Integrated control of bacterial soft rot of potato caused by *Pectobacterium carotovorum* subsp. *carotovorum* using bacterial biocontrol agents and lemongrass (*Cymbopogon citratus* L.) essential oil

By

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DISSERTATION SUMMARY

Potato (*Solanum tuberosum* L.) is an economically significant staple crop widely grown and consumed worldwide. It is a relatively perishable vegetable and prone to biotic factors during postharvest storage and transportation resulting in food insecurity and poor quality. Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is one of the most economically important diseases, causing significant losses of potatoes both preharvest and postharvest.

Preventative measures used to control the disease such as planting disease-free seeds, avoidance of contamination, and disinfection machines and tools are currently used. However, these control strategies are not effective in suppressing pathogen infection. On the other hand, chemical bactericides are not recommended to be used as one of the control methods due to their toxic effect, non-persistence and development of resistance by bacteria. Therefore, there is a crucial need for the development of natural, sustainable, environmentally friendly, and generally regarded as safe (GRAS) antimicrobials such as essential oils and biocontrol agents. Thus, the aim of this study was to investigate the effect of bacterial biocontrol agents and lemongrass essential oil, individually and in combination, to control *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) *in vitro* and *in vivo*.

A total of 150 bacterial isolates were isolated and screened against *Pcc* for their antibacterial activity using a disc diffusion assay *in vitro* and a preventative essay on whole potatoes. *Bacillus amyloliquefaciens* isolates (YMCB, BMO3, and MSAG) had the best antibacterial effect against *Pcc in vivo* and *in vitro*. They caused cell deformation of *Pcc* and leakage of its constituents while protecting the potato cell organelles.To evaluate the antibacterial effect of lemongrass (LE) on *Pcc*, LE concentrations LE0.125%, LE0.175%, LE0.5%, LE1.0%, LE2.0% and LE3.0% were prepared and tested against *Pcc in vitro* and on ‘Sifra potatoes. LE3.0% had the highest antibacterial activity (34.00 mm inhibition zone) against *Pcc in vitro* and invaded the plasma membrane of the *Pcc* cells, which resulted in *Pcc* cell deformation and leakage of the cell constituents. LE0.125% and 0.175% controlled bacterial soft rot incidence by 94.83% and 89.66%, respectively, on ‘Sifra’ potatoes compared to the control treatment (0.00%).TEM micrographs of potato wounds treated with

LE0.125% showed LE essential oil deposistion in the potato cell wall and inside the cell compared to the control that showed viable *Pcc* cells.

Furthermore, the antibacterial activity of the treatment combination was evaluated by using the best LE concentrations and biocontrol isolates and screened against *Pcc* using disc-diffusion and dipping methods *in vivo*. LE3.0% + *B. amyloliquefaciens* (YMCB) had the largest pathogen inhibition zone (30.00 mm) and caused cell deformation of *Pcc* cells. A treatment combination of LE0.125% + *B. amyloliquefaciens* (MSAG) showed the highest antibacterial effect and reduced bacterial soft rot incidence to 96.56%. The TEM micrographs of potato wounds treated with LE0.125% + *B. amyloliquefaciens* (MSAG) showed that there was an interaction between different antimicrobials from LE essential oil and *B. amyloliquefaciens* in the potato cell wall and amyloplast. In conclusion, the research findings from this study suggest that lower concentrations of lemongrass essential oil (LE0.125% and LE0.175%) can be combined with *B. amyloliquefaciens* strains to control bacterial soft rot of potatoes during storage.

PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Plant Pathology, School of Agricultural, Earth and Environmental Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by Potatoes South Africa.

DECLARATION

I, CAROLINE NOZIPHO MHLABA, declare that:

I. The research reported in this thesis, except where otherwise indicated, is my original work.

II. This thesis has not been submitted for any degree or examination at any other university.

III. This thesis does not contain other persons" data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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Caroline Nozipho Mhlaba (MSc candidate)

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Dr Nokwazi Carol Mbili (Supervisor)

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I would like to thank my late mother, Mrs V. Silva-Mkhathswa. I am grateful for the constant support, love, prayers, and motivation.

Finally, I thank God for all the strength He gave me to complete this study.

“But as for you, be strong and do not give up, for your work will be rewarded." 2 Chronicles 15:7

DEDICATION

I dedicate my work to my late mother, Mrs V. Silva-Mkhathswa.

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CHAPTER 1

DISSERTATION INTRODUCTION

Background

Potato (*Solanum tuberosum* L.) is South Africa's (SA) most important vegetable crop and the world's most widely consumed staple food (Devaux *et al*., 2014). It is an essential crop recommended as a food security crop by the United Nations (FAO STAT, 2023). Potato is an annual plant in the nightshade family (*Solanaceae*), grown for its starchy edible tubers, high in nutrients and low in fat. They are a rich source of potassium, copper, vitamin C, manganese, phosphorus, niacin, dietary fiber, pantothenic acid, and vitamins B1 and B6. Additionally, potatoes are extremely low in sodium (Beals, 2019).

China is the largest producer of potatoes worldwide, with about one-third of the world's potatoes produced in China and India. FAO estimated that in 2022, over 375 million metric tons of potatoes were produced worldwide, an increase from a production volume of 333.6 million tons in 2010. The total production of potatoes in SA was over 2.5 million metric tons in 2022 (Statista, 2023). The major potato-producing areas in SA are Limpopo (19%), Western Free State (16%), Sandveld area (15%), and Eastern Free State (11%). These four regions represent ± 61% of the total potato production in SA (DALRRD, 2022). Potatoes have high sugar and starch content, which makes them susceptible to bacterial and fungal diseases (Pérombelon, 2002).

Potato is a relatively perishable vegetable and undergoes various losses during postharvest storage and transportation, including biotic stresses that affect production quality and quantity (Alexios and Spyridon., 2021). Postharvest potato diseases remain a concern of the seed potato production sector in many potato-growing regions worldwide. Bacterial soft rot of potatoes is one of the most economically important diseases, causing a significant reduction in the yield of potatoes, resulting in economic losses of up to 60% during transit and storage (Abo-Elyousr *et al*., 2014).

Bacterial soft rot is a complex disease caused by Gram-negative and Gram-positive bacteria, with *Dickeya* and *Pectobacterium* being the most studied bacterial soft rot bacteria. In addition to soft rot, these bacterial pathogens cause potato blackleg

(Czajkowski *et al*., 2015). Soft rot bacteria were classified within the *Erwinia* genus and divided into species and subspecies based on molecular, biochemical, and host range differences (Wegener, 2001). Strains formally classified as *Erwinia carotovora* have been incorporated into the genus *Pectobacterium*, while those classified as *Erwinia chrysanthemi* are now assigned to the genus *Dickeya* (Czajkowski *et al*., 2015).

Pectobacterium carotovorum subsp. *carotovorum* (Jones) has a wide host range, causing soft rot disease in crops such as potatoes (*Potato tuberosum* L.), carrots(*Daucus carota)*, capsicum(*Capsicum annuum*), and calla lily *(Zantedeschia aethiopica*) (Pérombelon, 2002), and *P. atrosepticum* primarily infects potatoes, causing blackleg of the stem and tuber soft rot (Pérombelon, 2002). The most distinguishing feature of the pathogenicity of *Pectobacterium* and other soft rot bacteria is the production of various plant cell wall degrading enzymes (PCWDEs), such as pectinases, cellulases, and proteases, which destroy tissues and deprive the bacteria of nutrients (Davidsson *et al*., 2013). Tubers are susceptible to soft rot during harvest, storage, and transportation due to injury, poor ventilation, and high humidity. Infected seed tubers may fail to germinate, have blackleg, or wilt during the growing season, and in severe infections, progeny tubers may rot in the soil (Hadizadeh *et al*., 2019).

Methods for avoiding contamination and reliance on seed certification schemes are widely used and have been partially successful in soft rot management. Improved storage management can reduce bacterial load on tubers and tuber rotting (Czajkowski *et al*., 2011). Chemical bactericides are not recommended for use in controlling soft rot bacteria due to their non-persistence, adverse toxic effects, high cost, and bacterial population development of resistance (Abd-El-Khair *et al*., 2021).

