. show a consistent trend – at least with plants grown under field conditions. The field environment is subject to subtle differences which may affect the timing of tuberisation or water availability. For this reason if growers choose to produce their own seed crop it is essential that that the production area is subject to the same soil type, aspect, slope, and water availability and that care is taken to ensure that the tubers are all planted at the same depth and spacing and that the tubers are the same weight and age. Further investigations into the timing of fertilizer applications, in relation to growth stage, need to be completed so as to optimise calcium assimilation.

While calcium is essential to maintain normal structure and function in potatoes, clinical deficiencies are probably rare and it's usefulness in protecting a crop against Ec infection is questionable. Fertilizer trials indicate that it is possible elevate tuber calcium levels, however it is unlikely that Coliban tubers are able to mobilise adequate defences against Ec. once the bacteria have proliferated. The best insurance against Ec induced soft-rots is to eliminate or reduce the level of infection in the seed stocks and to avoid exposing tubers to conditions likely to favour rot development.

3.2 Record of Tuber Calcium Levels and Establishment Associated With Potatoes Sourced From Riverina and MIA Growers

Summary

No evidence was found to support the contention that tuber calcium levels are associated with increased establishment as the levels did not correlate with establishment success. The wide variation observed in the range of seed tuber Ca levels suggests that it is amenable to manipulation. Variation in establishment levels between the growers samples suggests that infection with Ec bacteria prior to the trial is the strongest predictor of loss.

Introduction

Poor crop establishment due to loss of the mother tuber from *Erwinia carotivora* (Ec.) induced soft-rot periodically devastates ware potato crops in the Riverina district of New South Wales. It is generally believed that low tuber calcium levels make tubers more susceptible to the action of Ec. (Cother & Cullis 1992). This study seeks to investigate the levels of calcium present in the tubers of seed potatoes and to correlate these with the field establishment rates.

Materials and Methods

Samples of seed potatoes were obtained directly from Riverina and MIA growers prior to crop planting in February 1999 and 2000. Tuber samples were identified (cultivar, owner and source i.e. graded from previous crop, cool stored certified or purpose grown material) and subjected to the following procedures:

1. *Tuber Calcium determination*

   In 1999, ten tubers from each sample were selected for Ca determination. Each tuber was washed, weighed and given an identifying number. Each tuber was then peeled and the peel weight recorded. A five gram sample of peel was finely ground in liquid nitrogen and suspended in 5 mL of Hepes buffer (Sigma) as the buffer should only affect sulphur and sodium levels. The suspension was then hard centrifuged (10 000 *g*) for 10 min) and carefully decanted, retaining both the supernatant and the pellet so that any calcium free in the cytosol could be extracted separately to that bound into the walls. Each pellet was then dried at 60^{o}C for 48h, weighed and digested in 5mL of 70% nitric acid (Sigma) at 140^{o}C for 1.5h. These digests and cytosol extracts were then made up to 25ml with water. Determination of calcium levels in the sample extracts and digests was done by ICP-OES though a commercial laboratory (Zarcinas *et al.* 1987. Calcium levels were calculated as μmol/g (FW).

2. *Crop Establishment*

   Thirty tubers from each sample were used to determine their field establishment potential. Tubers were planted into conventional hills at Yanco Agricultural Institute during late February and establishments counts recorded after two months.

Results and Discussion

Tuber Calcium determination

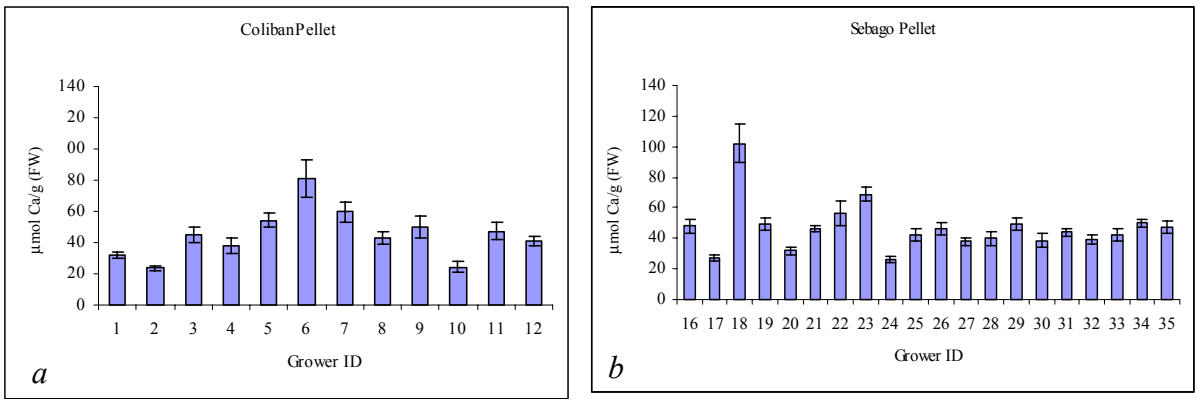


Figure 3.2.1 Cell wall calcium levels measured in seed tubers taken from commercial growers for the crop sown in February 1999. *a,* Coliban cell wall and b, Sebago cell wall. Standard errors determined from 10 tubers.

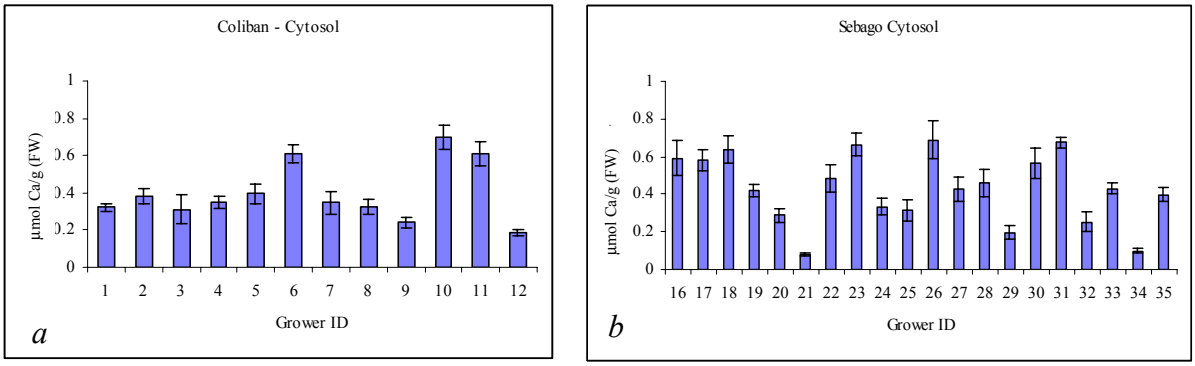
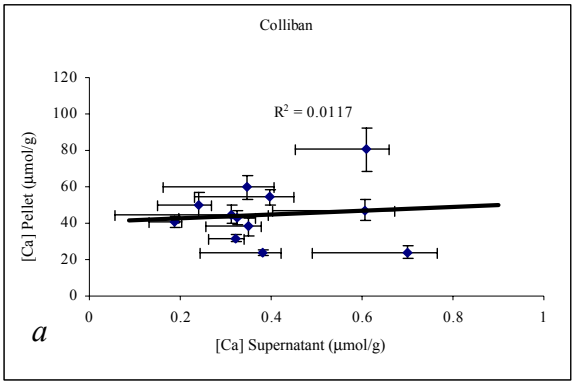


Fig 3.2.2 Cytosol calcium levels measured in seed tubers taken from commercial growers for the crop sown in February 1999. *a,* Coliban cell wall and b, Sebago cell wall. Standard errors determined from 10 tubers.

Levels of Ca varied according to both cultivar and source. Typically Coliban cell walls contained about 42 (± 2 SE) and Sebago 44 (± 1 SE) µmol/g FW and the cytosol less than 1/100 of the cell wall value. As can be seen in Fig. 3.2.1, the Ca content of tubers from some growers was significantly higher than others, the wall contents varying from 13-182 µmol/g in Coliban and 12-142 in Sebago. Cytosol levels shown in Fig 3.2.2 are similar for both Coliban and Sebago at around 0.41 (± 0.02 SE) µmol/g FW and ranging from 0.1-1.3 µmol/g FW in Coliban and 0.0-1.4 µmol/g FW in Sebago. The level of Ca free in the cytosol is roughly two orders of magnitude smaller than that contained in the cell wall although there was no correlation found between the level of calcium in the cytosol and that of the cell wall fraction as shown in Fig 3.2.3. Since the variability of Ca levels between samples was greater than that between tubers in a sample it appears likely that Ca level are amenable to manipulation.



