STUDIES OF Erwinia SOFT ROT DISEASE ON POTATO

IRDA SAFNI, SP
(Sarjana Pertanian, University of North Sumatra, Medan, Indonesia)

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Department of Applied and Molecular Ecology
Waite Campus
Adelaide University
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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Irdia Safni

July 2001
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Abstract

*E. carotovora* subsp. *carotovora* (*Ecc*), the soft rot bacteria of potato, survives for a long period in lenticels and suberized wounds during storage. The disease is more severe when the environmental conditions favour its development, therefore, managing soft rot disease is a difficult task.

Washed and brushed potato tubers, which were artificially inoculated using different inoculation methods, were used in this study to detect the population of *Ecc* in tuber. The number of *Ecc* were assessed by a plate assay on selective agar medium (CVP). This study also highlights the relationship between artificial tuber inoculation and the population of *Ecc* in the tubers. The effect of chlorine solution and drying treatment were also examined as a means to reduce soft rot development on potatoes.

Although low numbers of *Ecc* were found in the tubers, the lenticel sampling method could detect the bacteria more effectively than the peel method. However, both methods were time consuming and labour intensive. It is suggested that serological methods of detection should be investigated in further studies.

The result of experiments quantifying the colonization of soft rot bacteria revealed a poor correlation between the inoculation level of *Ecc* and the population of *Ecc* in the tubers. This study showed a slightly higher number of *Ecc* were re-isolated from tubers inoculated with 10^6 cfu/ml, both by the dipping and by the infiltration methods. This level of inoculation was used as the preferred inoculation level of *Ecc* for further experiments.
This study revealed that chlorine could not eliminate *Ecc* from the lenticels, both on washed and brushed potato tubers. Similarly, when chlorine was used in combination with drying *Ecc* was not eliminated from the tubers. Nonetheless, either chlorine or drying treatment could reduce the potential of soft rot development of brushed potato tubers. The effect was most dramatic with the drying treatment.

Overall the results suggest that disease development did not correlate with the number of bacteria in lenticels. It seemed difficult to eliminate all bacteria from lenticels. Even if the number of *Ecc* present were low, but the conditions for their growth were ideal (i.e. a moist environment), disease would rapidly develop.
## Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>CO₂</td>
<td>carbon dioxide</td>
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<td>cfu</td>
<td>colony-forming units</td>
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<td>CVP</td>
<td>crystal violet pectate</td>
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<td>cm</td>
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<td>°C</td>
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<td>d.f</td>
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<td>d</td>
<td>diameter</td>
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<td>Eca</td>
<td><em>Erwinia carotovora</em> subsp. <em>atroseptica</em></td>
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<td>Ecc</td>
<td><em>Erwinia carotovora</em> subsp. <em>carotovora</em></td>
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<td>LSD</td>
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<td>NA</td>
<td>nutrient agar</td>
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<td>PM</td>
<td>Peter Murphy</td>
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<td>ppm</td>
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<td>percentage</td>
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<td>SARDI</td>
<td>South Australian Research and Development Institute</td>
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SD
SDW
s.s
v.r
w/v

standard error of means
sterile distilled water
sum of squares
variance ratio
weight per volume
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CHAPTER 1.

LITERATURE REVIEW

1.1 Introduction

Roots and tubers of many species have been part of human diets since the development of food-gathering and hunting society (Burton, 1989). Amongst approximately 2,000 species of tuber-bearing genus *Solanum*, only potato (*Solanum tuberosum* L.) originating in the Andean highlands of South America is extensively and widely cultivated as an important food crop in about 140 countries located in tropical and subtropical zones (Beukema and van der Zaag, 1990; Hawkes, 1992). Potato is mainly utilized for human consumption because it produces more calories at a lower cost than other grain crops (Rubatzky and Yamaguchi, 1997). It is also used for fodder, starch and alcohol production (Beukema and van der Zaag, 1990; Horton and Anderson, 1992).

As an essential source of human nutrition, potato is ranked at the fourth in world production, after wheat, corn and rice (Rowe, 1993). In Australia, potato is the major horticultural crop grown, the total of production 1.37 million tonnes annually with a gross value of A$ 414 million (Australian Bureau of Agricultural and Resource Economics, 2000). The production has increased by approximately 500,000 tonnes per annum for the past 20 years (Williams *et al.*, 1996). This is due to the increasing potato demand, particularly for processed potatoes, since the early 1960s as per capita consumption increased (Bureau of Agricultural Economics, 1976).
Potato is susceptible to a large number of diseases, including bacterial diseases. They may lead to serious crop losses with both quality and quantity of the yield being affected. Amongst these diseases, bacterial soft rot is considered the most severe and common post-harvest disease of a wide range of fruits and fleshy vegetables, including potatoes (Gudmestad and Secor, 1993; Hooker, 1981; Rich, 1983). It affects the growing plant and the harvested crop during storage, transit, and marketing.

This disease is predominantly caused by two closely related species of *Erwinia carotovora*, *E. carotovora* subsp. *atroseptica* (*Eca*) and *E. carotovora* subsp. *carotovora* (*Ecc*). While *Eca* strains occur in cool temperate zones with restricted host distribution (only potato crop), *Ecc* strains are pathogenic to a wide host range in both temperate and tropical zones including in Australia (Cother and Sivasithamparam, 1983; Pérombelon and Kelman, 1980). The disease shows latent infection, which is a common soft rot phenomenon, because the pathogen can survive for a long time either in the intercellular spaces of suberized wounds or lenticels (Pérombelon and Salmond, 1995).

The economic importance of losses due to soft rot disease varies widely from country to country because of the differences in environmental conditions. Furthermore, it is difficult to estimate the amount of losses as they can occur sporadically (Ruehle, 1940). However, it is estimated that approximately $50-$100 million annually in the world potato production (Pérombelon and Kelman, 1980).

This review will describe the soft rot disease on potato, which includes the pathogen, the disease symptoms, host-pathogen interaction, epidemiology, and the disease cycle, as well as the control measures that can be applied. Its objective is to give a detailed background for this study.
1.2 Soft Rot Disease on Potato

1.2.1 The pathogen

The predominant pathogen that causes soft rot disease on potato is *Erwinia carotovora* (Jones) Bergey et al., which is also the main organism causing post-harvest diseases of a wide range of vegetables (De Boer, 1994; Lund, 1971; Rich, 1963). *E. carotovora* belongs to the kingdom *Procaryotae*, family *Enterobacteriaceae* (Buchanan and Gibbons, 1974). On one hand, it has been proposed that the soft rot bacteria be placed in a separate genus, *Pectobacterium* (De Boer and Kelman, 2001) containing two or three separate species (Lazar and Bucur, 1964). On the other hand, most researchers maintain *E. carotovora* as a single species that can be divided into several subspecies with the taxonomic grouping being based mainly on biochemical reactions and not on host specificity (Burkholder and Smith, 1949; Dye, 1969; Buchanan and Gibbons, 1974; Pérombelon and Kelman, 1980). However, the former opinions have been generally rejected since they have lacked support (De Boer and Kelman, 2001).

The two most important organisms that cause potato soft rot disease are *E. carotovora* subsp. *atroseptica* (van Hall) Dye (*Eca*) and *E. carotovora* subsp. *carotovora* (Jones) Bergey *et al.* (*Ecc*) (Lelliott, 1974; Gudmedstad and Secor, 1993). *E. chrysanthemi* (Burkholder *et al.*). (*Echr*) is another soft rot pathogen, but it less commonly causes soft rot. Several other pathogens can also be associated with soft rot destruction including pectolytic *Pseudomonas* *spp.*, *Bacillus* *spp.*, *Clostridium* *spp.*, and *Flavobacterium pectinovorum* (Hooker, 1981; Lovelock, 1979). Of these, *E. carotovora* subsp. *carotovora* is the most common bacteria that cause soft rot on potato tubers (Cother and Sivasithamparam, 1983).

Member of the genus *Erwinia* are gram-negative, non-spore forming, non-capsulate, not acid fast, and facultative anaerobes (Dye, 1969; Pérombelon and Kelman, 1980; Rehle, 1940). The pathogen is short rod-shaped,
approximately 1.5-3.0 µm long and 0.6-0.9 µm wide, rounded at the end, motile with two to many petrichous flagella, and occurs singly or sometimes in short chains (Dye, 1969; Hooker, 1981; Rich, 1983).

*E. carotovora* can be easily distinguished from other pathogens on selective media that contain polypectate, such as Stewarts MacConkey-pectate medium or crystal violet-pectate medium (CVP), because it forms deep pits or cavities after 48 hour incubation at 27 °C (Hooker, 1981; Pérombelon and van der Wolf, 1998).