1.2 Problem statement

Potato soft rot is caused by different bacteria worldwide, such as *P. carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum* and *Dickeya* species (Van der Merwe., 2009). *P. carotovorum* subsp. *carotovorum* has been reported as the most common causative agent of bacterial soft rot and is among the top ten plant pathogenic

bacteria with a wide range of hosts in tropical and subtropical regions, infecting plant species belonging to different prominent plant families (Czajkowski *et al*., 2015). Soft rot bacteria mainly infect potato tubers that have been damaged by mechanical injury or the presence of other diseases (Khlaif and Wreikat, 2018). The pathogenicity of *Pectobacterium* is linked to its ability to produce extracellular enzymes such as pectate lyases and other pectinases, cellulases, and proteases, which degrade cell wall elements and cause tissue maceration or rot symptoms (Baz *et al*., 2012).

1.3 Justification

The management of potato soft rot is essential to reduce the effect of the disease. Several strategies have been investigated to control tuber soft rot, but the level of success has varied.

Seed certification programs, washing and disinfection of machines used when planting, spraying, haulm flailing, harvesting, and grading in-store help reduce risks of introducing soft rot bacteria in pathogen-free crops(Charkowski, 2018). Chemical bactericides are not recommended for use in controlling soft rot bacteria due to their non-persistence, adverse toxic effects, high cost, and bacterial population development of resistance (Rahman *et al*., 2012). Therefore, biological control is a potential method to control bacterial soft rot disease. Biological control has been and is still being attempted with some success (Salem *et al*., 2018). Biological control agents are environmentally friendly, pose no threat to human health, and do not develop resistance compared to bactericides (Lahlali *et al*., 2022).

The strategy for biological control of plant diseases involves the use of antagonistic microorganisms before or after the infection. Commercial biological control agents are available for seed treatments and soil amendments to protect plants against pathogens (Heimpel and Mills., 2017). Currently, bacterial microorganisms such as *Bacillus subtilis* and *Pseudomonas spp*. and the fungi *Gliocladium spp.* and *Trichoderma spp*. are the organisms mainly used for biological control strategies (Tariq *et al*., 2020). Previous studies reported that some fluorescent *Pseudomonas* and *Bacillus* strains can act as biological control agents against soft rot pathogens (Vanneste and Yu, 1995; Algeblawi and Adam, 2013; Salem *et al*., 2018). *Bacillus*

possess various biocontrol properties and a unique ability to multiply quickly. It releases various antimicrobial substances by producing compounds, such as volatiles, and induce defence and growth responses (Fira *et al*., 2018). Some antagonistic yeasts have also been reported to inhibit postharvest decay of fruits and vegetables effectively and have shown potential as an alternative to synthetic fungicides (Spadaro and Gullino, 2004; Droby *et al*., 2009; Sharma *et al*., 2009).

Chemical fungicides may be replaced by natural plant protectants such as essential oils (EOs) and their important constituents. The EOs are naturally occurring antioxidants that have no lasting effects on fresh food and are well-known for their antibacterial and biodegradable qualities (Sivakumar and Bautista-Baños, 2014). EOs and their components are gaining popularity due to their volatile nature, which facilitates using safe, small concentrations for consumption. They are also harmless to the environment and are known as reduced-risk pesticides. The generally recognized as safe (GRAS) compounds status of EOs favors their application as biopesticides to control pests and diseases and provide safe food (Assadpour *et al*., 2023). Lemongrass (*Cymbopogon citratus)* is an essential oil with a growing reputation due to its antimicrobial properties. The effects of lemongrass on various pathogens have been investigated in several studies, and it has antimicrobial, antibacterial, and anti-fungal properties (Plotto *et al*., 2003; Tzortzakis and Economakis., 2007; Ali *et al*., 2015; Mbili *et al*., 2017).

Integrating biocontrol agents with other control strategies to improve their efficacy may be the best option in large-scale applications, with the goal of minimizing the quantity of fungicide required to manage postharvest diseases of essential fruits and vegetables (Arrebola et al., 2010). More research has focused on controlling bacterial pathogens using biocontrol agents or essential oils alone. Few studies have investigated the integration of EOs and biocontrol antagonists against bacterial pathogens. Therefore, this research investigates the effect of integrating biocontrol agents and lemongrass essential oil.

1.4 Research aim and objectives

This study investigates the effect of bacterial biocontrol agents integrated with lemongrass essential oil on bacterial soft rot of potatoes *in vitro* and *in vivo*. The specific research objectives were:

- To investigate the effect of bacterial biocontrol agents against *P. carotovorum* subsp. *carotovorum in vitro* and *in vivo.*
- To investigate the effect of lemongrass essential oil on *P. carotovorum* subsp. *carotovorum in vitro* and *in vivo*
- To investigate the integration of *B. amyloliquefaciens* and lemongrass essential oil *in vitro* and *in vivo.*

1.3 Thesis outline

Chapter 1: Dissertation Introduction

Chapter 2: Literature Review

Chapter 3: *In vitro* and *in vivo* effect of bacterial biocontrol agents against *Pectobacterium carotovorum* subsp. *carotovorum* of potato

Chapter 4: *In vitro* and *in vivo* antibacterial activity of *Cymbopogon citratus L.* essential oil against *Pectobacterium carotovorum* subsp. *carotovorum* causing bacterial soft rot of 'Sifra' potato tubers

Chapter 5: The antibacterial effect of *Cympopogon citratus* L. and *B. amyloliquefaciens* on postharvest potato soft rot caused by *Pectobacterium carotovorum* subsp*. carotovorum*

Chapter 6: Thesis Overview

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Potato (*Solanum tuberosum.* L) is grown worldwide. It is the fourth most important crop in the world after rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*), with a global production of 375 million tons in 2022 (FAO STAT, 2023). Potatoes are a versatile root vegetable and a staple food in many households (DAFF, 2013). Approximately 22% of potatoes are lost per year due to viral, bacterial, fungal, and insect pest attacks on potato tuber and potato plant, incurring an annual loss of over 65 million tons and bacterial soft rot alone accounts for 30–50% of this vast loss (Umunna and Anselem, 2014).

Potato soft rot caused by *Pectobacterium. carotovorum* subsp. *carotovorum* is one of the essential diseases of potatoes, causing a significant reduction in yield and economic losses in the field during transit and storage, causing losses of up to 60% (Khlaif and Wreikat, 2018). Soft rot bacteria mainly infect potato tubers that have been damaged by mechanical injury or the presence of other diseases.*P. carotovorum* subsp. *carotovorum* pathogenicity is linked to their ability to produce extracellular enzymes such as pectate lyases and other pectinases, cellulases, and proteases, which degrade cell wall elements and cause tissue maceration or rot symptoms (Baz *et al*., 2012).

Soft rot symptoms can develop during storage and in the field where early decay of seed tubers or seed pieces can result in the non-emergence of potato plants. Traditional phytosanitary and cultural practices are the foundation for controlling the bacterial soft rot of potatoes (Khlaif and Wreikat., 2018). There are no efficient bactericides used to protect potatoes against *Pectobacterium spp*. (Latour *et al*., 2008). Biological control is receiving increasing attention as an alternative means of disease control, both pre-harvest and postharvest, especially where disease resistance or chemical control is unavailable.

Biological control of plant pathogenic bacteria could be an alternative to chemical and physical control. Biological control of plant diseases is the suppression of populations of plant pathogens by living organisms (Heimpel and Mills, 2017). Previous studies

reported that some *Pseudomonas, Trichoderma and Bacillus* sp*.* strains could act as biological control agents against soft rot pathogens (Abd-El-Khair *et al*., 2021).

2.2 Potatoes

The potato plant (*Solanum tuberosum.* L) is a member of the *Solanaceae*, or nightshade family of flowering plants. Potatoes are rhizomes (modified stems) attached to the root system (de Haan and Rodriguez, 2016). They are covered by an outer skin called the periderm. Inside is the cortex, which serves as a storage area for protein and starch. The skin varies from brownish white to deep purple; the starchy flesh ranges typically from white to yellow, but it can also be purple (Millam, 2006). Potato cultivars available in South Africa can be divided into three groups according to the length of their growing periods. Early cultivars (less than 100 days) with 'Vanderplank' as the most popular cultivar, and medium-growing season cultivars (100 to 120 days), of which 'BP1' and 'Up to Date' are currently the most popular cultivars, longer growing seasons (longer than 120 days) such as 'Sackfiller', 'Late Harvest', 'Kimberley's Choice' and 'Cedara' (DAFF, 2013).