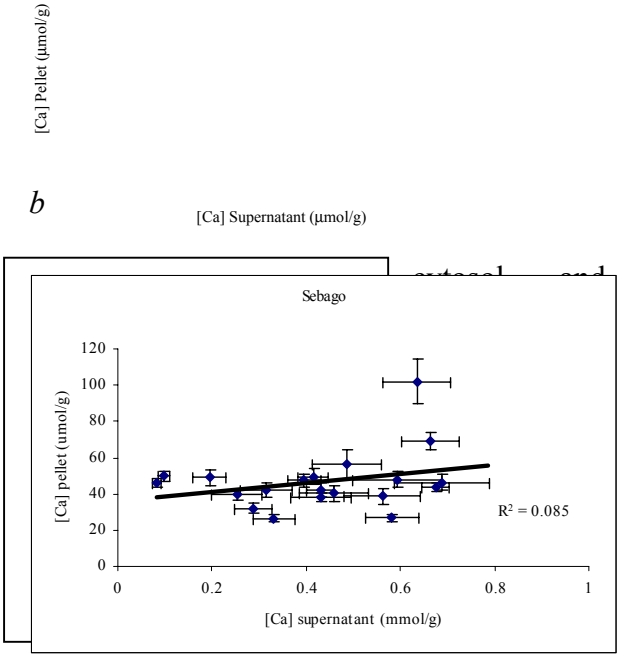


Fig 3.2.3 Correlation of calcium levels in cell walls in a) Coliban and b) Sebago seed from commercial growers for the crop sown 1999. Standard errors from 10 determinations.

Crop Establishment

Figures 3.2.4 and 3.2.5 show establishment grower collected seed for the summer 1999 and 2000 respectively. Generally more the seed planted established a plant after 2 months. Few growers used certified seed, however the use of certified seed was associated with establishment rates above 80% except in the case of Desiree in 1999 where only 60% of the sample established. More samples of certified seed are needed to conclusively state that the use of such seed protects against establishment loss, however it likely to be the case as it has had less exposure to possible sources of contamination.

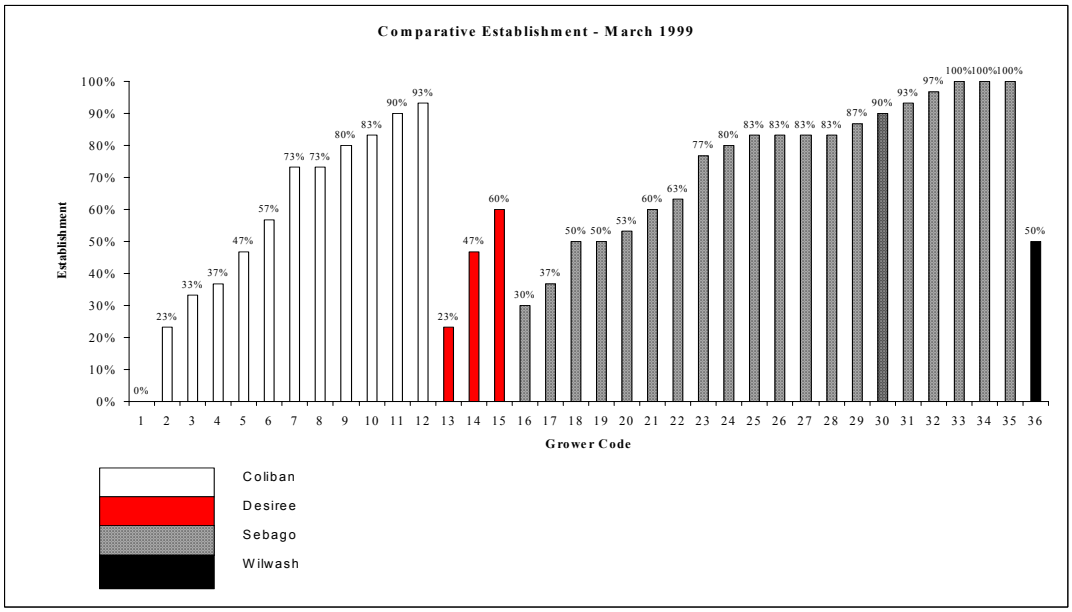


Figure 3.2.4 Establishments rate for Growers seed from a trial planted in February 1999 at Yanco. NB Certified seed was supplied by growers : 11, 12, 15, 27, 33.

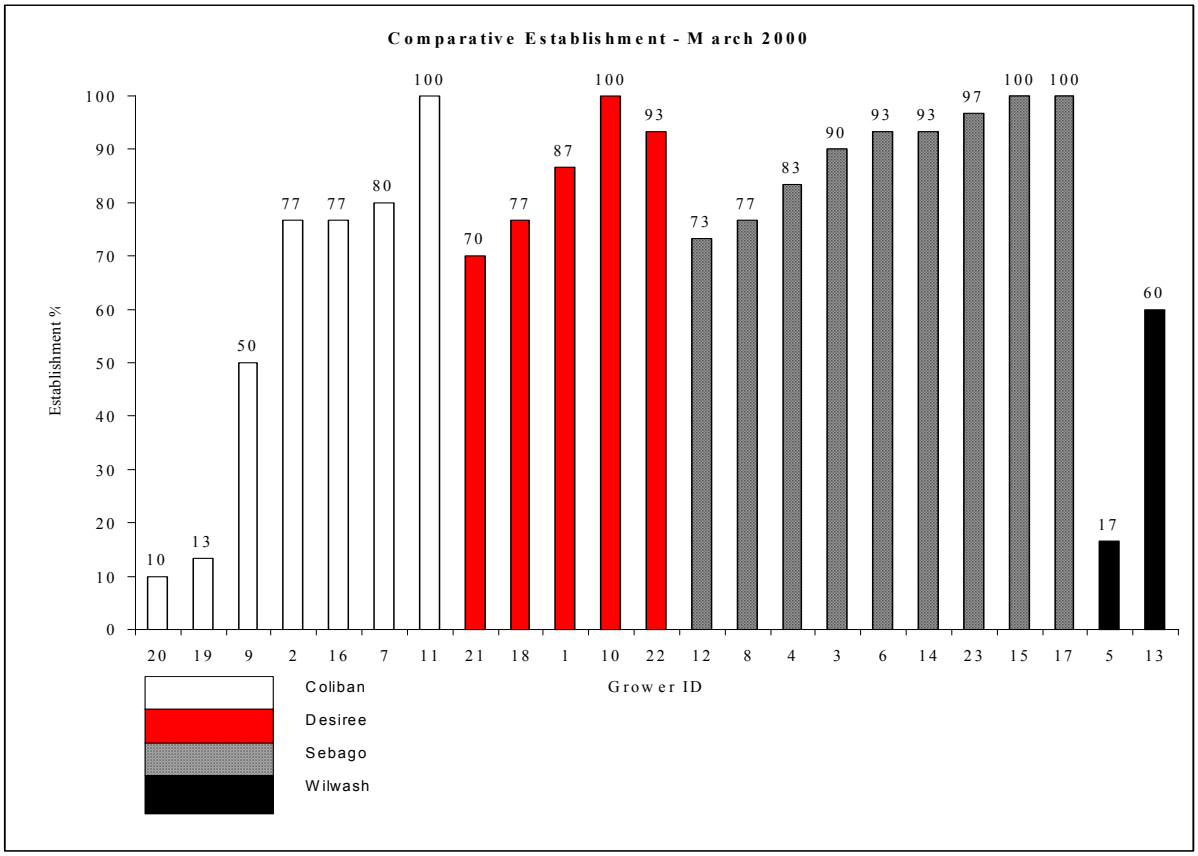


Figure 3.2.5 Establishments rate for Growers seed from a trial planted in February 2000 at Yanco. NB Certified seed was supplied by growers: 10, 11, 15.

Grower produced seed was able to match the performance of certified seed in Coliban and Sebago however the variability of the establishment rates observed between growers indicates that some factor associated with individual growers may influence the health of the crop produced. These factors are not necessarily associated with a grower's expertise and infrastructure (eg hygienic handling, irrigation scheduling, production site characteristics or storage facilities) but may reflect the level of inoculum brought in with the original certified seed and then amplified through grading (see Section 3.4).

The expected positive correlation between tuber calcium levels and establishment did not occur under the conditions of this trial. As shown in Figs 3.2.6 and 3.2.7, neither increasing levels of cell wall or calcium correlate with increased establishment. The direction of the correlation between calcium level and establishment is negative, however it is so weak (<0.09) that it is not significant for either Coliban or Sebago. These observations suggest that factors other than tuber calcium levels could predict susceptibility to establishment losses.