### 1.2.2 Disease symptoms

The soft rot bacteria can affect the tubers and above-ground parts of plants, but it mostly causes decay to the tubers (Lund, 1979). External symptoms may not be seen and the tubers seem to be healthy, but the pathogen can become active and infect the plant tissue as soon as the conditions favour its development (Pérombelon and Kelman, 1987). The infection of *Ecc* is the result of parenchymatous tissue maceration of the affected organ. The colour of infected tissues varies considerably, depending on temperature, moisture, and light condition as well as the presence of other pathogens (Pérombelon and Kelman, 1987). The decayed tissues are initially cream to tan coloured, soft, and watery and have a slightly granular consistency under moist conditions (Cother and Sivasithamparam, 1983) and normally separated from the healthy tissue by brown or black margins (Williams *et al.*, 1985). In this condition, the infected tissues can excrete a clear and yellowish-brown coloured liquid, particularly from cracks or wounds (Ruehle, 1940). However, they appear chalky-white if the air humidity is low (Rich, 1983). The soft rot can be accompanied by a foul and offensive odour, which usually develops from other secondary soft rot pathogens such as *Clostridium spp.*, but it is generally odourless in the early stages of natural infection (Gudmestad and Secor, 1993).
Fig. 1. Lenticel infections with circular water-soaked spots and cream to tan colour of rotten tissue (Williams et al., 1985)

Fig. 2. Advanced tuber decay with black margins surrounding the infected tissue (Williams et al., 1985)
Similarly, infections associated with the lenticels appear as yellow to cream colour, circular water-soaked spots around the lenticels, 0.25 inch in diameter and 0.25 – 0.5 inch in depth (Johnson, 1999). These spots will rapidly develop into soft rot under moist condition; however, the lesions become sunken, irregular in shape, dry and hard in dry environment (Hooker, 1981; Gudmestad et al., 1996).

The soft rot that appears in the storage and transit begins its preliminary symptoms in the field (Rich, 1983). The soft rot infection is generally developed from other primary diseases such as late blight and Fusarium tuber rot (Ruehle, 1940).

1.3 Plant-pathogen interaction

1.3.1 Degradative enzymes

The soft rot Erwinia are characterized by producing an array of pectolytic enzymes, including depolymerase (Hooker, 1981), endopolygalacturonate trans-eliminase (Strarr and Moran, 1962), phosphatidase (Tseng and bateman, 1968), proteinase (Friedman, 1962), pectin methyl esterase, and pectin lyase (Hooker, 1981). These enzymes not only macerate the parenchymatous tissue by breaking down the middle lamella, but also cause cell leakage and cell death (Cabezas De Herrera and Maeso, 1978; Pérombelon and Kelman, 1980). Moreover, tissue degradation may result in the disorganization of the cytoplasm that occurs because of the enlargement of the microbodies (Fox et al., 1972).
1.3.2 Natural defense mechanisms

As a response to pathogen attack, plants can generally defend themselves by forming structural defences that act as physical barriers and inhibit the pathogen invasion, and biochemical reactions that can be toxic or inhibit the growth of the pathogen (Agrios, 1997). Likewise, when the soft rot bacteria invade the tissue through lenticels or wounds, the tuber forms suberin as a barrier to the infection (Fox et al., 1971). Polyphenol oxidase and melanin have also been proposed to play important roles as internal infection barriers (Lovrekovich et al., 1967). However, it has been suggested that those related substances are likely to be a side effect of infection (Fox et al., 1972). Moreover, the bacterial infection is restricted to the cell walls and intercellular spaces, while some of these substances are intracellular (Fox et al., 1971).

The suberization of fresh wounds and lenticels is affected by environmental factors including temperature and oxygen supply in storage (Fox et al., 1971; Fox et al., 1972). The presence of a film of water and oxygen depletion damages the suberized layer of cells of the lenticels; and therefore, infection occurs (Pérombelon and Kelman, 1980).
1.4 Epidemiology

Plant disease epidemics develop as a result of the interactions of susceptible host plants, virulent pathogens, and suitable environmental conditions for a long period of time (Agrios, 1997). It involves not only the pathogen life cycle, but also populations of the pathogen associated with the population of host plants (Griffiths, 1978).

1.4.1 Survival of *E. carotovora* subsp. *carotovora*

There is controversy in the literature on the epidemiology of soft rot disease. Some researchers have reported that *Ecc* is not considered to be free living in soil (Lazar and Bucur, 1961; Graham and Harper, 1967). However, soil could become an important source of infection for the bacteria when the debris of diseased plants has not completely decayed (Lazar and Bucur, 1961). In contrast, others are generally agreed that *Ecc* is a soil-borne pathogen, which could survive in soil and the rhizosphere, or in association with infected plant materials such as roots or other parts of plants in soil (Pérombelon, 1974; Maneley and Stanghellini, 1976; Burr and Schroth, 1977; Powelson and Apple, 1984; Pérombelon and Hyman, 1989).

1.4.1.1 Survival in soil

The bacteria can be found in some agricultural soils or in the rhizosphere of weeds and crops. The presence of *Ecc* seems unlikely due to the colonization of the soil alone, but the result of the movement of bacteria from the rhizosphere to other parts of the soil (De Boer *et al.*, 1978). However, the existence of soft rot bacteria cannot be detected in fallow soils of potato growing areas where weeds and plant debris are absent (Burr and Schroth, 1977). It is suggested that this may be because of low population levels of *Ecc* (Burr and Schroth, 1977) or insufficient techniques for detection of small bacteria populations in soil (Meneley and Stanghellini, 1976; De Boer *et al.*, 1978).
Nonetheless, *Ecc* is not viable in soil for extended periods (Pérombelon and Hyman, 1989). The pathogen is killed rapidly in soil, particularly in non-sterilised soil (Lazar and Bucur, 1964; Graham and Harper, 1967).

Several factors influence the longevity of soft rot bacteria in soil. The population numbers of *Ecc* drop significantly at high soil temperature (more than 25 °C) (Pérombelon, 1976; Pérombelon and Hyman, 1989). However, the bacteria cannot survive for long at temperatures below 0 °C (Pérombelon and Kelman, 1980). Similarly, soil moisture is one of the factors that result in being short-lived *Ecc* in soil, although it affects less important than soil temperature (Lazar and Bucur, 1964). Like most bacterial pathogens, *Ecc* grows well in nutrient-rich plant tissue, and it is killed rapidly in a low nutrient environment. In their experiment, Klein and Casida (1967) showed that the population numbers of *Ecc* increased considerably when nutrients were added, even when the numbers of indigenous bacteria also multiplied. The antagonistic affect of other microorganisms such as bacteria, actinomycetes, fungi, and nematodes can also be involved in the short-lived nature of *Ecc* in soil (Pérombelon, 1976). They compete for the utilization of soil nutrients or grazing soil microorganisms kill *Ecc* (Armon *et al.*, 1995).

### 1.4.1.2 Survival in the seed tubers

It is generally accepted that mother tubers (seed tubers) are important and major source of soft rot contamination in potato growing areas (Pérombelon, 1972(b); Pérombelon, 1974; Pérombelon and Kelman, 1980; Powelson and Apple, 1984). As the disease can develop during the storage period, the seed of planting material can be easily contaminated. After the mother tubers are infected, the numbers of *Ecc* increase, and this is usually followed by the progeny tubers being contaminated (Lund, 1979). The bacteria are likely to move through the soil and infect the progeny tubers from healthy mother tubers (Pérombelon, 1974).
The movement of bacteria in soil and the level of tuber contamination during the growing season is mainly affected by soil water, which is correlated to soil moisture, the amount of rainfall, and soil temperature (Pérombelon, 1976). Elphinstone and Pérombelon (1986) reported that there was less mother tuber contamination in dry soils. Furthermore, the bacterial movement in soil can also be facilitated by other soil microorganisms, such as larvae of Hylemyia cilicrura and H. trichodactyla, nematodes, and earthworms (Pérombelon, 1974). However, it is unknown how far the bacteria can move in soil, the distance likely depending upon the soil type, drainage and rainfall (Graham and Harper, 1967).

1.4.1.3 Survival in plant debris

Like other plant pathogenic bacteria, the soft rot pathogen can also overwinter in debris that contains parts of infected plants (Lund, 1979; Agrios, 1997). Some studies showed that high numbers of Ecc were present in plant debris left in the field after harvest (Pérombelon, 1975; Pérombelon et al, 1979). Moreover, Ecc can persist particularly on potato leaves, and it multiplies there in the presence of the proper conditions to cause rotting (Pérombelon, 1978; Elphinstone and Pérombelon, 1986). The bacteria will survive in the plant debris for an extended period as long as the plant material is not completely decomposed (De Boer et al., 1979).