2.2.1 Origin of potatoes

The potato is native to South America, specifically the Andean Mountains. The Incan people domesticated the potato between 3700 and 3000 BC (Yin, 2016). The Incans introduced their potato to Spanish explorers in the 1500s, who, in turn, took it to Europe. China is the biggest producer of potatoes worldwide, with about one-third of the world's potatoes produced in China and India (Devaux *et al*., 2021).

2.2.2 Potato production in South Africa

Potato production in South Africa has increased dramatically in recent decades, from 1.2 million tons in 1990 to 2.5 million tons in 2021 (Potatoes SA, 2021). Potato cultivation area fell from 63,000 ha to 52,000 ha within the same period. Potato production can be done under irrigation or on dry land. According to (DALRRD, 2022), the major potato-producing areas in South Africa are the Limpopo (19%), the Western

Free State (16%), the Sandveld area (15%), and the Eastern Free State (11%). These four regions represent ± 61 % of total potato production in the country.

2.2.3 Uses and importance of potatoes

Potatoes are an essential crop recommended as a food security crop by the United Nations (Devaux, 2014). Potato is a globally crucial high-yield crop that produces nutrient-rich tubers. They provide high food energy and complex carbohydrates while taking up a smaller unit of land than other alternatives. Potatoes are an incredibly versatile root vegetable consumed in various dishes worldwide. It is also used as an industrial source for adhesives, binders, and texture agents and potentially for fuel-grade ethanol production (Srichuwong *et al*., 2009).

Potatoes have many health benefits. They contain iron, phosphorus, calcium, magnesium, and zinc, all of which aid in forming and maintaining bone structure and strength (Beals, 2019). The potato is also high in potassium, calcium, and magnesium, which naturally lower blood pressure. The potato's fibre, potassium, vitamin C, and vitamin B6 content, as well as its lack of cholesterol, all contribute to good heart health (Beals, 2019). Choline is an essential and versatile nutrient found in potatoes that aids muscle movement, mood, learning, and memory (Camire *et al.,* 2009). Potatoes are high in folate, which is involved in DNA synthesis and repair. Hence, it helps to inhibit the formation of many cancer cells caused by DNA mutations (Camire *et al.,* 2009).

2.2.4 Pathogen taxonomy and morphology

Pectobacterium carotovorum is classified under the family Enterobacteriaceae, Genus *Pectobacterium*, and the binomial name is *Pectobacterium carotovorum* subsp. *carotovora*um (Jones, 1901) Waldee 1945. *P. carotovorum* subsp. *carotovorum* is a Gram-negative, rod-shaped, non-sporulating facultatively anaerobic bacterium. The bacterial cells are typically 0.5-1.0 x 1.0-3.0 µm rods that are single-celled and motile with peritrichous flagella (Figure 2.1A; Niemi *et al*., 2017). *P. carotovorum* produces plant cell wall-degrading enzymes, including cellulase, pectinase, and polygalacturonase, which cause plant tissue maceration during infection (Lee *et al*.,

2014); *Pectobacterium* can be grown on nutrient agar or potato dextrose agar and form cream white colonies after three days at 26°C.

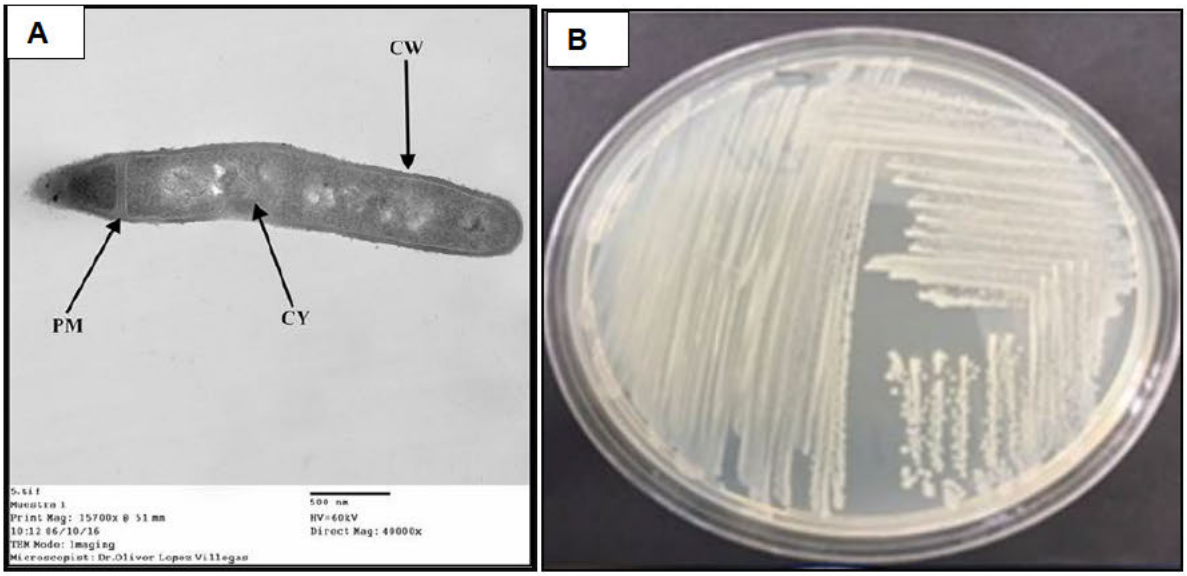

Figure 2.1: Transmission electron microscopy (TEM) micrograph of *Pectobacterium. carotovorum* subsp. *carotovorum* shows cell wall (CW), plasma membrane (PM) structures intact and continuous, and the cytoplasm (CY) enclosed within the cell (A) (Sotelo-Boyás *et al*., 2019). *P. carotovorum* subsp. *carotovorum* on nutrient agar media showing cream white colour colonies after three days at 26°C (B) (Cinisli *et al*., 2019).

2.4 Host range

Soft rot bacteria commonly affect plant storage organs, such as tubers, rhizomes, and bulbs (Ma *et al*., 2007). The bacteria can also be found in fleshy plant parts such as succulent stems, leaves, and vegetables with densely packed leaves like lettuce heads (Toth *et al*., 2003). *Pectobacterium* causes soft rot in various commercially significant vegetables, including cucumber (*Cucumis sativus*), radish (*Raphanus sativus*), carrot (*Daucus carota* subsp. *sativus*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), onion (*Allium cepa* L.), garlic (*Allium sativum*), potato (*Solanum tuberosum*), cabbage (*Brassica oleracea* var. *capitate*), sweet potato (*Ipomoea batatas*), tomato (*Solanum lycopersicum)*, and squash (*Cucurbita moschata*) (Opara and Asuquo, 2016).

2.5 Symptoms of bacterial soft rot

Soft rot symptoms include soft, wet, rotted, tan, or cream-coloured tissues. Rot begins on the tuber surface and progresses inward (Holmes,1997; Figure 2.2 B). Infected tissues are sharply delineated from healthy tissue by dark brown or black margins (Taylor, 2017; Figure 2.2 C). Shallow necrotic spots on the tubers result from infections through lenticels. Rotting tissue is usually odourless in the early stages of decay but develops a foul odour as secondary organisms invade infected tissue. Soft rot can also infect wounded stems and roots. Bacterial soft-rot symptoms are caused mainly by secreted pectinases, which degrade pectin in the middle lamella and primary plant cell walls, macerating the plant tissues and generating a wet, frequently foul-smelling rot of the plant organ (Charkowsk, 2018). The bacteria can cause necrosis in vascular tissues when it reaches primarily concentrated ions in the xylem.