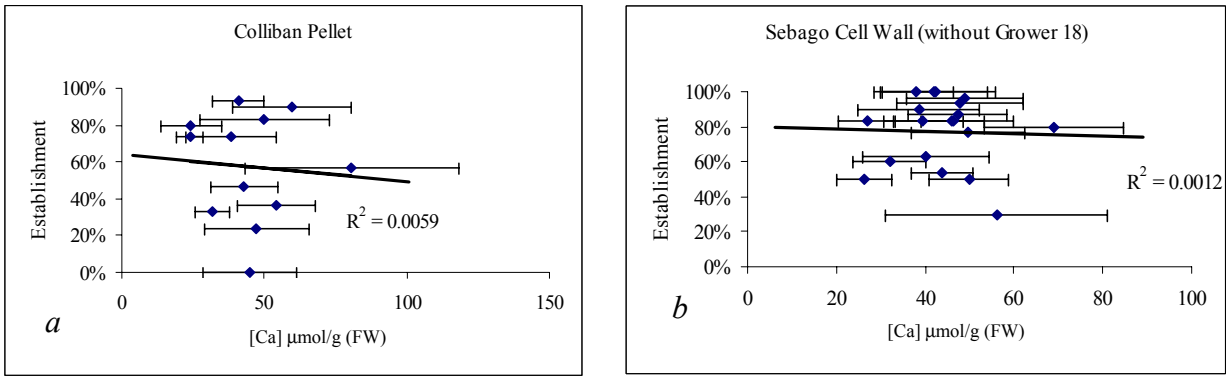


Figure 3.2.6 Association between observed crop establishment and levels of calcium from the cell walls of a) Coliban and b) Sebago seed tubers collected from Riverina growers from their February planting in 1999.

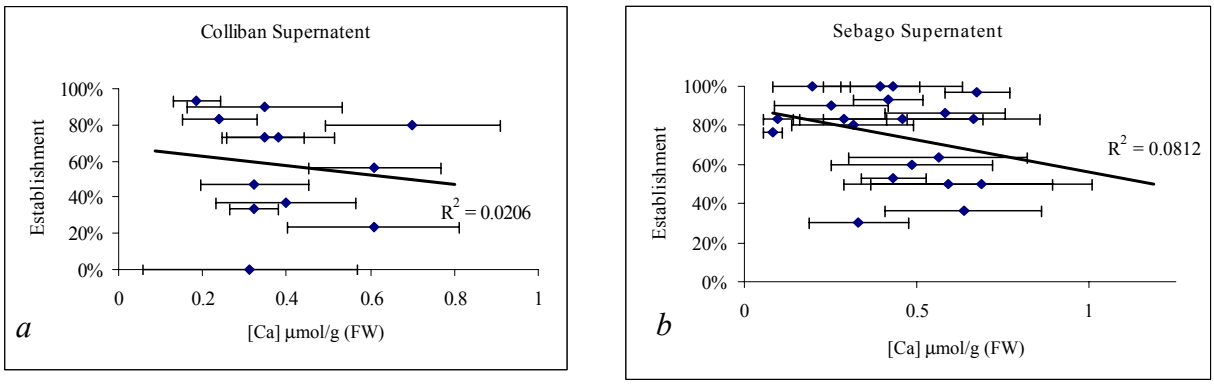


Figure 3.2.7 Association between observed crop establishment and levels of calcium from the cytosol of a) Coliban and b) Sebago seed tubers collected from Riverina growers from their February planting in 1999.

Seed source has a stronger influence on a sample's predisposition to rot than does the level of calcium either in the walls or free in the cytosol. The most likely reason for loss of the seed tubers is determined prior to planting by the presence of pathogens already on or in the tubers

Conclusion

Given that Ca plays an important role in the structural integrity of plant cell walls (Bain *et al* 1996, Bush *et al* 2001, Parker *et al* 2000), tolerance to heat stress (Jiang & Huang 2001), intra-cellular signalling (Reddy *et al* 2002) and the regulation of defence mechanisms (Kim *et al* 2002) it is likely that deficiency would predispose affected tubers to loss by soft rot infections. The observed variability in tuber calcium levels suggests that manipulation of Ca levels is possible, however there is no evidence from this trial to suggest that enhancing tuber calcium beyond the levels encountered would protect the tubers from rot.

White (2001) concluded that functional separation of available Ca^{2+} into an apoplastic pool, for transfer to the shoot, and a symplastic pool, for root nutrition and cell signalling, means that roots can supply both pools without competition or interference for the resource. Physical symptoms of deficiency were not apparent in either the tubers or the crop so there is nothing to suggest that any nutritional deficiency is responsible for predisposing tubers to soft rot. Factors other than calcium appear to have influenced the establishment of the tubers collected from the Riverina farms in this study.

It could be argued that increasing the sample size of the tubers collected and planting them in a replicated randomised block would help eliminate the possibility that spatial factors (eg soil variation or proximity to a sprinkler head) may have influenced establishment, however except in extreme circumstances the tuber's water and carbohydrate reserves are all that is needed to produce and establish a shoot.

Pathogens carried either on or in tubers are probably the most important factor in the development of soft rot. Rot organisms such as *Erwinia* are near ubiquitous in the production environment and are often imported with the original seed (Pérombelon & Kelman 1980, DeBoer 2002). Typically growers do not use seed more than one generation away from a certified generation and in this study establishment rates for certified seed exceeded 80%. The very low levels of establishment observed in some grower produced seed (<20%) implies that farm practices may be involved. It is known that rough handling and practices such as seed cutting or washing are responsible for spreading and amplifying Ec. in tubers (Pérombelon & Kelman 1980, Pérombelon 2000), and anecdotally it seems most growers are unaware of this. The most widespread practice for seed selection and retention involves grading out the larger tubers for the market and keeping the smaller tubers as seed for the next crop. It is proposed that this process may amplify the proportion of tubers carrying infections. This mechanism is elaborated in Section 3.3.

3.3 Filial Transmission of *Erwinia* - Its Consequences and Implications for the Management of Soft Rot in Potatoes

Introduction

Tuber soft rots attributed to *Erwinia carotivora* (Ec.) are frequently imported with seed (Helias *et al* 2000, Luamb *et al* 1986, Pérombelon 2000, Pérombelon & Kelman 1980). The seed potato certification scheme exists because there is high risk of transmitting diseases in vegetatively propagated crops as the crop and the germ-line are one and the same and so are subject to the same diseases (Janse & Wenneker 2002), however certified seed potato tubers are frequently infected with Ec. (Pérombelon *et al* 1976). Currently commercial growers buy certified seed for spring planting and following a mid-summer harvest, grade out tubers for planting 6-8 weeks later, when environmental conditions favour rot development. Although the routes of transmission for Ec. are well documented (Pérombelon 2000, Pérombelon & Kelman 1980), just how the incidence of soft-rot disease can amplify within an affected population has not been adequately investigated by researchers.

Many bacterial genera exist as endophytes within potato tubers and some are capable of producing soft-rots (Pérombelon 2000, Pérombelon & Kelman 1980, Sturz *et al* 1999). The question as to whether Ec could be counted amongst these is not widely supported by the literature and is viewed as unlikely by Cother (2002 pers com). Helias *et al* (2000) however was able to isolate Ec from within symptomless plants throughout their life cycle, their study suggests that transmission of latent infections can occur through a systemic pathway. Pérombelon (2000) notes that Ec. are found in the transmitting tissue of even healthy plants but that quiescent colonisation of a daughter tubers lenticels is the result of bacteria washed onto it from a rotting seed and may not experience the right conditions to manifest rot. In his review, Pérombelon (2000) acknowledges the opportunistic nature of Ec. as a pervasive, facultative pathogen/saprophyte, so it is not inconceivable that it exists and is transmitted internally. Amongst asymptomatic tubers, it has been reported that Ec. is detected more frequently from the haulm ends rather than the terminal ends of tubers (De Boer 2002) – this strongly suggest that infections are passed systemically from one generation to the next.

It is obvious that when Ec. infected tubers rot before emergence that no daughter tubers will be set however the consequences for daughter tuber formation, after the vines become established and suffer seed piece loss has received scant attention. An investigation by Bohl *et al*. (2001) of Russet Burbank yields for U.S. No. 1 grade tubers (i.e. those free from external defects, with a minimum diameter of 4.5cm) found that the maintenance of the seed tuber positively contributed to yield but that the effect tapered off towards flowering. An earlier study by Denney (1929), also noted a decline in yield disparity in the cultivar Irish Cobbler following matriotomy (i.e. excision of the mother or seed tuber) the later into the season the procedure was performed and at some point matriotomy actually increased yields of the variety Bliss Triumph.

It is known that even latent Ec. infection of seed affects yield (Pérombelon 2000) and that grading seed from one-off certified crops is near universal practice. It is therefore hypothesised that a differential develops between the tuber yield components of infected and uninfected plants in the category of tubers chosen for seed. Thus the objectives of the present

study were to look for evidence of filial transmission (i.e. from seed to daughter tuber) and to document and understand the effect of infection on the components of tuber yield in terms of the demographics of daughter tuber weight distributions. The fresh market variety Coliban was chosen as a model for this study as it is very susceptible to Ec. and is in high demand by Australian consumers.