1.4.1.4 Survival in other sources

Besides those sources above, Ecc can also survive well in aquatic environments in many regions (De Boer, 1994). The presence of Ecc could be detected in water of drains, ditches, streams, rivers, lakes, sea, underground, even in winter snow (McCarter-Zorner et al., 1982; McCarter-Zorner et al., 1984) as well as in irrigation water during the growing seasons (Powelson and Apple, 1984). The numbers of Ecc isolated from many rivers ranges from $10^3$ cells/l in temperate regions to more than $10^6$ cells/ml in warmer regions.
(Pérombelon, 1992). The bacteria are indigenous to certain rivers and proliferate in the bottom of sediments (Cother and Gilbert, 1990).

Some researchers consider soft rot Erwinia as an airborne pathogen since it is found in air and insects, which have a role as vectors of Ecc (Pérombelon et al., 1980). The survival of Ecc in air is influenced by environmental conditions including relative humidity and temperature (Pérombelon, 1976).

1.4.2 Dissemination

The dissemination of pathogens within and between crops generally results in plant disease outbreaks (Agrios, 1997). Similar to other pathogens, the movement of soft rot bacteria over long distances is mainly facilitated by aerosols, insects, or farm equipments (Burr and Schroth, 1977).

The dispersal of Ecc in air is generated by windblow rain in moist air conditions (De Boer, 1994). The release of Ecc in aerosols occurs when potato stems are pulverized prior to harvest (Pérombelon et al., 1979). Although the bacteria cannot persist for long time in air (only 50% of the pathogen is still alive for 5-10 minutes), it can be spread by rain for several hundred meters before disposition occurs (Pérombelon, 1992).

Additionally, the role of insects as vectors of soft rot pathogens has been recognized for a long time (Pérombelon and Kelman, 1980). Dipterans, particularly Drosophila spp. and Leptocera spp., are considered to be the most important agents for transmitting the bacteria to the crops (Harrison et al., 1977). The insects are likely to transmit the bacteria into damaged stem tissue (Pérombelon and Kelman, 1980); however, the bacteria carried by the insects may survive on undamaged tissue until injuries occur (Harrison et al., 1977).
Farm equipment is also responsible for the dispersal of the bacteria. This is due to the ability of the bacteria to move from tuber to tuber when the seeds are cut before planting, the bacteria can also be spread by the wheels of tractors during the growing season as well as by harvest machinery (Pérombelon and Kelman, 1980).

1.5 Disease cycles

As previously mentioned, soft rot bacteria can survive in soil, seed, and plant debris as well as in insects. The bacteria are released into the soil in large numbers decayed seed as soon as the crops are planted (Hooker, 1981). The soft rot infections develop on the tubers, rhizomes, bulbs, and stem (Agrios, 1997) entering through lenticels, wounds, or from the point of attachment of the stolon (Lund, 1979).

Figure 3. Disease cycles of bacterial soft rot (Agrios, 1997)
Insects play a very important role in inoculating the bacteria into the plant tissue both in the field and in storage (Pérombelon and Kelman, 1980). The bacteria can persist in all stages of pupae and larvae of many insects, which also transmit them to potato tubers (Harrison et al, 1977). When the insects move on the decayed seed tubers, not only do they carry the bacteria to the crops, but also deliver the bacteria into the wounds or lenticels, which then develop soft rot disease (Agrios, 1997).

As soon as the bacteria enter the wounds or lenticels, they multiply, invade, and rot the tuber when the proper environmental conditions including temperature, relative humidity, and free moisture are present (Lund, 1979). The bacteria can infect out of xylem vessels under wet conditions, but they only macerate the tissue in the vascular system when the condition is dry (Pérombelon, 1992). However, the soft rot bacteria can become dormant when the conditions are unfavourable to disease development (Pérombelon and Salmond, 1995). They then become active, rot the tuber tissue as well as complete the annual cycle of infection when the contaminated tubers are planted and food materials are translocated under favourable environmental conditions (Graham and Harper, 1967; Pérombelon and Kelman, 1987).

1.6 Disease Controls

Soft rot disease results in yield losses of 5-10%, or downgrading and rejection of seed crops for certification. Moreover, it can depress crop growth when it is still in latent infection (Pérombelon, 1992).

Controlling soft rot is quite difficult as environmental factors play such a prominent role in disease development. No completely satisfactory control of soft rot disease, either by chemicals or breeding for resistance has been achieved (Graham and Harper, 1967; Pérombelon, 1992; Pérombelon and Salmond, 1995). Chemical treatments have failed to reduce soft rot contamination because the bacteria are well protected in the suberized
lenticels and wounds (Pérombelon, 1992), and the chemical residues may stay in the tubers (Ranggana et al., 1998). However, sodium hypochlorite has been added to water used to machine wash potatoes in order to reduce the inoculum level of soft rot. Although this solution can minimize the potential of soft rot (Bartz and Kelman, 1986), its effect has been temporary (Scholey et al., 1968). Thermal treatment may be another viable alternative to control post-harvest soft rot. Mackay and Shipton (1983) reported that Ecc on the surface and in lenticels of infected tubers could be killed when the tuber were dipped in circulating water at 53 °C for 5 min or 51 °C for 10 min. In another study, Ranggana and coworkers suggested treatment at 57.5 °C for either 20 or 30 min, and after this treatment, the potatoes could be stored for at least 12 weeks at either 8 or 18 °C (Ranggana et al., 1998). However, it has not been broadly applied since it is costly in practice (Pérombelon, 1992). Consequently, control of soft rot disease is mainly based on a thorough sanitation program in the field and in storage.

In potato growing areas, some attempts that aim to reduce the spread of the pathogen can be applied. As soft rot is a seed-borne disease, the use of clean seed is worthwhile to minimize the initial amount of contamination (Pérombelon, 1992). Seed tubers should be warmed to 12.7-15.6 °C before planting and treated with a fungicide early in the season of planting because soft rot is a secondary disease of several fungal pathogens (Gudmestad and Secor, 1993). Some biological control agents, such as antagonistic bacteria and plant growth-promoting rhizobacteria, have also been applied to the seed tubers before planting (Agrios, 1997). The crops should be planted in well-drained areas to maintain the surface moisture by creating sufficient space among plants and excessive irrigation should be avoided (Agrios, 1997). Excessive fertilizer use, particularly nitrogen should also be avoided (Floyd, 1989). Removing the infected stems and rotted tubers from the field can also reduce disease incidence as long as it is done frequently and at the right time (Graham and Harper, 1967).
Managing soft rot disease is primarily focused on the handling of the crop during harvest and storage. Irrigation systems should be monitored close to harvest time to avoid excessive soil moisture that can result in lenticel infection (Hooker, 1981). The potato stems should not be pulverized before harvest as they generate large numbers of aerosols containing the pathogen, which can infect other crops and leave the debris in which the pathogen can multiply easily (Pérombelon, 1992). Another way is harvesting only the mature tubers, which have better skin set that resists wounding or harvesting, when the soil temperatures are less than 20 °C (Gudmestad and Secor, 1993). Moreover, care should be taken to minimize bruising and other mechanical damage during harvesting and handling (Hooker, 1981).

In storage, managing the storage conditions is important to prevent formation of condensation on the tubers (Lund, 1979). The tubers are cooled to 10 °C or lower as soon as they are harvested and left for 10-14 days to promote wound healing (Gudmestad and Secor, 1993). After this they are stored at 5-10 °C in a relative humidity of 85-95% (Hooker, 1981). In addition, good ventilation is necessary to prevent the accumulation of CO₂ and moisture films (Gudmestad and Secor, 1993). Storing wet tubers should be avoided as it favours soft rot development; therefore, the washed tubers should be dried before transport (Lund, 1979).

1.7. Research objectives

Washed potatoes are normally contaminated after harvesting and during commercial washing (Morgan and Wicks, 2000). Hydrostatic pressure during the washing process forces the bacteria to infiltrate the tubers through lenticels and injury sites (Bartz and Kelman, 1984).

The soft rot infection mainly occurs through lenticels that are the main entry point of Ecc and the bacteria can overwinter in lenticels until favourable environmental conditions promote the bacteria to become active.
The main objectives of this study are:

- To compare and verify two detection methods for *Ecc*; the peel method and the lenticel sampling method, for tools to determine *Ecc* contamination.

- To examine the relationship between artificial tuber contamination and *Ecc* numbers in lenticels using two inoculation methods, the dipping and the infiltration method.

- To investigate the effect of chlorine solution and drying treatment on the *Ecc* numbers in lenticels as well as the potential of soft rot development using washed and brushed potatoes.
CHAPTER 2.  
MATERIALS AND METHODS

2.1 Potato tuber
2.1.1 Tuber Material

Two types of potato tubers used in this study, namely washed potatoes variety Coliban and brushed potatoes variety Coliban obtained from the retailed grocery stores.