The non-emergence of plants, wilting, browning of tissues, haulm desiccation, and plant death have all been linked to infection by soft rot bacteria (Elhalag *et al*., 2020). These symptoms are favoured by excellent, wet soils with temperatures of 10℃-15℃ at planting and above 20℃ after emergence. These conditions can result in blackleg (Ngadze, 2012; Figure 2.2 A), where the bacteria invade the internal vascular system of the plant and cause wilting (Elhalag *et al*., 2020). When infected tubers are cut, the margin of the rotted area will turn brown to black with a clear boundary between the rotted and healthy tissue (Holmes,1997; Figure 2.2 B). Infected potato tuber showing tissues are sharply delineated from healthy tissue by a dark brown or black margin (Taylor, 2017; Figure 2.2C). A mass of potatoes shows symptoms of bacterial soft rot in a commercial unit (Taylor, 2017; Figure 2.2D).

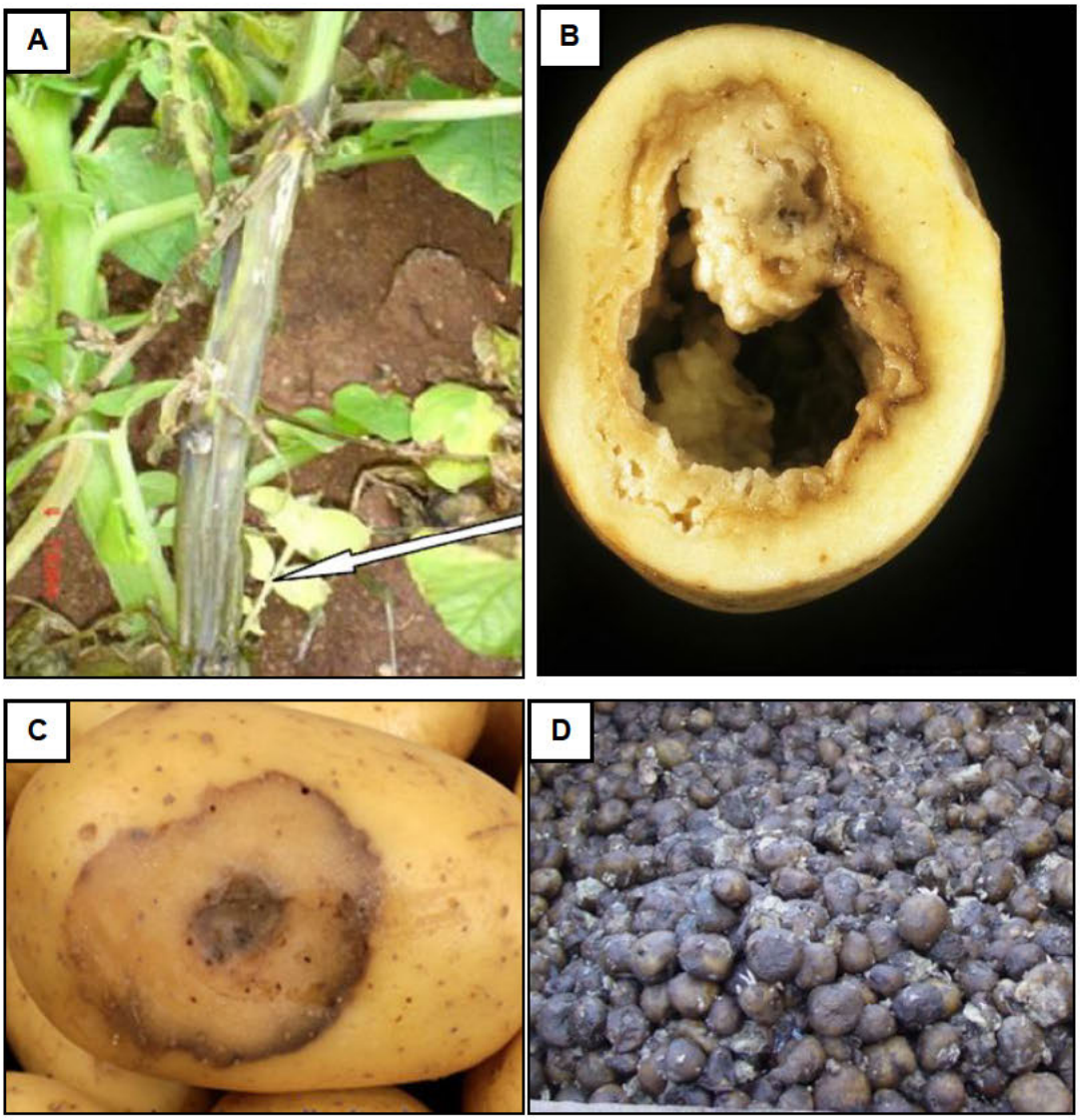

Figure 2.2: Symptoms of potato soft rot showing black leg and wilting on potato stem (Ngadze, 2012) (A), cut tuber showing soft rot and macerated tissue (Holmes,1997) (B), Infected potato tuber showing dark brown or black margin (Taylor, 2017) (C), Soft rot in the commercial units (Taylor, 2017) (D).

2.6 Economic importance and geographical distribution

P. carotovorum subsp. *carotovorum* causes tuber soft rot in stored farm produce and blackleg disease in the field. Soft rot causes heavy losses in stored potatoes if not correctly managed, creating a perception of poor quality in export seed potato markets (Abd-El-Khair *et al*., 2002). The non-emergence of plants, wilting, browning of plant tissues, stem desiccation, and plant death have all been linked to infection by soft rot

bacteria. *P. carotovorum* subsp. *carotovorum* has a broad host range worldwide. Potato soft rot is one of the most important diseases of potatoes, causing a significant reduction in yield, resulting in economic losses in the field during transit and storage, causing losses of up to 60% (Khlaif and Wreikat, 2018).

2.7 Disease epidemiology

Several species cause soft rot and blackleg in potatoes, and strains within each species differ in virulence features, making disease epidemiology extremely complicated (Colleta *et al*., 2014). The bacteria that cause soft rot can remain in potato plants and tubers without apparent symptoms. The symptoms of the soft rot bacteria only become apparent when the potato's natural resistance is overcome (Pérombelon, 2002). The leading cause of spread is wounds or damage to the potato tuber. These usually occur during harvesting and grading, allowing the bacteria to invade the tuber (Charkowski, 2018). When combined with water on the surface of the tuber, the bacteria can defeat the tuber's natural defences and start the tuber rot.

The soft rot bacteria do not overwinter in the soil. Survival in the soil is limited to one week to six months, depending on environmental factors such as soil temperature, moisture, and pH (Charkowski, 2018). The bacteria cannot survive in the soil in a 3 to 8-year crop rotation system. Soft rot can occur from as low as 16℃ to above 35℃ soil temperatures (Kucharek and Bartz,1980). High temperatures create ideal conditions as oxygen in the tuber is rapidly replaced by high carbon dioxide levels, causing stress on the tuber (Charkowski, 2018).

Airborne insects from infected plants can spread soft rot bacteria long distances and contaminate adjacent potatoes (Agrios, 2005; Figure 2.3). They can also be found in aerosols created by rain impaction on plants infected by blackleg and haulm pulverisation before harvest (Colleta *et al*., 2014). Exposure to sunlight also aids soft rot development by destroying the tuber cells. The development of secondary infections leads to increased infection. Therefore, bacterial soft rot should be controlled throughout the life of a potato to reduce its impact on future generations of potatoes.