Materials and Methods

Field Observations

A rot affected commercial Coliban crop was identified close to the Yanco Agricultural Institute in the Autumn of 2000. Thirty plants, in close proximity, were dug up five weeks prior to commercial harvest. The plants were classified as either possessing an intact (Healthy) or a rotted (sick) seed tuber. Individual tuber weights for each plant were then recorded. Each tuber was then assigned to one of five weight categories: 0-50, 50-100, 100-150, 150-200 or > 200g depending on the health of the seed tuber. Frequency distribution plots for the five tuber weight categories were then used to compare Healthy and Sick plants and an ANOVA used to determine the significance of the results.

Experimental manipulation

Early generation Certified Coliban seed tubers were obtained from the Crookwell Potato Association to reduce the risk of importing Ec onto an experimental site at YAI which had not previously been used to grow potatoes or any other vegetables. The seed tubers were divided into two treatments: either let alone (control) or injected with 5µl of Ec. (treatment). culture. In a second treatment, tubers were injected with only one then thousandth (10^{-4}) of the Ec. used in the previous inoculation. Both treatment and control tubers were allowed to cure for 24h under ambient conditions before planting in September 2001.

Yield components of ten treated and ten control plants were collected and analysed in a similar method as described for the Field Observations. These tubers were then individually wrapped in polythene film and incubated at 38^{o}C and 70%RH for 40 days to encourage native rots and the resultant rot development was recorded.

Following harvest of the experimental crop in late December 2001, tubers from the treatment and control plots were graded into small (<100g), medium (100-150g) and large (>150 g) tuber categories. Fifty tubers for each for the three size categories of both original treatment or control plots were then replanted in February 2002 and establishment levels recorded after 9 weeks, which were used as an indirect measure of disease incidence.

Results and Discussion

No significant yield differences, on a per plant basis, were detected within the commercial field crop or the experimentally manipulated crop between healthy and sick, or treatment and control plants (Figure 3.3.1). There was however a significant difference in the average tuber size between the two infection classes of both the field and experimental crops. A highly significant difference was found between the mean tuber weight for the healthy plants compared to sick plants (160g cf. 122g). Within the experimental crop the observed

difference (control=70 cf. treatment=84g) was only just significant at the 5% level. Factors such as the unknown and presumably unequal physiological age of the seed tubers in Expt 1 & 2 and their growth at different sites in different season may mean absolute comparisons unreliable; however the existence a differential between the two infection classes is evident.

Examination of the distribution of the 50g yield components as set out in Figure 3.3.2 shows why the difference in average tuber weights between the two infection classes has occurred. Large differences in the proportion of tubers in the classes 50-100, 100-150 were recorded between infection classes within the field and experimental site. Other differences such as the absence of tubers <50g within treated plants and the reduced number of tubers >200g at the field site were also noted.

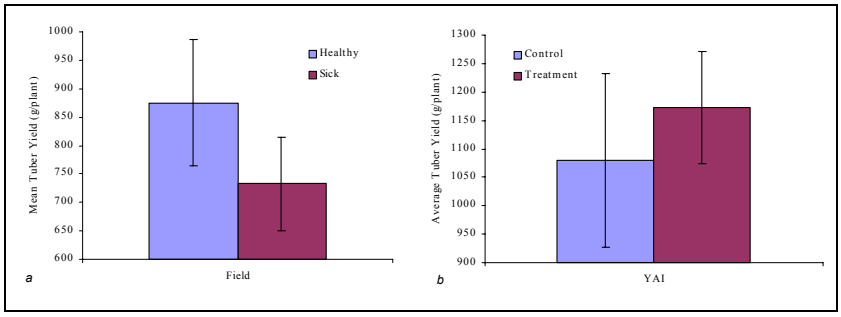


Figure 3.3.1. Yield differentials in ***a***, commercial Coliban crop displaying plants with intact (Healthy) seed tubers, compared to those with rotted (Sick) seed tubers and; ***b***,1-off Certified Coliban tubers where the Certified parent generation treatments were either let alone (Control) or infected with Ec. (Treatment). Standard Error bars shown.

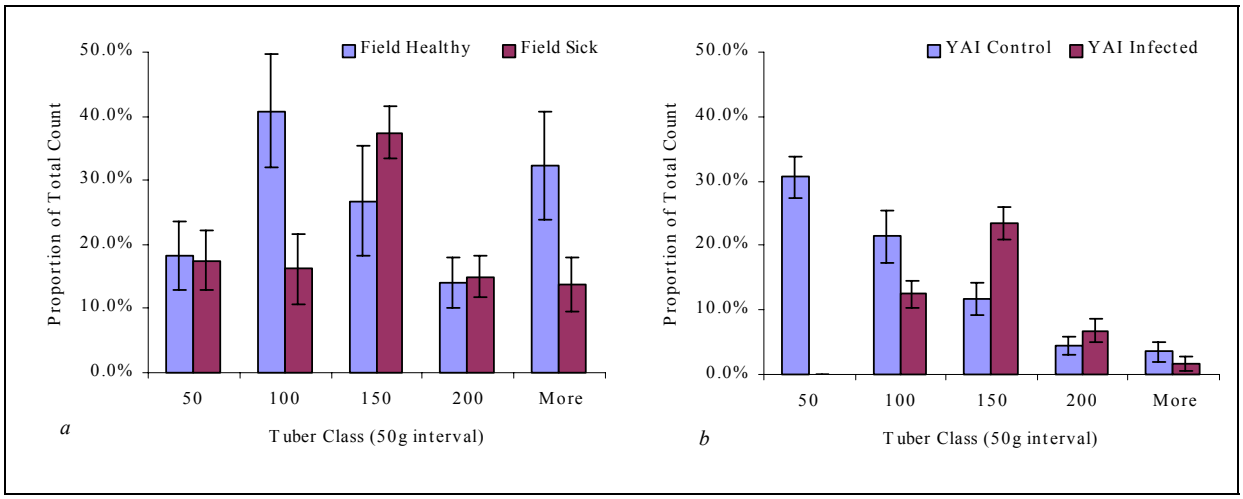


Figure 3.3.2. Weight-Class differentials for proportion of tubers in ***a***, commercial Coliban crop displaying plants with intact (Healthy) seed tubers, compared to those with rotted (Sick) seed tubers and; ***b***,1-off Certified Coliban tubers where the Certified parent generation treatments were either let alone (Control) or infected with Ec. (Treatment). Standard Error bars shown.

An analysis of variance of the proportion of tubers in the size class 100-150 at YAI show a highly significant difference between the numbers of control and treated (infected) tubers set. The difference for the same class of tubers within the commercial field crop was only just significant at the 5% level, however the pattern was the same - more tubers as a proportion of the total are set by infected plants. The tuber set differential is more easily seen in Figure 3.3.3 which has been standardised to the uninfected (Healthy & Control) treatments.

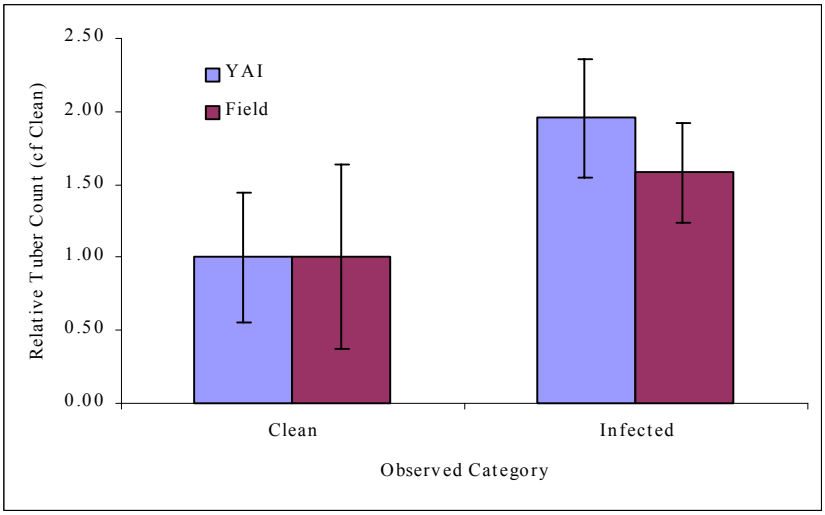


Figure 3.3.3. Counts conducted on deliberately infected stock (YAI) and within plants from a commercial crop (Field) comparing tuber set in clean and infected plants and standardised to the clean category for the tuber range 100-150g (95% CL shown).