2.1.2 Tuber Preparation

In order to encourage lenticels opening and ease of bacterial entry, all tubers were incubated in a plastic bag that had 100 ml of distilled water placed in the bottom and incubated at 20 °C for 24 hours before being utilized. The tubers, both control (without treatments) and treated tubers, were sterilized by spraying with 100% ethanol and flaming for approximately five seconds before treatments were applied.

2.2 Bacterial Isolate
2.2.1 Source of isolates

The pure culture used in this study, *E. carotovora* subsp. *carotovora* strain 15 (*Ecc* 15), (PM3048), was obtained from the culture collection of the laboratory of Horticultural Pathology Unit - SARDI. This strain along with four other *Ecc* strains were tested for their pathogenicity as measured by their ability to macerate the plant tissue. Below is the list of five strains of *Ecc* that were prepared for the pathogenicity test.
Table 2.1. Origin and source of *Ecc* strains

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Host</th>
<th>Date of collection</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ecc</em> 9</td>
<td>Potato tubers from the end line of the Washing Plant</td>
<td>15&lt;sup&gt;th&lt;/sup&gt; Oct 1998</td>
<td>Virginia</td>
</tr>
<tr>
<td>(PM3047)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ecc</em> 15</td>
<td>Potato tubers from the end line of the Washing Plant</td>
<td>9&lt;sup&gt;th&lt;/sup&gt; Nov 1998</td>
<td>Virginia</td>
</tr>
<tr>
<td>(PM3048)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ecc</em> 17</td>
<td>Potato tubers from the initial wash</td>
<td>22&lt;sup&gt;nd&lt;/sup&gt; Oct 1998</td>
<td>Blanchetown, Riverland</td>
</tr>
<tr>
<td>(PM3049)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ecc</em> 20</td>
<td>Potato tubers from the initial wash</td>
<td>22&lt;sup&gt;nd&lt;/sup&gt; Oct 1998</td>
<td>Blanchetown, Riverland</td>
</tr>
<tr>
<td>(PM3050)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ecc</em> 30506</td>
<td>Potato tubers</td>
<td>January 1978</td>
<td>Blighty, NSW</td>
</tr>
<tr>
<td>(PM3051)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Pathogenicity Test for tissue maceration

All *Ecc* strains to be used for the pathogenicity test were grown on Nutrient Agar (NA). Six colonies of each strain were taken and cultured on new NA plates and incubated at 28 °C for 24 hours. 200 μL of *Ecc* suspension (10<sup>6</sup> cfu/ml) of the cultures was inoculated onto sterilised potato slices that were placed in Petri dishes on moist sterile filter paper. The potato slices were prepared by disinfecting the tissue surface by immersing in a 10% White King® (42 g/l sodium hypochlorite) for 10 min. and air-dried. This surface sterilisation was repeated twice. The *Ecc* cultures were also tested on selective agar medium - crystal violet pectate (CVP) - that is described in Appendix A. 100 μl of *Ecc* suspension was spread over the plate by a sterile glass rod. The cultures, both on potato slices and CVP agar, were incubated at 28 °C for 48 hours. The soft rot development on potato slices was assessed by determining the occurrence of decay and tissue maceration of the tissue surrounding the inoculated site. The growth of *Ecc* on CVP plates was observed by the presence of deep cavities.
2.2.3 Preservation of bacterial culture

2.2.3.1 Short-term storage

After growing the culture at 28 °C for 48 hours, single culture isolates of *Ecc* were stored on a NA plate at 4 °C. The cultures remained viable for at least one month (De Boer and Kelman, 2001).

2.3.2 Long-term storage

A loopful of the bacterial culture from a 48-hour-culture on NA was inoculated into 5 ml Nutrient Broth (NB) in a McCartney bottle at 28 °C for 24 hours. 1 ml of the isolate(s) (PM3047- PM3051) was placed into 300 μl sterile glycerol stock and frozen by dipping into liquid Nitrogen for 2-3 minutes in a 1.8 ml Nunc® tube. The stock was then stored at -80 °C. To revive the glycerol stock, the frozen cells were removed and streaked with a loop onto a NA plate. A viable culture started to grow during overnight incubation at 28 °C.

2.2.4 Preparation of inocula

2.2.4.1 Soft rotted flesh Inocula

Potato tubers were inoculated by completely immersing in *Ecc* suspension for 15 min. The inoculated tubers were placed in a net plastic bag and incubated in a mist chamber at between 18 - 25 °C and 100% relative humidity in the dark for 4 days. To prepare inocula, 100 g of the rotted potato flesh was cut out and mixed in 10 l distilled water, which is equivalent to a cell density 10^5 cfu/ml.

2.2.4.2 Pure culture

To prepare inocula, the *Erwinia* isolate was sub-cultured onto NA plates and incubated at 28 °C for 24 hours. The cultures were flooded with 2 ml sterile distilled water (SDW), then the surface of the culture was gently rubbed with a sterile inoculating needle to dislodge the bacteria. The suspension was then measured with a spectrophotometer (Beckman Du®-68) and adjusted to
an absorbance of 1.0 at 590 nm, which is equivalent to a cell density 10⁹ cfu/ml. To obtain the required inoculum concentration, the suspension was diluted.

2.2.5 Enumeration of the *Erwinia*

The number of *Erwinia* in lenticels was assessed by using the plate-count technique. The dilution series was prepared in sterile 1.5 ml eppendorf tubes. 0.1 ml of bacterial suspension was transferred into a sterile eppendorf tube containing 0.9 ml of SDW, and successive dilutions made in a similar manner. A volume of 0.1 ml of bacterial suspension of the required dilution was then placed onto CVP plates and spread using a sterile glass rod, starting from the most dilute sample. The plates were incubated at 28 °C for 48 hours.

The following calculation was done to measure the numbers of *Erwinia* per ml on the CVP plates:

\[
\text{number of colonies that formed deep cavities} \times \text{dilution of sample} \times 10
\]

2.3 Comparing methods for detection of *Ecc*

Two methods for detection of *Ecc* numbers in lenticels were examined.

2.3.1 Peel Method

Washed potato tubers, which were prepared as described in section 2.1.2, were inoculated by dipping them into a suspension of soft rotted flesh inocula that was prepared as described in section 2.2.4.1 for 15 min. Following this, the tubers were air-dried for 10 min. Other tubers were prepared as controls, which were not inoculated with the bacterial suspensions.
30 g of peel tissue (5 g from each of six tubers) of potato tubers, was removed from tubers each weighing 100 – 200 g. The peel tissue was then homogenized with 300 ml SDW using a Waring Blender for 60 sec. varying the speed from slow to the maximum speed. The peel suspension was serially diluted comprising two tenfold dilutions ($10^{-1}$ and $10^{-2}$) as described in section 2.2.5.

2.3.2 Lenticel Sampling Method

Washed potato tubers, which were selected for size 100-200 g, were prepared as described in section 2.1.2. The tubers were inoculated by dipping the tubers into the suspension of soft rotted flesh inocula as described in section 2.2.4.1 for 15 min., and followed by air-drying for 10 min. Other tubers were prepared as controls, which were not treated with the bacterial suspensions.

20 lenticels per tuber were removed with a sterilized cork borer (d = 0.5 cm) and a sterilized scalpel and placed into a 1.5 ml eppendorf tube that was filled with 1 ml SDW. The lenticels were crushed with a small pestle and then left for 15 min. to allow the bacteria to diffuse out of the tissue. The $E_{cc}$ numbers were assessed by a tenfold dilution series comprising $10^{-1}$ and $10^{-2}$ as was described in section 2.2.

2.3.3 Experimental design

This experiment was arranged as a completely randomized design with three separate tubers as replicates. The Petri dishes were placed in the 28 °C incubator cabinet for 48 hours.

2.3.4 Statistical analysis

Test of comparison of means between the treatments was used in this experiment.
2.4 Quantifying Ecc contamination of tuber and testing inoculation method

Washed potatoes, which had equal-sized tubers (100 – 250 g) and lacked evidence of wounds, were used in this experiment. Preparation of tubers and Ecc inocula were as described in section 2.1.2 and 2.2.4.2 respectively. Ecc suspension was prepared as three inoculum densities, i.e. $10^4$, $10^5$, and $10^6$ cfu/ml. Two inoculation methods tested to measure Ecc numbers in lenticels are described below.

2.4.1 Dipping method

The tubers were dipped into each bacterial suspension and water for control for 15 min., followed by air-drying for 10 min.

2.4.2 Infiltration method

The tubers were inoculated with each bacterial concentration or water for control by vacuum infiltration with a vacuum pump at 15 Hg. for 20 min. Potatoes were completely submerged in a beaker containing either the bacterial suspension or water, which was placed in a vacuum chamber. The solution was stirred with a magnetic stirrer to prevent the inoculum precipitating (for the treatment with bacterial suspensions). The tubers were air-dried for 10 min.