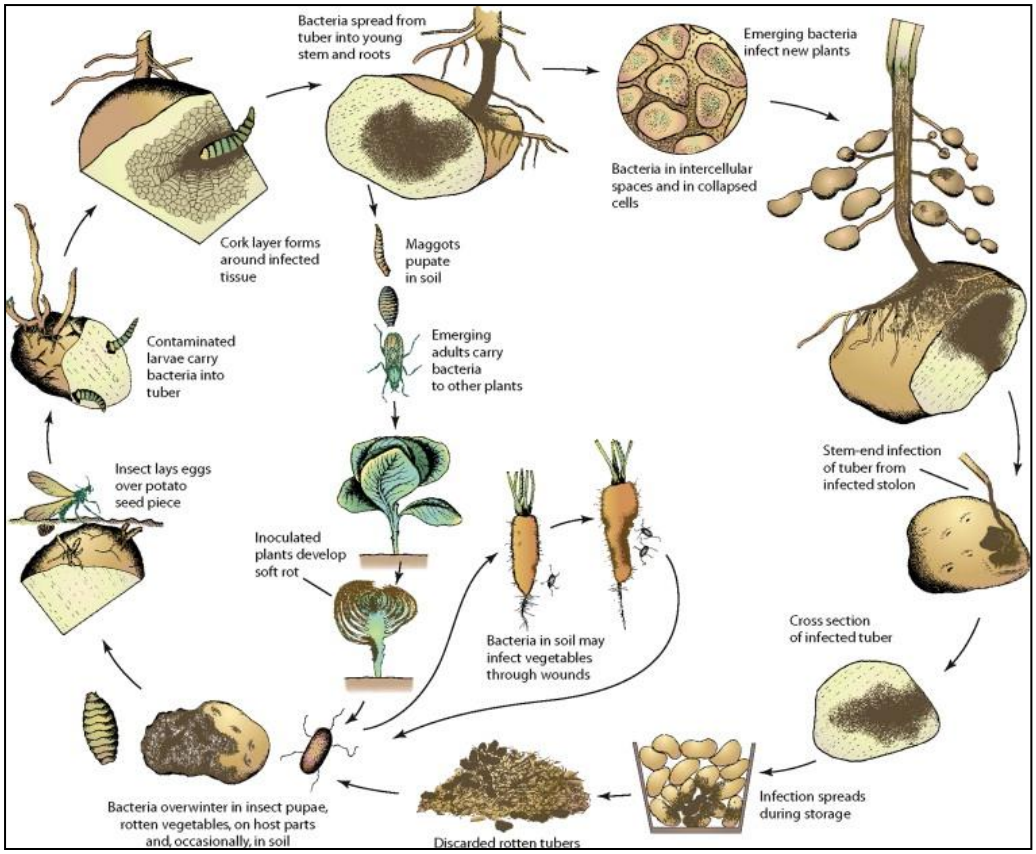

Figure 2.3: Disease cycle of bacterial soft rot of vegetables caused by soft rot bacteria sp. (Agrios, 2005).

2.8 Management of bacterial soft rot

2.8.1 Avoiding contamination

Washing and disinfection of machines used when planting, spraying, haulm flailing, harvesting, and grading help to reduce the risks of introducing soft rot bacteria in a pathogen-free crop (Pérombelon, 2002). During harvesting and grading, rotten tubers can be removed to reduce the spread and smearing of bacteria in a seed lot (Czajkowski *et al*., 2011). Correct machinery adjustment during harvesting and grading is critical to reduce the risk of wounding, as bacteria can survive after wound healing (Czajkowski *et al*., 2011). Wounding risks are reduced by using mature tubers with a well-developed periderm. Good storage management is critical to avoid tuber decay and increasing the tuber inoculum load, which would increase disease risks in the future.

2.8.2 Physical seed tuber treatment

The traditional method for preserving healthy seed tubers has been to store them in ventilated stores at low temperatures (5℃) (Fennir *et al*., 2005). These conditions are easily met in temperate countries, where storage temperatures can be regulated using cold outside air during the winter until planting time in the spring. However, storage is a significant issue in tropical and subtropical regions unless expensive refrigerated stores are available (Czajkowski *et al*., 2011). The disease can develop before cold storage or transit to the market, and specific treatments become helpful (Hadizadeh *et al*., 2019). Physical control, such as heat, is recognised as competitive with biological and chemical methods as it does not require registration and may be effective against a broad range of pathogens (Katan, 2000). However, physical procedures affect not only superficially located pathogens but also beneficial microorganisms and may negatively influence tuber emergence and health (Czajkowski *et al*., 2011).

The physical factors in controlling tuber soft rot infection involve hot water, hot air, UV, and solar radiation. Jeong *et al*. (2016) investigated the antibacterial efficacy of gamma irradiation *in vitro* and *in vivo* against *P. carotovorum* subsp. *carotovorum* and discovered that gamma irradiation in a bacteria cell suspension significantly dropped viable counts. Scanning electron microscopy of irradiated cells revealed substantial damage to the surface of most bacterial cells. Johnson (1966) investigated the effect of hot water treatment and chloride on soft rot bacteria. Hot water treatment at temperatures 52℃-53℃ reduced soft rot incidence on long-stemmed bell peppers inoculated with *P. carotovorum* subsp. *carotovorum* after 6 hrs of inoculation.

2.8.3 Chemical control of bacterial soft rot of potatoes

Chemical control strategies for bacterial diseases are based on pathogen eradication and creating unfavourable environmental conditions for disease development. Disease control is limited once the disease has begun due to the rapid multiplication and spread of bacteria and the inability of chemicals to penetrate the inner tissues (Czajkowski *et al*., 2011). Chemical control has limitations because bacterial pathogens are usually well protected in lenticels, suberised wounds, and the vascular system. There are no systemic bactericides for the management of bacterial soft rot;

even if available, they would be ineffective postharvest due to the lack of vascular activity in harvested tubers (Bonde and Souza, 1954).

Streptomycin was once considered as a promising control agent for potato blackleg and soft rot diseases (Bonde and de Souza, 1954). Before planting, seed tubers were immersed in a mixture of streptomycin and oxytetracycline hypochlorite or streptomycin and mercury compounds, which reduced the incidence of blackleg and tuber decay in storage (Bonde and de Souza, 1954). However, even though antibiotic treatments showed promise, larger-scale field studies are no longer permitted due to the risks of introducing resistance to human or animal bacterial pathogens(Czajkowski *et al*., 2011).

Abd-El-Khair and Karima. (2007) studied the *in vitro* and *in vivo* effect of bactericides and bioagents on *P. carotovorum* subsp. *carotovorum* of potatoes. Starner and streptomycin sulfate reduced the cellulolytic enzyme of the bacterium, and the plant tubers yielded, and the average tuber yield increased when the bactericides were applied.

Mahovic *et al*. (2007) showed that within 24 hrs of inoculation on tomato fruits, bacterial soft rot was observed on >80% of the nontreated tomato wounds. Tomato wounds exposed to an atmosphere containing up to 99 mg chlorine gas(ClO_2) for a 2- or 24-hrs period remained firm and dry without bacterial soft rot symptoms**.**

2.8.4 Biological control of Bacterial soft rot of potatoes

Biological control is one of the most essential methods for controlling bacterial soft rot disease (Algeblawi and Adam, 2013). Biological control of plant pathogenic bacteria could be an alternative to chemical and physical control. Biological control of plant diseases is the suppression of populations of plant pathogens by living organisms (Heimpel and Mills, 2017). Biocontrol strategies comprise antagonists affecting pathogen populations directly or via antibiosis, competition for nutrients, or induction of plant systemic resistance (Osei *et al.,* 2022; Figure 2.2).

The potential of biological control of bacterial soft rot with *Bacillus spp., Pseudomonas spp., and Trichoderma spp.* in different crops has been reported (Table 2.1). Microbial antagonists and their bioactive substances have gained attention as recommended and less harmful alternatives to chemical pesticides for eco-friendly plant disease

management. *Bacillus* and yeast species are used as biocontrol agents to control bacterial pathogens.

Table 2.1: Bacterial and fungal antagonists tested for their antimicrobial efficacy and mode of action against *P. carotovorum* subsp. *carotovorum* on different crops.