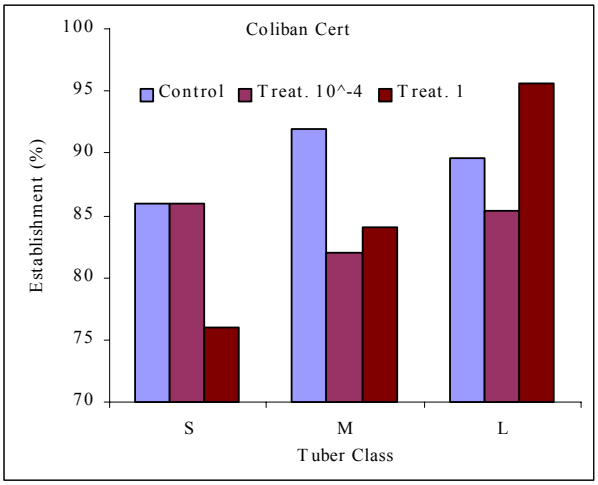


Figure 3.3.3. Comparison of Coliban crop establishment following grading procedure for crops initially grown from untreated (Control) or Ec infected seed (Treat 1 & 10^{-4}). Grade classifications: Small (<100g), Medium (100-150g), and Large (>150g).

When placed into conditions likely encourage rot, none of the tubers from the ten control plants rotted whereas 7.1% of tubers originating from 6/10 plants grown from infected seed-tubers rotted. In another trial, sourced from seed tubers infected with only one ten

thousandth (10^{-4}) of the concentration of Ec. injected into the treated tubers, the . Tubers descended from the low level infection (10^{-4}) treatment, exhibited weight frequency distributions almost identical to the control, however 30% of these tubers representing tubers from all 10 treated plants rotted exposed to rot conducive conditions. As shown in Figure 3.3.3, when replanted into the field after grading 86% of small, 92% of medium and 89% of large control treated established compared to 76% of small, 84% of Medium and 96% of Large Treated (infected source) tubers established. The improved establishment associated with larger seed has also been associated yield increases (Divis & Barta J.(2001).

In addition to soft rot acquired though mechanical injury this study supports the view that *Erwinia* transmission from parent to progeny tubers does occur. In the absence of washing or cutting of seed it is probably the main conduit for disease transmission. Even when inoculum is present within the seed pieces at planting, crop establishment and yield potential may be unaffected as long as other risk factors such as high temperatures or low oxygen tensions do not coincide with this period. This is backed by the observation that during cool storage no tubers rotted and during the incubation of tubers larger ones appeared to be more susceptible to rot - presumably as oxygen diffusion into the tissue mass would be less because of their reduced surface area to volume (data not shown).

Increased yield, as a consequence of loss of the seed tuber, was recorded within the experimental crop but not in the commercial crop, however the results were not significant. We suspect that the effect does occurs under certain circumstances and has been reported in other varieties (Denney 1929) but this needs further investigation. Why this happens probably depends on the strength of antagonistic photosynthate sink signals originating from the various daughter tubers forming on the plant. In healthy plants, continuity between the shoot systems is maintained via the seed tuber until just before harvest when it rots away however this is not the case in plants where individual shoots prematurely separate because the seed tuber has rotted. In such disarticulated plants, each shoot effectively acts as a separate plant and the number or size of its tubers can not be affected by other tubers forming on stolons not directly connected to it. Work on processing potatoes by Tizio & Tizio (1981) and De-Bottini *et al* (1981) found that excised sprouts commenced tuberization much earlier than those remaining attached to the seed tuber and that the higher levels of gibberlic acid appeared to play a role in suppressing tuber formation in the intact plants.

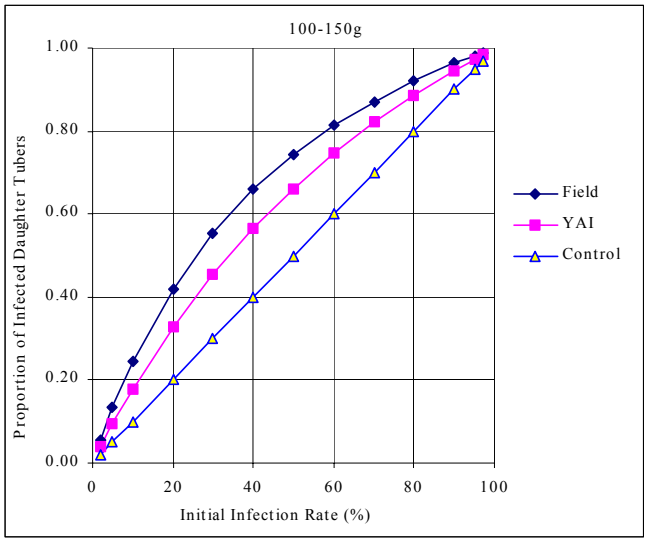


Figure 3.3.5. Infection amplification model based on observed differential tuber weight sets in soft-rot affected. Coliban crops at two sites: Field, a commercial crop and YAI, infection trial conducted at YAI. Model shows proportion of infected daughter tubers following grading (100-150g) from initial levels and a standardised Control.

There is strong evidence that the proportions set between the different weight categories of daughter tubers is affected by loss of the seed tuber. We suspect the variation observed between the field and experimental data sets can be explained by differences in the timing of seed tuber rots. If rot-conducive conditions occur at planting then there may be no subsequent crop and if they occur very late in the season then it's likely that no effect will be seen. If plants barely manage to establish then it is reasonable to speculate that only small tubers will be set - as occurs when plants are established from small cuttings. Intermediate transitional frequency distributions of tuber weights should also be expected. This assertion could be readily tested in a series of growth chambers running at different temperatures. In figure 3.3.5 these speculated changes are modelled using the Yanco field infection trial (Developmental Stage E).

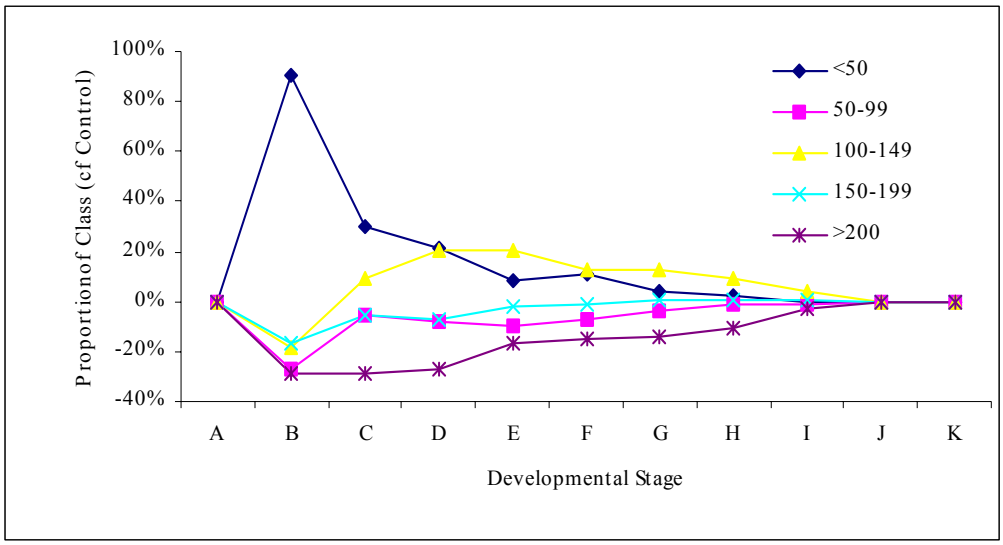


Figure 3.3.5 Speculated change in frequency distribution of 50g tuber weight interval classes associated with seed tuber rot at differing times/developmental stages.

The data gathered from this study strongly suggests that amplification occurs in the range 100-150g, however this range will probably change depending on the timing of the rot. Using the differential profiles gathered form this study it is possible to model the amplification of the diseased proportion of the crop, following grading of the 100-150g tuber category. Figure 3.3.6 show the modelled amplification occurring within the field and experimental crops under scenarios ranging from 0-100% initial infection levels of the seed.

The proposed differential tuber-set model makes the assumption that all tubers set by infected plants will carry the disease. This may be an oversimplification as not all the potentially infected tubers rotted when incubated or were field sown, however this may be a limitation of the methods used to elicit rot in the tubers. Significant consequences of the differential tuber-set model for the management of Coliban crops are that that there is a decrease in amplification relative the initial level of infection in the seed pieces for the range 100-150g (Figure 3.3.6). For the ranges 50-100g and >200g the converse is true (Figure 3.3.2). As shown in Figure 3.3.5, these weight classes in non infected plants decrease in proportion to the same weights in the infected plants - so growers should be able to actively select for reduced rot potential. Such predictions need to be tested using a field-scale experiment,

however care should be taken in assuming that the over-represented infected tuber weight-class will remain in the range 100-200g as this is likely dependant on the timing of the seed piece's rot.