Treatments used in the experiment were:

1. Ecc concentration $10^4$ cfu/ml with dipping
2. Ecc concentration $10^4$ cfu/ml with infiltration
3. Ecc concentration $10^5$ cfu/ml with dipping
4. Ecc concentration $10^5$ cfu/ml with infiltration
5. Ecc concentration $10^6$ cfu/ml with dipping
6. Ecc concentration $10^6$ cfu/ml with infiltration
7. Control with dipping
8. Control with infiltration
The lenticel sampling method as described in section 2.3.2 was used to detect the \textit{Ecc} numbers in lenticels. A dilution series as described in section 2.2.5 was used to determine the number of bacteria in the lenticels. For the treatment applying bacteria to the tuber at $10^4$ cfu/ml, $10^3$, $10^2$, $10^1$ dilutions were used; for the treatment applying bacteria at $10^5$ and $10^6$ cfu/ml to the tubers, $10^2$, $10^3$, $10^4$ dilutions were used; and for the control treatment $10^1$ and $10^2$ dilutions were used to assess the bacteria numbers.

2.4.3 Experimental design

This experiment was arranged as a completely randomised design with three separate tubers as replicates. The Petri dishes were placed in the 28 °C incubator cabinet for 48 hours.

2.4.4 Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Microsoft Excel® 2000 with Two-way ANOVA.

2.5 Effect of chlorine solution and drying on the \textit{Ecc} numbers in lenticels and the development of soft rot on potato tubers

This section consists of two independent experiments. The first experiment used washed potato tubers, whereas the second experiment used brushed potato tubers.

2.5.1 Experiment 1

Preparation of tuber materials and the bacterial isolate were as described in section 2.1.1, 2.1.2, and 2.2.4.2 respectively. The tubers used in this study were washed potato tubers, which were selected for size (100 – 250 g) and lacking evident wounds. Chlorine solution was prepared by diluting sodium hypochlorite-based-household bleach (White King®) with distilled water.
The following calculation was done to calculate the chlorine concentration in part per million (ppm) measurement:

\[ V_1 \cdot C_1 = V_2 \cdot C_2 \]

Where \( V_1 \) is the required volume of chlorine, \( V_2 \) is the volume of water (1l), \( C_1 \) is the concentration of sodium hypochlorite (42 g/l or 42,000 mg/l), and \( C_2 \) is the required concentration of water added.

15 l Ecc suspension, which was prepared as \( 10^8 \) cfu/ml, was mixed with 100 g sterilised potato crush. 15 ml of 0.1% w/v Triton X-100 was added to the bacterial suspension in order to promote inoculation by reducing the surface tension of the water. The final concentration of surfactant was 0.0001%.

Treatments used in measuring the Ecc numbers in lenticels were:

1. 50 ppm chlorine
2. 100 ppm chlorine
3. 1000 ppm chlorine
4. Control (immerse in distilled water)

The acidity level of each chlorine solution was measured with a pH meter, where the acidity level of 50 ppm, 100 ppm, and 1000 ppm chlorine was 9.84, 10.60, and 12.11 respectively.

All tubers were inoculated by the dipping method as described in section 2.4.1. The tubers of the chlorine treatments and control treatment were immersed in both the chlorine solution and water for 2 min. The lenticel sampling method, as described in section 2.3.2, was used to detect the Ecc numbers in lenticels. A tenfold dilution (\( 10^{-2} \)), which was prepared as described in section 2.2.5, was used to count the numbers of Ecc colonies.
Treatments used in assessing the soft rot development were:

1. 50 ppm chlorine with drying
2. 50 ppm chlorine without drying
3. 100 ppm chlorine with drying
4. 100 ppm chlorine without drying
5. 1000 ppm chlorine with drying
6. 1000 ppm chlorine without drying
7. Control (immerse in distilled water) with drying
8. Control (immerse in distilled water) without drying

For determining the soft rot development, after treatment with the chlorine solution and water, two of the four tubers were air-dried with a fan for 4 hours. All tubers were put in a net plastic bag and incubated in a mist chamber for 4 days.

The disease severity was assessed by using the scale that was adopted from Morgan and Wicks (2000):

0  =  no visual sign of soft rot
1  =  lenticel invasion only
2  =  < 25% surface area effected by soft rot
3  =  25 – 50% surface area effected by soft rot
4  =  50 – 75% surface area effected by soft rot
5  =  > 75% surface area effected by soft rot

2.5.1.1 Experimental design

For measuring the *Ecc* numbers in lenticels, the experiment was arranged as a completely randomized design with three tubers as replicates. The Petri dishes were placed in the 28 °C incubator cabinet for 48 hours. For determining the soft rot development, the experiment was also arranged as a completely randomized design with three replicates, which contained two
tubers. The net plastic bags were placed in the mist chamber (Figure 2.1) under maintained continuous misting at between 18 - 25 °C for 4 days.

2.5.1.2 Statistical analysis

Statistical analyses were performed using Microsoft Excel® 2000. Data were examined using Two-way of ANOVA and correlation coefficient analysis.

2.5.2 Experiment 2

In this experiment the tuber materials were replaced with brushed potatoes, which were selected for size (100 - 250 g) and lacking evident wounds. Tubers were rinsed to remove soil. They were incubated in a mist chamber for overnight to promote lenticels opening and facilitate bacterial entry. These tuber materials were not sterilized by ethanol spraying, which was done in the previous experiment. The bacterial isolates were prepared as described in section 2.2.4.2. Chlorine solution was prepared by diluting sodium hypochlorite-based-household bleach (White King® ) with distilled water.
Treatments used in this experiment were:

9. 50 ppm chlorine with drying
10. 50 ppm chlorine without drying
11. 100 ppm chlorine with drying
12. 100 ppm chlorine without drying
13. 1000 ppm chlorine with drying
14. 1000 ppm chlorine without drying
15. Control (immerse in distilled water) with drying
16. Control (immerse in distilled water) without drying

90 ml Ecc suspension was prepared as $10^6$ cfu/ml and added to 90 l distilled water. In contrast to the previous experiment, the bacterial suspension was not added with sterilized potato crush and surfactant.

All tubers were inoculated by the dipping method as described in section 2.4.1. In the previous experiment, the tubers were immersed in the chlorine solution and water. Alternatively, in this experiment the tubers of the chlorine and control treatment were sprayed with both the chlorine solution and water, which is the same treatment as in the commercial washing plant. The lenticel sampling method as described in section 2.3.2 was used to detect the Ecc numbers in lenticels. A tenfold dilution ($10^{-1}$ and $10^{-2}$), which was prepared as described in section 2.2.5, was used to count the numbers of Ecc colonies.

For determining the soft rot development, after treatment with the chlorine solution and water, five of the ten tubers were air-dried with a fan for 4 hours. All tubers were put in a net plastic bag and incubated in a mist chamber for 4 days.
The disease severity was assessed by using the scale, which was adopted from Morgan and Wicks (2000), as described in the previous experiment (section 2.5.1).

2.5.2.1 Experimental design

This experiment was arranged as a completely randomized design with five replicates, which comprised five tubers. The net plastic bags were placed in the mist chamber under maintained continuous misting at between 18 - 25 °C for 4 days.

2.5.2.2 Statistical analysis

This experiment was analyzed by using Microsoft Excel® 2000. Data were examined using Two-way of ANOVA, a Least Significant Difference (LSD) Test and correlation coefficient analysis.
CHAPTER 3.
RESULTS

3.1 Pathogenicity test for tissue maceration

3.1.1 Aim of experiment

The ability of soft rot bacteria to macerate the parenchymatous tissue of potato indicates their pathogenicity and determines the presence of pectolytic enzymes. However, it does not determine their pathogenicity in a natural environment because of the association of endophytic or epiphytic microorganisms with inoculated tissue (De Boer and Kelman, 2001). This test aimed to measure the ability of Ecc to macerate the plant tissue of potato tubers.

3.1.2 Result

The results shown in table 3.1 indicate that all colonies of Ecc strains could macerate the potato slices. Furthermore, all Ecc strains present in the CVP plates formed deep cavities. All strains performed identically in the pathogenicity tests and strain 15 was used in further studies.
Table 3.1. Pathogenicity test for *Ecc* strains

<table>
<thead>
<tr>
<th><em>Ecc</em> strain</th>
<th>Pathogenicity</th>
<th>CVP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potato slices</td>
<td>CVP</td>
</tr>
<tr>
<td>30506-1</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>30506-2</td>
<td>yes</td>
<td>yes</td>
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<td>30506-3</td>
<td>yes</td>
<td>yes</td>
</tr>
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</tr>
<tr>
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</tr>
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<td>9-1</td>
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</table>
3.2 Comparing methods for detection of Ecc

3.2.1 Aim of the experiment

Soft rot disease shows latent infection due to its ability to overwinter superficially in lenticels and suberized wounds during storage, where its development depends on favourable environmental conditions (Perombelon and Kelman, 1987; Perombelon and Salmond, 1995). Moreover, it has been considered that lenticels are the main entry points of Ecc to invade and cause decay on potato tubers (Perombelon, 1973).