Antagonist	Mechanism	Crop	References
Pseudomonas spp.	Antibiosis	Valerian	Ghods-Alavi *et al.* (2012)
P. putida	Mycoparasitism	Carrot	Sowmya *et al.* (2012)
P. fluorescens	Antibiosis	potato	Haas and Defago (2005)
P. aeruginosa	Antibiosis, toxins production	potato	Rahme *et al.* (1997)
P. brassicacearum	Antibiosis	potato	Nelkner *et al.* (2019
Bacillus simplex BA2H3	Antibiosis	potato	Khayi *et al.* (2015)
Bacillus spp. *B. pumilus*	Antibiosis	Pepper	Silva *et al.* (2014)
Trichoderma asperellum	ISR	Okra	Idowu *et al.*(2016)
P. fluorescens *B. megaterium* *B. pumilus* *T. harzianum* *T. viride* *T. virens*	Antibiosis, Production of siderophores	Potato	Abd-El-Khair *et al*.(2002)

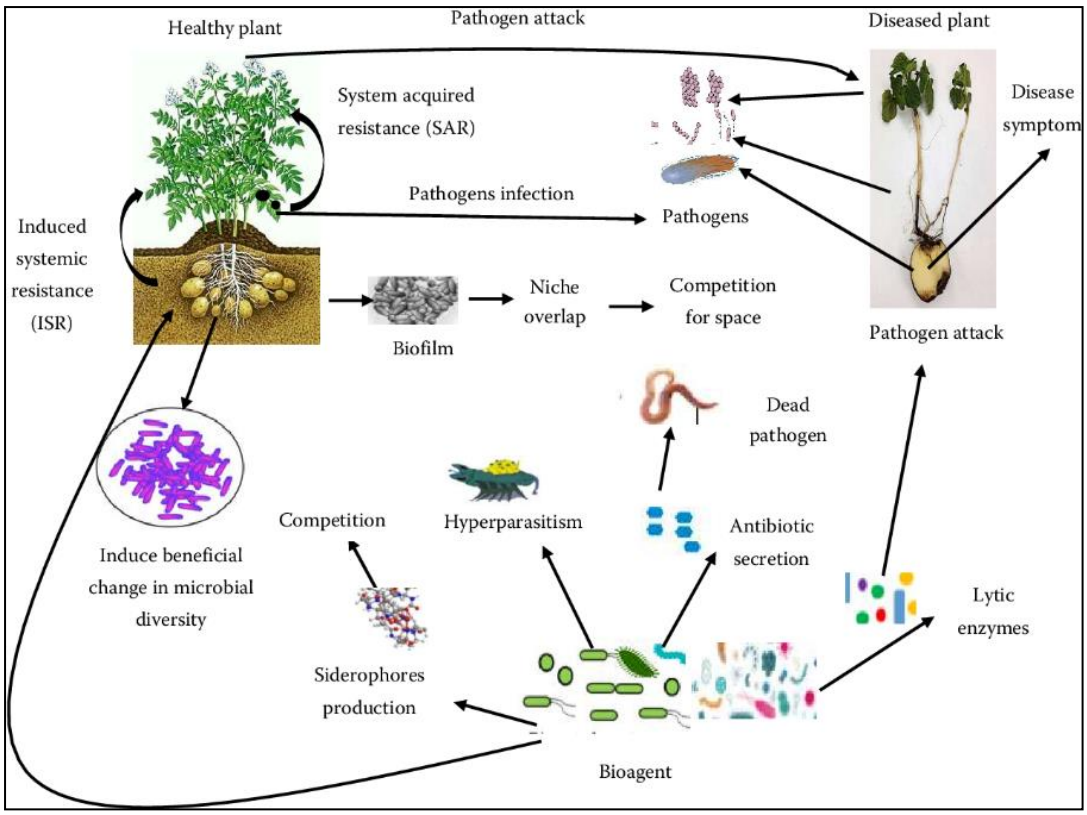

Figure 2.4: Mode of action of bioagents against potato soft rot (Osei *et al.,* 2022).

2.8.4.1 Bacteria as a biocontrol agent

Many researchers have investigated the effect of beneficial bacteria in enhancing crop growth and disease management. Currently, several bacteria of different genera, such as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Acinetobacter*, *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Rhizobium*, and *Streptomyces*, have been proven to be biocontrol agents for many economically significant plant diseases (Elshahat *et al*., 2016; Höfte, 2021; Etesami *et al*., 2023; Le *et al*., 2023)

Most *Bacillus spp.* are important microbiome members and play a significant role in plant protection and disease management (Dimkić *et al*., 2022). Using *Bacillus* spp. as a biological control agent against plant pathogens has many advantages. They are environmentally friendly (Serrão *et al.,* 2024). *Bacillus* have spore-forming characteristics that make them viable in hostile environments (Checinska *et al*., 2015) and can produce a wide range of antimicrobial compounds (Stoica *et al*., 2019).

The synthesis of antimicrobial compounds is considered one of the most significant biocontrol mechanisms for *Bacillus* spp. with space and nutrition competition coming

in second (Fira *et al*., 2018). *Bacillus* species can also be directly antagonists by causing plant systemic resistance ISR (Hossain and Chung, 2019). *Bacillus* spp. are known for producing a variety of antimicrobial compounds, including peptide and lipopeptide antibiotics (Tran *et al*., 2022). Peptides are divided into two categories based on whether they are synthesised ribosomal (bacteriocins) or n onribosomal (polymyxins and iturins) (Albayrak, 2019).

Many secondary metabolites from *Bacillus* spp., such as lipopeptides, polyketides, and volatiles, can cause (ISR) (Shafi *et al.,* 2017). According to Huang *et al*. (2012), Dimethyl disulfide (DMDS), isolated from the *B. cereus* C1L strain, induced ISR in tobacco and maize plants, suppressing grey mould and southern leaf blight disease. Etchegaray *et al*. (2008) investigated the antagonistic effects of iturin and surfactin derived from the *B. subtilis* OG strain on *Xanthomonas campestris* pv. *campestris* (Xcc), and *Xanthomonas axonopodis* pv. *citri* (Xac) cells, where lipopeptides induced morphological changes in Xcc cells, such as cell wall damage, distortion, and intracellular content leakage and caused swelling and roughness of the Xac cell surface.

Bacillus spp. produce many enzymes such as chitinases, glucanases, cellulases, xylanases, and proteases that control fungal pathogens. Morales-Ruiz *et al*. (2021) showed that *B. cereus* B25 significantly suppressed the maize pathogen *Fusarium verticillioides* through the release of chitinases, which are involved in pathogen control by degrading cell walls and limiting pathogen growth.

2.8.5 Plant essential oils (EOs)

Essential oils (EOs) are primarily colourless, lipophilic, volatile secondary metabolites derived from aromatic plants. Fruits, bark, rhizomes, roots, flowers, resins, seeds, leaves, and wood are among the plant organs that contain these essential oils (Moghaddam and Mehdizadeh, 2017). Various substances, including phenols, hydrocarbons, aldehydes, alcohols, methoxy derivatives, methylenedioxy compounds, and terpenes, are constituents of EOs (Sadgrove *et al*., 2022). Potential biological activities of these bioactive chemicals include antibacterial and antifungal effects. Due to the phenolic compounds in their structures, they can be used as a flavouring, preservative, and functional food additive (Abdi-Moghadam *et al*., 2016). In

comparison to other synthetic preservatives, EOs are gaining increasing popularity due to their alignment with the contemporary trends of “green,” “safe,” and “healthy” food additives.

2.8.5.1 Chemistry of essential oils

Some essential oils contain more than 300 distinct chemicals; however, most are between 20–60 components in varying quantities. However, two or three components are frequently present in considerable amounts (20-70%) compared to other constituents in modest concentrations (de Sousa *et al*., 2023). The primary constituents of EOs are responsible for their biological characteristics. Minor chemicals, however, may also be crucial to bioactivity, either by enhancing the effects of main components or by having additive or antagonistic effects (Carson and Hammer, 2011). Essential oil components possess various main metabolic precursors and are created through diverse biosynthetic routes. Terpenoids (the primary group) and non-terpenoids (mostly phenylpropanoids) are the two main classes into which they can be separated (Carson and Hammer, 2011). They can all be classified as aldehydes, ketones, alcohols, oxides, esters, amines, amides, phenols, nitrogen and sulfur compounds, and heterocycles (Başer and Demirci, 2007).

2.8.5.2 Mode of action of essential oils

Few studies have been done recently to illustrate fully the mechanisms of action of EOs against phytopathogens (Raveau *et al*., 2020). To achieve their antimicrobial actions, most EOs interact with processes occurring in bacterial cell membranes, such as phosphorylation, electron transport, ion gradients, protein translocation, and other enzyme-dependent activities (Faleiro, 2011). Different EOs may have different modes of action regarding their antibacterial activity. The EOs change the structure of polysaccharides, fatty acids, and phospholipid layers by penetrating the bacterium's cell wall and cytoplasmic membrane. Toxin secreted by the bacteria is inhibited by the EOs and enhances membrane permeability and cell membrane leakage of the bacterial cell (Jugreet *et al*., 2020).