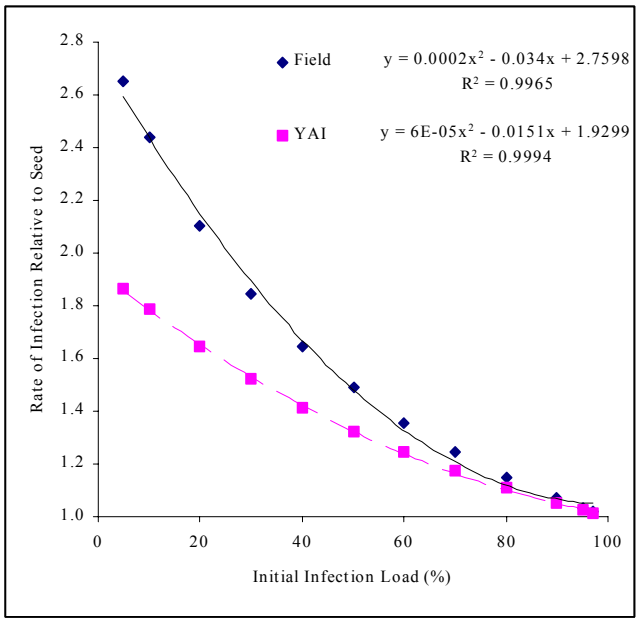


Figure 3.3.6. Modelled rate of change of infection based on differential tuber set (100-150g) between soft-rot affected and clean cv. Coliban crops at two sites.

Conclusion

The opportunity for disease amplification occurs when infected tuber classes are preferentially retained even where some original tubers carry sub-clinical infections - the *Erwinia* is passed onto the daughter tubers. Once infected, clinical or obvious symptoms (tuber rot) occur only when other parameters needed for the bacterial proliferation are met (principally a susceptible host, elevated temperatures, low oxygen tensions and time).

Under some conditions sub-clinical *Erwinia* infections of seed tubers may provide a yield bonus however tubers derived from these are potential time bombs, weather they are consumed or replanted. Any soft rot management strategy should aim to minimise the crop's inoculum load and not just clinical manifestations for the weeks following harvest. To do so is risks the loyalty of consumers to the product or alternatively gambles crop establishment costs against buying in clean seed.

There is some evidence that *Erwinia* exists endophytically within the tuber and is internally transmitted (Helias *et al* 2000, Pérombelon 2000) so that even the most rigorous surface disinfection techniques will be ineffective. If seed tubers are to be produced by ware growers then they need to manage a seed block rather than grade out seed pieces from their ware crops. Within defined seed productions area, infected plants could be rogued and at harvest tubers unsuitable for seed graded out. Seed producing areas within a production area have the advantage that immediate environmental conditions should be more or less uniform, so that plants within it aren't subjected to differing soils, irrigation patterns or aspects all of which could theoretically influence rot development.

3.4 Effect of Cool Storage, Temperature and Light Conditioning on the Subsequent Development of Soft Rot in Potatoes

Introduction

Episodic outbreaks of tuber decay attributed to the bacteria *Erwinia carotivora* can severely constrain the economic viability of potato growing operations in the Riverina and MIA regions of southern NSW. The retention of non-certified seed tubers from a previous crop for later establishment of the autumn crop has been cited as a major contributing factor to these outbreaks. As this is the hottest part of the year, infected tubers are at extreme risk of rotting before they are even planted if they are not cool stored.

Cool storage is an effective method of extending the useful life of seed potatoes and ensuring a quality product with minimal disease. As with cool-stored food, the activity of most spoilage and pathogenic organisms is greatly diminished and the metabolism of the tuber itself is also slowed so that vital processes like the amount of time taken to break dormancy is increased. The time taken to recover from extended periods in cool storage is much less for micro-organisms than it is for large multi-cellular organisms such as potato tubers. This recovery period creates a window of opportunity for any invading pathogens to proliferate before the tuber's defence mechanisms become fully operative. Handling operations such as grading, washing, cutting and planting place tubers at risk since they will inevitably receive mechanical injuries and will transfer pathogens from infected to clean tubers. One way to diminish the risk to cool stored tubers is to condition them to the ambient environment before handling them.

The practice of chitting (greening and spouting tubers prior to planting) has long been said to increase establishment rates (Valkonen *et al* 1996, Watts and Watts 1943). A proposed reason for this is the induction of gylcoalkaloid (GA) synthesis, through greening and sprouting (Percival *et al* 1998, Valkonen *et al* 1996). Elevated GA levels have been associated with reduced microbial and pest activity both in *vitro* and in *vivo* (Engstrom *et al* 1999, Gubarev *et al* 1998, Percival *et al* 1998, Rangarajan *et al* 2000, Rodriguez-de-Sotillo 1998, Saour *et al* 1999, Sarquis *et al* 2000, Valkonen *et al* 1996).

This study investigates the time taken for tubers to achieve maximum resistance to the action of *Erwinia carotivora* following deliberate inoculation following removal from cool storage. In addition to temperature conditioning, another protocol used sought to investigate the possible influence of illumination on the tuber's ability to resist rotting.

Materials and Methods

Effect of illumination on resistance to rot

Certified tubers of cv. Colliban were supplied from Crookwell NSW, 13 days after harvest. 100 tubers of 50-100g fresh weight were randomly selected and placed in a growth chamber at 25^{o}C, with half being placed in the dark and the remainder exposed for 12h/day to 80W fluorescent tubes. Batches of 20 tubers were removed from each treatment (light or dark) 1, 2, 4, 8 and 16 days after the initiation of the experiment. Ten tubers were analysed for glycoalkaloid content and the remaining ten were inoculated with *Erwinia carotovora*.

Ten tubers were soaked for 30 minutes in a 10% available chlorine solution (dilute calcium hyperchlorite). Each tuber was then stab inoculated with 20µL of a strong Ec. culture (see section 3.4 for preparation details), weighed, wrapped in polythene film, numbered and placed in a plastic container and incubated at 25°C for three days. After incubation the tubers were cut in half, the rotted material washed away and re-weighed.

Effect of temperature conditioning on resistance to rot

Two following harvest tubers of cv. Colliban were place into cool storage at 5°C for three weeks. 10 tubers were removed on each sample day (Day: 0, 1, 2, 4, 12, or 16) and placed into an unlit growth chamber with a 12h cycle of 15-25 °C. These tuber samples were then

Results and Discussion

Effect of illumination on resistance to rot

Figure 3.4.1 illustrates the resistance of tubers to Ec. No difference was found in rot resistance between light and dark treatments however significant changes to resistance were found over the duration of the conditioning period.

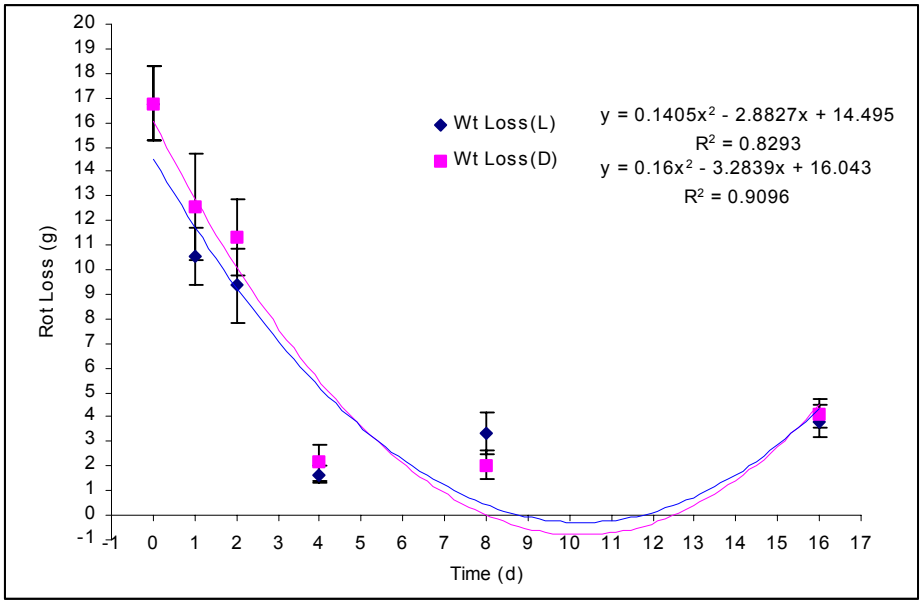


Figure 3.4.1 Resistance to *Erwinia* digestion of tubers placed either in the light or in the dark following removal from cool store.

The tubers subjected to 12h illumination per day for 16 days exhibited visible greening but this did not advantage the treatment over the control tubers. While elevated GA levels might be expected under illumination, selection pressure to reduce levels has probably pushed capacity below any therapeutic threshold in commercial varieties. GA levels encountered by consumers in Australian supermarkets have been found to be below the acceptable MRL even in green potatoes (San *et al* 1993). This area of investigation is in need of further study as the induction of GA in seed tubers of some varieties could possibly lead to a reduction in pest

and pathogen outbreaks however temperature conditioning had a much greater effect in Coliban than did any light effects.