Various techniques have been conducted to detect the soft rot pathogens on tubers, such as a wash method to estimate the level of surface bacteria (Chard and Oxley, 1989), a tuber incubation method (De Boer and Kelman, 1975), a peel method (Perombelon and Hyman, 1986; Perombelon et al., 1987), and a lenticel sampling method (Perombelon, 1973; De Boer and Kelman, 1975). Additionally, several studies have tested various serological methods based on the use of O-somatic antigens, fluorescence antibodies and immunodiffusion techniques to detect and quantify pectolytic Erwinia (Vruggink and Maas-Geesteranus, 1975; Allan and Kelman, 1977; Stanghellini et al., 1977; Vruggink and De Boer, 1978; Jones et al., 1994). Yet, none of the tests have been found to satisfactorily estimate the level of soft rot pathogen on potato tubers (Fraajie et al., 1996).

Consequently, this study aimed to compare and verify two detection methods for Ecc; the peel method and the lenticel sampling method, for their suitability and efficiency as simple reliable tools to determine tuber contamination. The number of Erwinia were assessed by determining the numbers of live pathogen cells in lenticels in samples of tuber plated on a diagnostic selective agar medium (CVP).
3.2.2 Result

There was a highly significant difference (P < 0.001) between the control lenticel sampling method and the control peel method in detecting *Erwinia* numbers in lenticels per tuber (Appendix 2). Likewise, the population of *Ecc* in lenticels varied between both detection methods for the inoculated treatment (P < 0.05).

The result shown in figure 3.1 indicates that a larger population of *Ecc* was detected by the lenticel sampling method than by the peel method, either in the control or inoculated treatments. As a result, the lenticel sampling method was used in the subsequent experiments.

![Log10 Ecc numbers in lenticels](image)

**Figure 3.1.** The number of *Ecc* cells in lenticels per tuber of two different detection methods of tuber contamination. Bars represent standard error of means of three replicates (SD).
3.3 Quantifying *Ecc* contamination of tuber and the testing inoculation method

### 3.3.1 Aims of the experiment

Previous studies have reported that there is a relationship between the level of soft rot *Erwinia* on tubers and soft rot disease development. Blackleg disease, caused by the closely related species, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *carotovora*, as well as the commercial seed stocks were more severe with increased number of bacteria on the seed tubers (Bain *et al.*, 1990). Likewise, increased bacterial soft rot occurred when the population of bacteria inoculated in the wash water increased during the washing process in a commercial washing plant (Bartz and Kelman, 1984; Bartz, 1999). In addition, the potential for soft rot bacterial development is affected by the number of *Erwinia* present in lenticels (Adam, 1975; Bartz and Kelman, 1985a), where the bacteria can persist during dormant periods.

Because of these reports, this study examined the relationship between artificial tuber contamination and the population of *Ecc* in lenticels using two inoculation methods. Since lenticels are considered to be the most likely entry point of soft rot *Erwinia*, such methods focused on the penetration of bacteria into the tubers through lenticels.

### 3.3.2 Result

The inoculation levels of *Ecc* and Log_{10} *Ecc* numbers in lenticels were not significantly correlated (*r* = 0.655 and 0.488 for dipping and infiltration method, respectively). Similarly, no differences between both inoculation levels of *Ecc* and inoculation methods were observed, nor was there any interaction between them (P<0.63, 0.24 and 0.14 for *Ecc* concentration, inoculation methods, and interaction between them respectively (see Appendix 3). Nevertheless, the largest number of *Ecc* re-isolated was achieved when the inoculation level was 10^6 cfu/ml, by both the dipping and
infiltration methods (figure 3.2). Therefore, the Ecc inoculation level of $10^6$ cfu/ml was used for the following experiments.

Generally, the dipping method resulted in slightly higher numbers of re-isolated Ecc. In the dipping method increasing the concentration of the inoculant slightly increased the number of Ecc re-isolated, but not more than the control (uninoculated) treatment. The infiltration method gave more variable results as the concentration of inoculant varied, but once again was rarely higher than the uninoculated control.

![Graph](image)

**Figure 3.2.** The number of Ecc cells in lenticels per tuber of different inoculation levels of Ecc using different inoculation methods. Bars represent SD of three replicates.
3.4 Effect of chlorine solution and drying on the *Ecc* numbers in lenticels and soft rot development

3.4.1 Aims of experiment
Managing bacterial soft rot on potatoes has been difficult since environmental factors, in general, influence the disease development. Neither various chemicals or breeding for resistant have not achieved satisfactory control of the bacterial infection (Graham and Harper, 1967; Pérombelon, 1992; Pérombelon and Salmond, 1995). Nevertheless, several studies have used chlorine, which is the principle biocide applied to sanitize fruit and vegetable packing-houses (Robbs *et al.*, 1995), to reduce losses from the post harvest decay of soft rot. However, the results are still inconsistent. Additionally, another experiment has also applied thorough air-drying to reduce the soft rot contamination as soft rot development is likely to occur when the tubers are wet (Burton and Wigginton, 1970).

This experiment, therefore, aimed to investigate the effect of chlorine solution and drying treatment on the population of *Ecc* in lenticels and the effect on potential soft rot development using both washed and brushed potatoes. The relationships between the population of *Ecc* and the disease development were also investigated.

3.4.2 Result

3.4.2.1 Experiment 1
Due to the low number of *Ecc* achieved in the previous experiment, sterilized potato crush and surfactant were added to the bacterial suspensions in order to promote bacterial inoculation. While the potato crush was assumed to be a nutrient source for the bacteria, the surfactant is likely to reduce the surface tension of water, which results in facilitating the bacterial infiltration and increase the number of bacteria in potato tubers.
Figure 3.3. The numbers of Ecc cells in lenticels per tuber at different levels of chlorine concentration without drying treatment on washed potato tubers. Bars represent SD of three replicates.

Figure 3.3 shows that chlorine solution did not reduce the population of soft rot bacteria in lenticels of washed potato tubers. The analysis of variance (ANOVA), shown in Appendix 4, indicated that no significant difference existed between treatments ($P < 0.07$).

Comparing the controls from figure 3.2 and figure 3.3 indicates the number of Ecc re-isolated was not substantially increased by adding the potato crush and the surfactant. The levels of Ecc remained in the range 3.5-4 $\log_{10}$ cells per tuber. Furthermore, dipping the potatoes in 50 – 1000 ppm chlorine did not affect the bacterial numbers re-isolated from lenticels.
Results in figure 3.4 show that chlorine solution is broadly ineffective at reducing soft rot development on washed potato tubers. No significant differences between chlorine treatments and disease severity ($P<0.43$) were observed (Appendix 5). Similarly, the interactions between chlorine and drying treatments did not affect disease development ($P<0.96$). It is noteworthy that replicates were highly variable in this experiment resulting in very large error bars.

In general, there was no relationship between the population of *Ecc* re-isolated from tubers and soft rot severity for all chlorine concentrations and the control. However, for 100 ppm chlorine treatment there was a significant correlation ($r = 0.965$). The coefficient of correlation for other treatments was 0.869, 0.921, and 0.913 for 0 (control), 50, and 1000 ppm chlorine, respectively.
3.4.2.2 Experiment 2

The previous experiments indicated that the addition of sterilized potato tubers and surfactant into the bacterial suspension did not increase the population of *Ecc* in the tubers. As a result, they were no longer used in this experiment, which used brushed potato tubers as distinct from washed potatoes in the previous experiment.

Disinfecting the potato tubers with chlorine solution (spraying as opposed to dipping as in experiment 1) and thorough air-drying did not eliminate *Erwinia* from the tubers (figure 3.5). The ANOVA shown in appendix 6 also indicated that the chlorine and the drying treatments had no effect on the population of soft rot bacteria in lenticels (P< 0.20 and P< 0.18 for chlorine and drying treatments, respectively). The interaction between chlorine and drying treatments also did not significantly affect the *Ecc* numbers in lenticels (P< 0.09).

![Graph showing Log₁₀ Ecc numbers in lenticels of 2nd experiment](image)

*Figure 3.5. The numbers of Ecc cells in lenticels per tuber with different levels of chlorine concentration and drying treatment on brushed potato tubers. Bars represent SD of five replicates.*
Results shown in figure 3.6 indicate that the combination between chlorine solution and air-drying reduced soft rot development. The ANOVA also indicated that there was a significant difference between the interaction of chlorine and drying treatments ($P < 0.04$) (see appendix 7). Generally, the disease severity of potato tubers, which were sprayed with chlorine solution without air-drying, decreased as the chlorine concentration was increased (table 3.2). However, there were no significant differences between control and 50 ppm chlorine. On the other hand, all chlorine concentrations combined with air-drying did not significantly affect the severity of bacterial soft rot, although less disease was found.