Essential oils have insecticidal, antibacterial, antifungal, and antiviral functions because they effectively kill various pests and pathogens under the influence of

various functional groups such as alcohols, aldehydes, phenols, terpenes, ketones, and other antibacterial compounds (Başer and Demirci, 2007). EOs antibacterial mechanisms might include loss of integrity of the cell walls and membranes, leakage of electrolytes, loss of intracellular constituents, an increase of bacterial electrical conductivity and nucleic acid concentration in cell suspension, and inhibition of respiratory metabolism decrease bacterial metabolic activity (Jugreet *et al.*, 2020). Gram-negative bacteria are less sensitive to EOs than Gram-positive bacteria because their outer membrane is rich in lipopolysaccharides which limits direct contact between the cytoplasmic membrane and the EOs (Faleiro, 2011). In addition, an outer membrane also restricts direct contact between the cytoplasmic membrane and the EOs, making them less vulnerable to EOs than Gram-positive bacteria.

2.8.5.3 Lemongrass EOs

Lemongrass (*Cymbopogon citratus*) is a fragrant, evergreen, perennial grass that forms clumps and grows to a height of about 1.5 meters (Abdulazeez *et al*., 2016). It produces several stiff stems that emerge from a short rhizomatous rhizome. It does not often produce flowers (Abdulazeez *et al*., 2016). The leaves are linear in shape, flat, erect, and blue-green. When crushed, they release a distinct lemon flavour (Majewska *et al*., 2019). *C. citratus* is a member of the *Poaceae* family, which has over 635 genera and 9,000 species (Sell, 2020). This herb plant grows widely all over the world. About 140 species of *Cymbopogon* are known to exist; 52 of these species are found in Africa, 45 in India, 6 in Australia, 6 in South America, 4 in Europe (but only in Montenegro), 2 in North America and other species are found in South Asia. The main species extensively grown for their EOs in various parts of the world are *C. exuosus* and *C. citratus* (Majewska *et al*., 2019).

About 60-80% of lemongrass EO is composed of neral, mineral, geranial, iso geranial, geraniol, geranyl acetate, citronellal, citronellol, germacrene-D, and lemon, which are the main constituents of most lemongrass species (Mukarram *et al*., 2021). Camphene, pinene, limonene, linalool, citronellyl acetate, element, and caryophyllene oxide are the remaining ingredients, also referred to as minor ingredients. Different functional groupings in lemongrass EO components show varying antibacterial

activity; aldehydes and phenols have the highest activities, while hydrocarbons and esters have the lowest (Kalemba and Kunicka, 2003).

Lemongrass EO has antimicrobial properties against many pathogens and has been used as an antiviral, antifungal, and antibacterial agent. It has been suggested that lemongrass EO induces the destruction of bacterial biofilms and hinders further bacterial growth and development (Mukarram *et al*., 2021). Furthermore, its components can weaken lipid bilayer bonds and destroy bacteria by disintegrating the membrane. Various studies have demonstrated the effectiveness of lemongrass EO in combating various pathogens, such as yeast, fungus, and gram-positive and gram-negative bacteria (Inouye *et al*., 2001; Naik *et al*., 2010; Mbili *et al.*, 2017).

According to Mbili *et al*. (2017), all EO treatments (lemongrass and citral) reduced *B. cinerea* in the volatile phase method at doses from 0.016 to 1.0% compared to the control treatment. Light and scanning electron microscopy studies revealed morphological damage to *B. cinerea* hyphae, such as vesiculation, cytoplasmic disintegration and collapsed hyphae, after exposure to lemongrass EO. Popović *et al*. (2018) reported the antibacterial activity of lemongrass EO against *E. amylovora* (fire blight), *Xanthomonas campestris* pv. *campestris* (black rot) and *Pseudomonas syringae* pv*. syringae* (bacterial canker and leaf spot) *in vitro*.

2.8.6 Integrated control

This method integrates different control methods such as timeous use of chemicals, biological control, plant quarantine, seed certification, and resistant varieties in a manner where the benefits of each strategy are synergistic (DALRRD, 2015). Integrating different approaches could be the key to establishing safe and sustainable solutions for efficient postharvest disease management (Wisniewski *et al*., 2016). No studies have been reported on integrating biocontrol agents and essential oils to control bacterial soft rot of potatoes. The integration of EOs and biocontrol agents has been reported to have successful results in some fungal and bacterial plant pathogens (Arrebola *et al*. 2010; Abdel-Kader *et al*., 2013; Abo-Elyousr *et al*., 2014; Zamani-Zadeh *et al*., 2014).

Arrebola *et al*. (2010) investigated the effect of combined treatment of *bacillus* PPCB004 and lemongrass EO and *bacillus* and thyme. Disease incidence was reduced by 50% for *B. cinerea*, 42% for *P. expansum* and 82% for *R. stolonifer*, which indicated a synergistic effect between PPCB004 and LO in inhibiting the disease incidence.

Abo-Elyousr *et al*. (2014) investigated the effect of *G. mosseae*, thyme(*Thymus vulgaris*) EO and peppermint (*Mentha piperita*) EO on their disease efficacy against tomato bacterial wilt disease under greenhouse conditions. *G. mosseae*(MZ) treatment resulted in a 22.2% reduction of tomato bacterial wilt, while combining thyme oil and peppermint oil reduced the disease by 72.2%. Furthermore, combining thyme oil + peppermint oil + MZ reduced disease incidence by 27.8%.

Zamani-Zadeh *et al*. (2014) found that combining *L. plantarum* A7 with thyme and cumin (*Cuminum cyminum*) essential oils effectively controlled *B. cinerea* in strawberries. The results demonstrated that combining *L. plantarum* + cumin oil and *L. plantarum* + thyme oil completely reduced pathogen mycelia growth *in vitro*. Combining *L. plantarum* + cumin oil (50 µL/L) and *L. plantarum* + thyme oil (100 µL/L) on wounded strawberries significantly enhanced Botrytis infection control compared to using *L. plantarum* A7 or essential oils alone.

2.9 Conclusion

Bacterial soft-rot pathogens are diverse and ubiquitous in the environment. They significantly impact food security and sustainability since they cause loss in producing vegetables and ornamental plants. Alternative management strategies that are both cost-effective and ecologically sustainable are needed to reduce the problem of resistance to chemicals by bacterial soft rot pathogens. The method of combining biocontrol agents with essential oil agents against soft rot bacteria can be implemented to prevent bacterial soft rot on potatoes.

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Chapter 3

In vitro and *in vivo* effects of bacterial biocontrol agents against *Pectobacterium carotovorum* subsp. *carotovorum* of potato

Abstract

Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is one of the most common bacterial diseases of potatoes during preharvest and postharvest. The aim of this study was to investigate the antibacterial activity of biocontrol agents in the management of *Pcc in vitro* and *in vivo*. A total of 155 bacterial isolates were isolated from different plant species and screened *in vitro* against the pathogen using the disc diffusion method and incubated at 25℃ for 48 hrs. The ten best Isolates that inhibited *Pcc* with the highest inhibition zones from (15 mm to 22 mm) *in vitro* were further screened for their antibacterial activity on 'Sifra' potatoes. The tubers were sterilized, wounded and inoculated with 1×10^4cells/mL of the pathogen suspension and stored at 25℃ for 24 hrs. After 24 hrs, the tubers were inoculated with 1×10^8 cells/mL of the bacterial isolates and stored at 25℃, 95% relative humidity for 21 days. The *in vivo* results showed that MSAG, YMCB and BMO3 isolates reduced soft rot incidence to (13.33%, 15%, and 16.67%, respectively) compared to the control with (96.67%) soft rot incidence. BLAST prediction identified the best 3 isolates as follows: MSAG as *Bacillus amyloliquefaciens* NR112685.1, YMCB and BMO3 as *B. amyloliquefaciens* NR117946.1. *B. amyloliquefaciens* treatment resulted in cell wall disruption, plasma membrane and cytoplasm leakage of *Pcc in vitro.* Protection of the potato cells and amyloplasts was observed in treated potato wounds. The antibacterial activity of *B. amyloliquefaciens* could be due to different mechanisms of action of *Bacillus,* such as the production of different antimicrobial compounds.