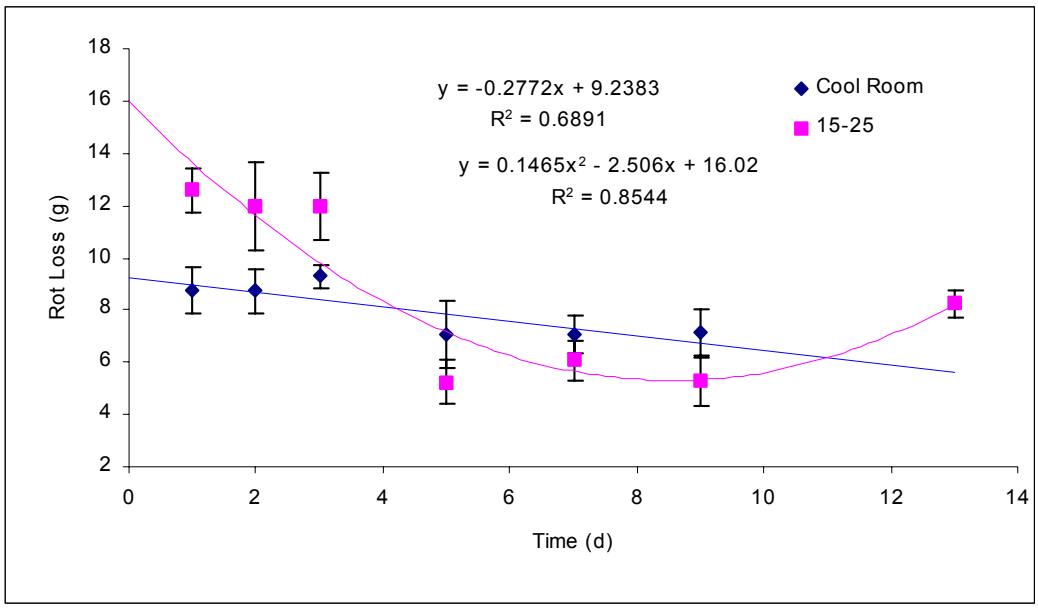


Figure 3.4.2 Resistance to *Erwinia* digestion of tubers exposed to simulated ambient temperature conditioning (12h @ 15 ^{o}C : 12h @ 25 ^{o}C) or left in a cool storage (5 ^{o}C).

The rot resistance of tubers removed from the cool room was consistantly found to increase over time. Figure 3.4.2 compares changes in rot resistance in tubers exposed to simulated ambient temperatues with those remining in the cool room. The rot resistance of tubers left cool storage held more or less constant over 9 days in which resistance was measures and no statistically significant difference could be found over that period. However for the simulated ambient treatment the 68% relative decrease in resistance over time (days 1-3 cf. days 5,7,9) was highly significant (P= 0.001, CI=1%, DF=43). The observed rot at time 0 of 13.31g (±1.13 SE) was excluded from the model because the true rot potential will have been compromised by the 3d incubation period. The incubation period needed to judge resistance partly confounds the results by intoducing a 3d contitioning period to all treatments. Measures to eliminate conditioning during incubation (eg by irradiating the tubers) would probably be biologically meaningless as neither bacterial digestion or induced resistance happen instantly.

Comparing the fitted line models (figures 3.4.1 and 3.4.2) for the observed decrease in rot resistance over time, it can be seen that maximum resistance occurs some time after day 8. Other trials (data not shown) also show that this point may be reached up to 11 days after removal from the cool room.

The resistance to rot observed during our trials reflect changes to tuber metabolism during a conditionsing period which quickly brings them to ambient temperature. In large bulk stored tubers, the much larger thermal mass would greatly increase the energy required to increase their temperature. Without active measures (eg forced air warming) bulk tubers will take

much longer to condition (i.e reach ambient temperature) than the isolated tubers used in our trial. Allied with this is the danger of atmospheric water condensing on tubers brought straight out of the cool-store into ambient conditions. Tubers subject to such conditions may develop a film of water over their surfaces, restricting respiration and thereby greatly favouring rot development.

Conclusion

Tubers removed from cool storage need time to be conditioned to ambient temperatures before being handled or they are at increased risk of rot should they carry or come into contact with pathogenic *Erwinia* spp and/or be exposed to conditions which favour their development (i.e. hot, wet, & anoxic). In the case of isolated tubers, maximum resistance is reached sometime between 8 and 11 days after removal from the cool room. To ensure that the centres of bulk stored tubers have achieved ambient temperature and thus attracting aviod condensation, it is recommended that gradual warming over a two week period, be done in conjunction with temperature monitoring.

3.5 *Invitro* Screening for Microbial Antagonists against *Erwinia carotivora*

Introduction

Competition between tuber rotting organisms is to some degree an arms race. Toth *et al*. 2003) demonstrated that antibiotics produced by *Erwinia carotivora* ssp. *chrysanthemi* (Ech) inhibit the growth of potential competitors, so it may be that competitors also have similar chemical defences. Reiter *et. al*. (2002) reported that as many as 38% of bacterial endophytes did inhibit Ec. in tissue culture. Since tuber soft-rots caused by *Erwinia carotivora* (Ec.) start at or near a potato tuber's surface, either on wounds or within lenticels (Scott *et al* 1996, Pérombelon 2000) we decided to investigate if superficial infections could be amenable to topical application of an antagonistic microbial agent. For various reasons, treatment with antibiotics is undesirable and unlikely to be cost effective for the treatment of Ec. on potatoes. Biological control provides a more desirable choice for the treatment of Ec. in potatoes, however no specific agents have been released. Successful control of other bacterial, fungal and nematode diseases has been gained with preparations of *Streptomyces* sp, but no efficacy claim has been made against Ec. .

Materials and Methods

Two sites were surveyed for possible microbes antagonistic to Ec. Site 1; experimental potato plots at Yanco Agricultural Institute and Site 2; potato waste disposal area of a commercial farm at Euroley. Both sites are located on sand hills, formed from the red sandy loams which are typical of the potato growing soils of the Riverina. Site 1 was chosen because it was close to the laboratory and had a number of potato cultivars growing at the time (Coliban, Desiree, Purple Congo, Sebago). Site 2 was chosen because it was close to the laboratory and it was thought that the waste area would support a large number of microbes which would have to strongly compete for the available resources.

Sources of microbes

Erwinia carotivora

The Ec. culture used in these experiment was grown on sterile Nutrient Broth (Oxoid Chemical Company) incubated in a shaker bath for 48 h at 27^{o}C. The original Ec. mother culture (DAR 33861) was obtained from the bacteria collection of NSW Agriculture at Orange Agricultural Institute, Orange, NSW 2800.

Soil & decaying tubers

Two gram samples were placed into 20ml of sterile distilled water and the suspension shaken vigorously. From this stock a 200µL sample was taken and smoothed over the surface of a ¼ PDA petri-dish and, incubated at 21^{o}C for 3-4 days. Once visible, colonies were picked off and purified before testing on an Ec. lawn. Alternatively the soil suspensions would be streaked directly onto the Ec. lawn and evaluated for inhibitory effects. Five plates were made from each soil sample.

Tuber Skin

Tubers were washed and excised sections of peel were placed on to the surface of Ec. lawn cultures (details under *In vitro* testing for inhibition), incubated at 21^{o}C and examined daily for signs of microbial growth and inhibition of the surrounding Ec. lawn.

Isolation of purification of cultures

Bacterial colonies suspected of inhibiting Ec. were streaked onto sterile ¼ PDA (Sigma Chemicals) in order to separate the constituent cells. Streaked plate cultures were incubated at 21^{o}C for 48h to allow visible colonies to develop. The separated colonies were then evaluated and obviously different colonies were individually picked off and smeared onto the surface of another sterile ¼ PDA plate to create a pure culture. These pure cultures were again tested on an Ec. lawn for inhibition of the background colony. Non –inhibiting cultures were discarded.

Fungal colonies generally grew rapidly under the conditions of the culture, engulfing nearby bacterial colonies making it difficult to separate them. Subculturing from the edge of mycelium once or twice was generally enough to separate contaminant bacteria which were themselves tested for their ability to inhibit Ec.

Evaluation of microbes

Invitro testing for inhibition

Lawn cultures of Ec. were established on sterile ¼ PDA in petri – dishes, 24h prior to the introduction of potential antagonists. Lawns were made by placing 200 µL of Ec. culture onto a sterile ¼ PDA plate and smoothing it over the surface with a glass rod. Cultures were then incubated overnight at 21^{o}C before being used.

Invivo testing for inhibition of Ec.