![Soft rot disease severity on potato tuber of 2nd experiment](image)

**Figure 3.6.** Soft rot disease severity with several chlorine concentrations and drying treatment on brushed potato tuber. Bars represent SD of five replicates.
Table 3.2. Severity of bacterial soft rot treated with combination of chlorine and drying treatments

<table>
<thead>
<tr>
<th>Chlorine concentration</th>
<th>Disease severity&lt;sup&gt;x&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No drying</td>
<td>Drying</td>
</tr>
<tr>
<td>0 ppm (control)</td>
<td>3.16 a</td>
<td>1.12 a</td>
</tr>
<tr>
<td>50 ppm</td>
<td>3.04 a</td>
<td>1.00 a</td>
</tr>
<tr>
<td>100 ppm</td>
<td>2.36 b</td>
<td>0.80 a</td>
</tr>
<tr>
<td>1000 ppm</td>
<td>1.88 c</td>
<td>0.72 a</td>
</tr>
</tbody>
</table>

<sup>x</sup> Each value is the average of 5 tubers. Values followed by the same letter were not different (p = 0.05)

Table 3.3. Correlation between Log<sub>10</sub> Ecc numbers in lenticels and disease severity on brushed potato tubers (* = significant, NS = not significant)

<table>
<thead>
<tr>
<th>Correlation</th>
<th>r</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at control (+drying)</td>
<td>0.92</td>
<td>*</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at control (-drying)</td>
<td>0.79</td>
<td>*</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 50 ppm (+drying)</td>
<td>0.88</td>
<td>*</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 50 ppm (-drying)</td>
<td>0.47</td>
<td>NS</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 100 ppm (+drying)</td>
<td>0.67</td>
<td>NS</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 100 ppm (-drying)</td>
<td>0.75</td>
<td>NS</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 1000 ppm (+drying)</td>
<td>0.74</td>
<td>NS</td>
</tr>
<tr>
<td>Log&lt;sub&gt;10&lt;/sub&gt; Ecc numbers and disease severity at 1000 ppm (-drying)</td>
<td>0.71</td>
<td>NS</td>
</tr>
</tbody>
</table>
Generally, the population of *Erwinia* was not correlated significantly with disease severity (table 3.3). However, the control for both drying and no drying treatments, as well as 50 ppm chlorine with drying, showed a highly significant correlation (r = 0.92, 0.79, and 0.88 for control with drying, control without drying, and 50 ppm chlorine with drying respectively) between *Ecc* numbers and disease development.
CHAPTER 4.
DISCUSSION

This chapter describes the findings of experiments that had been designed to:
- compare and verify the suitability and efficiency of two detection methods for Ecc.
- examine the relationships between artificial tuber contamination and the numbers of Ecc in lenticels by comparing two inoculation methods.
- investigate the effect of chlorine solution and drying treatment, as a means to manage the soft rot disease and determine the effect on the population of Ecc in lenticels and the effect on soft rot development, as well as to examine the relationships between the population of Ecc and soft rot development.

Both detection methods estimated the population of Ecc based on a subcuticular count since lenticels are important sites of infestation of soft rot bacteria (Pérombelon, 1973). The lenticel sampling method was confirmed as an effective detection method for Ecc colonization because larger Ecc numbers were detected using this method than with the peel method. Although live Ecc cells can be easily distinguished and enumerated on selective agar medium (CVP), both methods were generally labour intensive and time consuming. This is in agreement with previous studies conducted by De Boer and Kelman (1975) and Chard and Oxley (1989). However, there is an advantage in detecting the population of Ecc in potato tubers using the lenticel sampling method. The number of Ecc can be detected in each lenticel because 20 lenticels per tuber were sampled. On the other hand, the population of Ecc using the peel method cannot be counted in each lenticel as the number of lenticels cannot be determined precisely.
This study found low numbers of Ecc detected in the tubers that were artificially inoculated. It is likely that using soft rotted flesh is not sufficient as inocula to cause soft rot disease. Additionally, it is not acceptably good science to use an undefined mix containing many different and unknown microorganisms. Moreover, Jones et al. (1994) reported that dilution plating using the CVP colony count method cannot be relied upon for its selectivity, as E. carotovora subsp. atroseptica (Eca) can also grow on this medium. Therefore, pure cultures of Ecc instead of soft rotted flesh were used for all further experiments.

Another reason for the low numbers of Ecc detected in tubers may be because washed potato tubers were used as the tuber samples, where these tubers are likely to have been treated with chemicals during the washing process in the commercial washing plant. This condition does not facilitate the bacterial entry to lenticels.

In the experiment that quantified the contamination of soft rot bacteria and tested the appropriate inoculation method, the result showed that increasing the inoculation level of Ecc did not increase the population of Ecc in the tubers. Subsequently, a poor correlation between the inoculation level of Ecc and Ecc numbers in lenticels was obtained. These findings contrasted with previous studies, which found a good relationship between potato tuber contamination with E. carotovora subsp. atroseptica (Eca), the closely related species of Ecc, and disease development (Wale et al., 1986; Bain et al., 1990). Another study suggested that the level of inoculation contributed to the severity of blackleg disease, caused by Eca (Robinson and Foster, 1987). Nevertheless, a slightly higher number of re-isolated Ecc was achieved at inoculation level of $10^6$ cfu/ml per tuber, both by dipping and by the infiltration method. As a result, $10^6$ cfu/ml was used as the preferred inoculation level of Ecc for further experiments. Such evidence supports the
view that $10^5$ and $10^6$ cells/ml were sufficient for disease development (Hall, 1986).

Furthermore, none of the inoculation methods achieved satisfactory result. However, the dipping method, which gave slightly higher numbers of re-isolated Ecc, can be regarded as the method of choice for subsequent experiments. Variation in the population of Ecc in lenticels was found when the tubers were vacuum infiltrated. This result is in agreement with a previous study that confirmed failure of such a method to give consistent result (Bain and Pérombelon, 1988). However, another study reported a satisfactory result, where the disease development was more severe after vacuum infiltration of Ecc into the potato tubers (Hélias et al., 2000). Additionally, the contamination level of Erwinia on the tubers, which were vacuum infiltrated, was greatly influenced by the number of entry points, which were open lenticels (Lapwood and Read, 1986). Although the tuber samples of this study were incubated in wet condition to encourage lenticel opening, this effort failed to increase the population of Ecc in the tubers. These unsatisfactory results may due to the use of washed potato tubers as tuber samples, which are likely to be treated with chemicals. This is presumably because chemical compounds from the washing process still protected the tubers from other pathogens, including soft rot bacteria. Another possibility is that the potato tubers used in this study may be resistant cultivars, where the origin of the tubers were unknown.

In addition, although pure cultures of Ecc replaced soft rotted inocula, it still did not facilitate a high contamination level. Consequently, either a higher inoculation level of Ecc or the inoculation method does not assist the infiltration of the bacteria into the lenticels of potato tubers.
In the experiment investigating the effect of chlorine solution and drying treatment, two types of potatoes were compared, i.e. washed and brushed potatoes. Interestingly, when using washed potato tubers, the level of Ecc of in the control treatment did not substantially increase even though sterilized potato crush (which is assumed to be a nutrient source for the bacteria) and surfactant (which is supposed to promote bacterial infiltration) were added. This finding with contrasted with previously published work that confirmed the effectiveness of surfactant. Bartz (1999) reported that soft rot severity increased when the surfactant was added to the Ecc suspension. Similar to previous experiments, this result might be due to the use of washed potato tubers that were presumably protected by chemicals.

Dipping the washed potato tubers with different concentrations of chlorine solution did not effectively eliminate the number of Ecc, nor reduce the soft rot severity. Increasing the chlorine concentration resulted in higher populations of Ecc in lenticels. This suggests there is phytotoxic effect of high concentrations of chlorine. The chlorine solution appeared to damage the tubers by creating more infection sites, where the bacteria could invade after the evaporation of the chlorine, at the same time as killing the surface cells of the tuber (Scholey et al., 1968; Bartz, 1999). Furthermore, treating the tubers by dipping (for 10 min.) in the chlorine solution also facilitated the phytotoxic effect, because the duration of contact between the chlorine solution and the tubers was too long. Consequently, the tubers were disinfected by chlorine by spraying in the subsequent experiment.

On the other hand, there was large amount of variation occurring in the experiment that examined the effect of chlorine and drying treatments on the potential for disease development. The results displayed a high degree of variability between experimental replicates, which resulted in large error bars. However, a later experiment utilizing brushed tubers did not show as much variation between replicates. Perhaps, not all the washed tubers had
been treated in the same manner when the tubers were washed through the commercial washing plant. While some of them may have been protected by the chemicals, others might not be covered by the chemical treatment. Therefore, the bacteria in some tubers could not be killed by the chlorine solution.

Likewise, with the experiment with brushed potato tubers, the same result was found. Either chlorine or drying treatments and the interaction between them had no effect on the population of Ecc re-isolated from lenticels. Large variation of replicates reflects the fact that experimental errors occurred in some treatments, particularly for the drying treatment. When tubers were wet, increasing chlorine concentration significantly reduced disease severity, however, when tubers were dry was much less disease symptoms and chlorine had no effect on the disease development.

These findings suggest that the effect of chlorine is likely temporary. This is in agreement with Scholey et al. (1968) who reported that the addition of sodium hypochlorite gave no long-term benefit and removing the surface cells could produce more sites for the bacteria to invade. Bartz (1999) also added that the highest chlorine concentration could not eliminate soft rot bacteria and damaged the tubers. More importantly, the effectiveness of chlorine as an antibacterial compound largely depends upon several factors, including concentration, pH, temperature, and organic content of the solution (Dychdala, 1983). It assumes that an increase in chlorine concentration will enhance the antibacterial activity. Nevertheless, this hypothesis is probably accurate as long as other factors are constant (Dychdala, 1983).
Similarly, the drying treatment gave inconsistent results. It is presumed that drying the tubers removes films of water from the surface of tubers, which eradicate a major tendency for soft rot development. Despite *Erwinia* cells on the tuber surface being susceptible to desiccation, as reported by Bartz and Kelman (1985a), a certain percentage of these bacteria are likely to survive for long periods in protected positions, particularly in lenticels (Pérombelon, 1973; Pérombelon, 1980). Furthermore, thorough air-drying did not prevent subsequent condensation or counteract previous mishandling of tubers (Bartz and Kelman, 1986). The soft rot symptoms continued to develop in dry tubers if the bacteria were present in the lenticels or the tubers were damaged (Bartz and Kelman, 1985b). Another study found that drying fresh washed potato tubers could reduce the soft rot development (Bartz, 1999). Accordingly, the drying treatment is more likely effective on washed tubers than brushed tubers.

In general, comparing two experiments that used washed and brushed potato tubers, the results showed that brushed tubers were more susceptible to *Ecc* contamination, which were artificially inoculated. This contrasted to previously published work that observed washed tubers, which had passed through a washing process in a commercial packing plant, were more likely to cause soft rot than brushed tubers (Lund and Kelman, 1977). However, it presumes that brushed tubers have not been treated, therefore; they are more susceptible to the bacteria.

Overall, the population of *Ecc* was not correlated with the potential of soft rot development, either on washed or brushed tubers. Only some treatments showed a significant correlation. This view contrasted with some reports, which found that the initiation and spread of soft rot were likely to be influenced by the number of soft rot bacteria (Lund and Kelman, 1977). Another study also found that soft rot development could be influenced by
the population of *Erwinia* and the frequency of the bacteria (Sturz and Matheson, 1996).

In summary, it has been found that chlorine solution is broadly ineffective in eliminating *Ecc* re-isolated from the lenticels of washed and brushed potato tubers. Likewise, a combination of chlorine and drying treatments also failed to decrease the population of *Ecc* in brushed tubers. However, the potential of soft rot development of brushed potato tubers could be slightly reduced by disinfecting the tubers with chlorine as well as treating with thorough air-drying.

Further work is required to look for better approaches to manage post harvest soft rot disease. Continued detection of *Ecc* in tubers using the lenticel sampling method and plate assay are generally time consuming and labour intensive, therefore; other detection methods should be investigated, in particular, serological methods.
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spread of Erwinia carotovora var. atroseptica into potato tubers. Potato
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carotovora var. atroseptica. Potato Research 15: 130-145.

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tubers derived from inoculated tubers of Solanum tuberosum L.

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weekly virulent radiation induced strain of Erwinia carotovora.
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APPENDICES

Appendix 1. Culture media

1. Nutrient agar

This medium was used as a non selective medium for sub-culturing the bacteria and storage. Medium contained nutrient agar (Gibco BRL, 23 g/l).

2. Nutrient broth

This medium was used as a general bacterial growth medium for scale up of bacterial cells for storage. Medium contained nutrient broth (Oxoid, 13 g/l).

3. Glycerol

Glycerol (BDH) was used as a freezing medium for long-term storage of the bacteria

4. Crystal violet pectate, selective for isolating pectolytic Erwinias (Cuppels and Kelman, 1974)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>1.0 g/l</td>
</tr>
<tr>
<td>Tri-sodium citrate</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>2.0 g/l</td>
</tr>
<tr>
<td>CaCl₂.2H₂O (10% aqueous solution)</td>
<td>13.6 ml/l</td>
</tr>
<tr>
<td>Crystal violet (0.075% aqueous solution)</td>
<td>2.0 ml/l</td>
</tr>
<tr>
<td>agar</td>
<td>4.0 g/l</td>
</tr>
<tr>
<td>Sodium polypectate (Sigma,citrus colloids)</td>
<td>18.0 g/l</td>
</tr>
</tbody>
</table>
Appendix 2. Test of comparison of means between lenticel sampling and peel method

<table>
<thead>
<tr>
<th>Treatment</th>
<th>F pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Inoculated</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Appendix 3. Analysis of variance table for the population of Ecc re-isolated per tuber of different inoculation levels of Ecc using two inoculation methods

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculation method</td>
<td>1</td>
<td>0.33135</td>
<td>0.33135</td>
<td>3.044913</td>
<td>0.100</td>
</tr>
<tr>
<td>Ecc concentration</td>
<td>3</td>
<td>0.16755</td>
<td>0.05585</td>
<td>0.513229</td>
<td>0.679</td>
</tr>
<tr>
<td>Inoc.method . Ecc concentration</td>
<td>3</td>
<td>0.87195</td>
<td>0.29065</td>
<td>2.670904</td>
<td>0.083</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>1.741133</td>
<td>0.108821</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>3.111983</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Appendix 4. Analysis of variance table for the population of Ecc in lenticels per tuber at different levels of chlorine concentration without drying treatment on washed potato tubers

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine treatment</td>
<td>3</td>
<td>0.249625</td>
<td>0.083208</td>
<td>0.534157</td>
<td>0.672</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>1.2462</td>
<td>0.155775</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>1.495825</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix 5. Analysis of variance table for the severity of soft rot with several chlorine concentrations on washed potato tubers.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying treatment</td>
<td>1</td>
<td>7.59375</td>
<td>7.59375</td>
<td>7.923913</td>
<td>0.012</td>
</tr>
<tr>
<td>Chlorine</td>
<td>3</td>
<td>2.78125</td>
<td>0.927083</td>
<td>0.967391</td>
<td>0.432</td>
</tr>
<tr>
<td>Drying.chlorine</td>
<td>3</td>
<td>0.28125</td>
<td>0.09375</td>
<td>0.097826</td>
<td>0.960</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>15.33333</td>
<td>0.958333</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>23</strong></td>
<td><strong>25.98958</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Appendix 6. Analysis of variance table for the population of Ecc in lenticels per tuber with and drying treatment and several chlorine concentrations on brushed potato tubers.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying</td>
<td>1</td>
<td>11395563</td>
<td>11395563</td>
<td>5.896989</td>
<td>0.021</td>
</tr>
<tr>
<td>Chlorine</td>
<td>3</td>
<td>2542688</td>
<td>847562.5</td>
<td>0.438598</td>
<td>0.727</td>
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<tr>
<td>Drying.chlorine</td>
<td>3</td>
<td>14363188</td>
<td>4787729</td>
<td>2.47756</td>
<td>0.079</td>
</tr>
<tr>
<td>Residual</td>
<td>32</td>
<td>61838000</td>
<td>1932438</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>39</strong></td>
<td><strong>90139438</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Appendix 7. Analysis of variance table for the severity of soft rot with several chlorine concentrations on brushed potato tubers.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying</td>
<td>1</td>
<td>28.9</td>
<td>28.9</td>
<td>199.3103</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Chlorine</td>
<td>3</td>
<td>4.56</td>
<td>1.52</td>
<td>10.48276</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Drying.chlorine</td>
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<td>1.356</td>
<td>0.452</td>
<td>3.117241</td>
<td>0.0396</td>
</tr>
<tr>
<td>Residual</td>
<td>32</td>
<td>4.64</td>
<td>0.145</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>39</strong></td>
<td><strong>39.456</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>