Potato tubers or tuber slices were inoculated with the alleged antagonistic and were then either challenged with *Erwinia* and the progress of the soft-rot recorded or they were examined for signs of pathogenicity attributed to the alleged antagonist

Tubers of Cv Coliban were cleaned and surface sterilised by washing under running water and then placed in 10% chlorine bleach for 30 min before rinsing in tap water. The tubers were allowed to drip dry and, while working aseptically in a laminar flow hood the tubers were thickly sliced (~6mm) with a sterile knife. Thirty slices were placed on damp paper-towelling in trays. For each alleged antagonist, 1/2 of the slices on a tray were inoculated with the alleged antagonist by placing a drop of liquid culture onto the surface of the potato slice or the case of fungi a section of agar (~3mm^{3}) was placed onto the surface of the tuber slice and the treatments identified. Trays were then covered with polythene film, taking care not to let the film come in contact with the slices and incubated at 25^{o}C for three days to allow the colony to establish. Following the incubation and pre-treatment with the alleged antagonist the trays were uncovered and challenged with Ec. or a control. Under aseptic conditions, a drop of either live or boiled liquid Ec. culture was placed onto the centre of the

slices. Boiled Ec. culture was used as a control to guard against the possibility that the culture medium itself may have been phytotoxic. The trays were then recovered in polythene film and placed into an incubator for 48h and any lesions noted.

Inv*ivo* testing for pathogenicity of likely Ec. inhibiting microbes

Potato tubers or tuber slices underwent a stab inoculation with the alleged antagonist, were covered and incubated at 21oC for a minimum of 4 day before evaluation. Visual signs of pathogenicity (eg rots) attributable to the alleged antagonist were recorded.

Results and Discussion

Site 1. No inhibition was observed in any 20 streaked plates from the 4 soil samples taken. No inhibition was observed around skin pieces from the cultivars Coliban, Sebago, Purple Congo (2 tubers per variety, X 5 plates/tuber and 3 pieces of skin per plate). Hyphae originating from the peel of one of the Desiree tubers did show inhibition of the Ec. lawn surrounding it. The hyphae, transferred and grown in pure culture were typical of *Fusarium* spp. When placed onto a freshly cut surface the fungus established and formed a dry-rot lesion. If challenged with Ec, inoculation was unable to prevent the progress of bacterial soft-rot however this may be a function of the level of inoculum used since the number Ec. cells applied may be more than is usually encountered in a new infection. While further work could have been done to investigate this possibility, the fact that the proposed organism caused dry rot excluded it as a candidate as a biological control

Site 2. Soil was taken from on two separate occasions, with 4 + 7 soil/composting potato samples taken respectively. Five plates were taken from each sample and from these 230 separate bacterial colonies and 4 fungi were screened against Ec. As a result of this a single bacterial culture was identified which inhibited Ec. in vitro. When inoculated into whole tubers or onto slices no adverse affects were noted however such inoculation was unable to prevent the establishment of a subsequent Ec. infection.

Conclusion

While no suitable soil or epiphytic microbes were identified by this study, the relative ease with which *invitro* studies can be done show that it is a useful technique for screening large number of bacteria and soil samples. Work on the transmission of Ec., undertaken as part of this study suggests Ec. is systemically transmitted to tubers making topical applications of antagonist ineffective against deeply seated infection. Other researchers have isolated potential Ec. antagonists from amongst the endophytic community of bacteria (Reiter *et. al*. 2002, Sturz *et. al*. 1999). A survey of the range and evaluation tuber endophytes in Australia may well identify a suitable biological control agent. Suitable bacteria could be used to inoculate mother stocks in the certification program. Such an action would not only help ware and seed growers by decreasing the proportion of seed carrying Ec. but would protect tubers once in the hands of consumers.

3.6 Prospects for the control of *Erwinia* in potatoes

It is doubtful if elimination of *Erwinia* induced seed piece decay is possible given that the causal organisms are ubiquitous in the growing environment and because individual growers are unable to control either the climate or the market with regard to cultivar and timing of demand. Management of the disease to limit its impact on enterprise profitability is something that growers have always done but the risk never eliminated. Ensuring that growers have access to the knowledge and clean seed supplies is the most potent tool available. In the future the potato industry might well take advantage of the emerging technology. These are principally:

- Improved Resistance
  - Constitutional (barrier defences)
  - Reactive (acquired defences)
- Lower inoculum load in seed
  - Selective retention of larger seed
  - PCR detection
  - Inoculation with endophytic antagonists

Opportunities for Further Research

The most important discovery from this project was that plants infected with Ec. have a propensity to set more tubers in the smaller class sizes than non-infected ones. Thus if smaller tubers are retained to establish a subsequent crop then the potential for disease can be amplified. This finding needs to be further examined and if possible exploited to find how selective retention could be used to lower disease potential. The economics of such a decision might not always be compatible to the best clinical outcome since disease incidence is episodic and the retention of larger seed would impact on storage requirements, subsequent planting densities, adjustment of planting equipment and fuel consumption. Better models of the relationship between selective retention and disease incidence need to be produced for different cultivars and which incorporate climatic data so that a decision support system can be produced.

A number of approaches have been made to increase the ability of tubers to resist *Erwinia* by priming natural defence systems. Conventional strategies, such as PT98011, seek to enhance constitutional defences while others have attempted to turn on reactive systems, most often phytoalexin production (i.e. the plants chemical defence system). Without major improvements to the genetic potential of potato plants, to increase the rate of calcium translocation, it appears unlikely that increasing calcium fertilization levels alone will ever strengthen cell walls enough to resist the action of *Erwinia*. The complex interplay of multiple genes (host and pathogen) with the environment makes the proposition for genetic improvements a long term goal. Increasing the flow rate of Ca by selecting for increased xylem diameters, would place the plant at increased risk of death during periods of water deficits and decrease overall water use efficiency. The alternate solution of slowing calcium sequestration during tuberisation would increase the time needed to mature the crop and thus increase its risk of exposure to *Erwinia* or other disease and also decrease water use efficiency.

The production of phytoalexins can be stimulated through wounding, exposure to pathogens, or the phytoalexin precursor molecules and even the simulation of these events (Metraux *et.*

al. 2002). The latter has been achieved by genetically engineering tubers to express changes in biochemistry similar to that seen when the tuber is under attack. Because expression of the transformed system is unregulated in these tubers they do not perform as well as the non-transformed plants so there is no advantage except when under attack (Linke *et al.* 2002). The introgression of resistance genes from wild germplasm carries a similar risk of depressed yield and vigour however careful selection and backcrossing can be undertaken to bring the yields of resistant lines into line with their commercial parents.

Genetically engineering a plant in order to permanently activate its existing defence systems is fraught with many perceived and real problems but it not the only method available. It has been demonstrated that it is possible to activate plant defences by exposing them to some of the chemicals produced naturally by plants after they have been challenged with a pathogen. In potatoes successful treatment of some foliar and tuber diseases has been achieved by treating plants with benzothiadiazole and acetylsalicylic acid (Bokshi *et.al*. 2003). In other plants, induction of Acquired Systemic Resistance has been achieved by exposure to jasmonic acid (Heil & Bostock 2002) and this approach needs further investigation in potato. *In vitro* tuberisation studies have shown that application of jasmonic acid may have positive effects on both yield and tuber quality (Pruski *et. al*. 2002)

The microbial screening undertaken during the present study did not produce a suitable candidate for biological control of *Erwinia* and in light of the fact that *Erwinia* infections do not always occur on the tuber surface any topical application of such a bio-control agent is likely to be ineffective. Inoculation of tubers with an endophytic, antagonistic, bacteria, which is transmitted in a similar way as *Erwinia,* from mother to daughter tubers is a strategy which is more likely to succeed. Identifying a suitable endophyte would be more difficult since not all bacteria are suited to in *vitro* culture as they may not grow on the media.

The use of certified seed by ware growers will reduce the chances experiencing tuber loss however new methods need to be developed to detect and eliminate *Erwinia* from certified seed stocks. Since many *Erwinia* infections are latent, growers may be unaware of the level of contamination in their stocks until planting coincides with conditions conducive to rot. Polymerase Chain Reaction (PCR)-based screening technology offers the best chance of detecting even trace contamination of *Erwinia*, so that affected stock can be culled or delivered to environments where the disease is unlikely to proliferate. PCR detection relies on the amplification of fragments of DNA specific to *Erwinia* but that are not found in potato or any other organism likely to contaminate a sample. Such tests are highly specific, and relatively fast and cheap when one developed. *Erwinia*-specific gene primers have been developed and PCR techniques have been used to detect Ec. in asymptomatic tubers (*Helias et al*. 1998 & 2000). If the current primers are suitable then the development of sampling protocols to identify commercially meaningful levels of *Erwinia* contamination also needs to be undertaken.

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ACKNOWLEDGEMENTS

I thank the following people for their skills and cooperation in realizing this project:

Mr Alan Boulton
Mr Jason Hammond
Mr Mark Hickey
Ms Linda Magee
Mr Stuart McVittie
Mr Tony Napier
Ms Karen Ryan
Ms Meryl Snudden
Mr Joseph Valenzisi
Mr Steven Wade
Mr Andrew Watson