Keywords: *Pectobacterium carotovorum* subsp. *carotovorum*, potatoes, postharvest, *B. amyloliquefaciens*

3.1 Introduction

Potatoes (*Solanum tuberosum* L.) are important energy sources due to their high protein content (Zaheer and Akhtar, 2016). They can be utilized as a food security crop, animal feed, cash crop, and for various industrial purposes due to their nutritional content (FAOSTAT, 2023). Pests, diseases, and changes in the climate are the most significant factors that affect the potato industry (Chakrabarti *et al*., 2022). One of the most common diseases that damage potatoes in the field and during storage is bacterial soft rot, caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). Bacterial soft rot causes significant losses in various vegetables in fields, storage, and transit, ranging from 15% to 30% in harvested vegetables and up to 60% in stored potatoes (Czajkowski *et al*., 2011).

Traditional phytosanitary and cultural methods serve as the foundation for reducing bacterial soft rot in vegetables (Charzowski *et al*., 2011). Chemical bactericides are not effective in controlling bacterial soft rot due to their short lifespan, adverse side effects, high cost, and the evolution of resistance in bacterial populations (Abd-El-Khair and Karima, 2007). Therefore, microbial antagonists and their bioactive compounds have received much attention as safer and more environmentally friendly alternatives to chemical bactericides for plant disease management. *Bacillus* spp., *Pseudomonas* spp., *Gliocladium* spp. and *Trichoderma* spp. have been tested with successful results as biological control agents of plant diseases in various crops (Abd-El-Khair *et al*., 2002; Ghods-Alavi *et al*., 2012; Khayi *et al*., 2015; Idowu *et al*., 2016; Nelkner *et al*., 2019).

The use of *Bacillus* spp. for biocontrol of various postharvest diseases in a wide range of fruits and vegetables has been reported (Calvo *et al*., 2017; Zhang *et al*., 2019; Chen *et al*., 2020; Bu *et al*., 2021; Taha *et al*., 2023). The efficacy of *Bacillus* depends on different mechanisms, such as induction of systemic resistance, antibiosis, siderophores production, and hydrolytic enzyme secretion (Li *et al*., 2014; Li *et al*., 2015; Mora *et al*., 2015; Rani *et al*., 2019). *Bacillus* is also an effective biocontrol agent because of its genetic variety, capacity to sporulate and persist in challenging conditions, and fast colonization enabled by flagellar and non-flagellar motility (Wu *et al*., 2015). Currently, no biological control agent is registered to control

bacterial soft rot in South Africa. Thus the aim of this study was to investigate the effect of bacterial biocontrol agents against *Pcc in vitro* and *in vivo.*

3.2 Materials and methods

3.2.1 Pathogen inoculum preparation

A pure culture of *Pcc* (Accession number: CP003776) used in this study was purchased from the Agricultural Research Council-Plant Health Protection (PHP), Pretoria, South Africa. The bacterial culture was stored in the refrigerator in 70% glycerol (v/v) at -80℃. Fresh cultures of the pathogen were prepared by inoculating potato dextrose agar (PDA) Petri plates with 450 µL of the pathogen suspension and incubated for 48 hrs at 25℃. *Pcc* colonies were then streaked into fresh PDA plates and incubated at 25℃ for 48 hrs. PDA plates with *Pcc* colonies were washed with sterile distilled water, and the pathogen suspension was adjusted to a concentration of 1×10^4 cells/mL using a hemocytometer.

3.2.2 Pathogenicity test of *Pcc*

Potatoes cv. 'Sifra' were harvested at Swayimane (Pietermaritzburg, South Africa) and surface disinfected with 70% ethanol (v/v) for 1 min, rinsed with sterile water, and air-dried at room temperature for 1 hr. Four artificial wounds (1.5 cm in depth and 2.5 mm in diameter) were made along the equatorial zone of each potato using a sterile scalpel. Each wound was inoculated with 20 µL of the pathogen suspension (1×10^4 cells/mL and 1×10^3 cells/mL), and the control treatment was inoculated with sterile distilled water. There were three replicates of five potatoes per replicate in a completely randomized design (CRD). The inoculated potatoes were stored in carton boxes at 25°C with 95% relative humidity (RH) for 21 days. The soft rot incidence was recorded after 7, 14, and 21 days post-inoculation. The soft rot incidence was recorded after 7, 14, and 21 days post-inoculation and calculated using the formula:

$$\%DI = \frac{\text{The number of infected potato wounds}}{\text{Total number of wounds in a potato}} \times 100$$

The concentration of *Pcc* suspension, which exhibited the highest percentage of soft rot incidence, was further used throughout the *in vivo* trials.

3.2.3 Isolation of bacterial isolates and inoculum preparation

Bacterial isolates were isolated from the leaves of the plants collected at Mangweni (Mpumalanga, South Africa) and Scottsville (Pietermaritzburg, South Africa) (Table 3.1). Plant samples were sterilized with 70% ethanol (v/v) for 1 min, washed with distilled water, and allowed to dry in a paper towel. They were then cut, weighed to a mass of 10 g, and placed into sterile bottles with 90 mL of distilled water. The sterile bottles were placed into a hot water bath at 80℃ for 1 hr. Serial dilutions of 1×10^{-1}, 1×10^{-2}, 1×10^{-3} and 1×10^{-4} of the inoculum were prepared. A volume of 1 mL of each dilution was pipetted and spread on Petri dishes containing PDA and incubated for 4 days at 25℃. Single colonies of bacterial isolates were picked and streaked on fresh PDA plates and incubated at 25℃ for 4 days. Pure isolates were stored on PDA in Eppendorf tubes with 30% glycerol (v/v) at -80℃ for use throughout the. The inoculum of bacterial isolates was prepared by streaking pure cultures streaked on PDA plates and incubated for 48 hrs at 25˚C. The PDA plates with bacterial isolates were washed with distilled water and a hemacytometer was used to adjust the pathogen suspension to a concentration of 1×10^{8} cells/mL.

Table 3.1: Bacterial isolation from different plant species.

Plant species	Source	Number of bacterial isolates
African potato (*Hypoxis hemerocallidea)*	Scottsville	6
African wormwood (*Artemisia afra*)	Scottsville	8
Aloe barbadensis miller (*Aloe vera*)	Scottsville	7
Avocado (*Persea americana*)	Scottsville	8
Bladdernut (*Staphylea trifolia*)	Scottsville	9
Cashew nut (*Anacardium occidentale* L.)	Mangweni	8
Forest elder (*Nuxia floribunda*)	Scottsville	8
Ginger bush (*Tetradenia riparia*)	Scottsville	9
Guava (*Psidium guajava*)	Scottsville	8
Gum tree (*Eucalyptus mannifera*)	Scottsville	7
Lavender tree (*Lavandula angustifolia*)	Scottsville	6
Lemon bush (*Lippia javanica*)	Scottsville	7
Lemon (*Citrus* × *limon*)	Scottsville	6

Macadamia nut (*Macadamia integrifolia*)	Scottsville	8
Mango (*Mangifera indica*)	Mangweni	9
Marula (*Sclerocarya birrea*)	Mangweni	8
Moringa (*Moringa oleifera*)	Mangweni	7
Natal Mahogany (*Trichilia dregeana*)	Scottsville	6
Papaya (*Carica papaya*)	Scottsville	7
White pear (*Apodytes dimidiate*)	Scottsville	8
Total		150

3.2.4 *In vitro* screening of bacterial isolates against *Pcc*

PDA was prepared, and a volume of 20 mL was poured into sterile Petri plates and allowed to solidify. PDA plates were inoculated with 0.4 mL of *Pcc* suspension (1×10^4 cells/mL) and spread using a glass rod. Sterile antibiotic discs (9 mm diameter) impregnated with the suspension of 1×10^8 cells/mL of bacterial isolates were placed at the center of PDA plates inoculated with the pathogen using sterile forceps. Antibiotic discs inoculated with sterile distilled water were used as a control. Inoculated PDA plates were incubated at 25℃ for 48 hrs. The antibacterial activity of bacterial isolates was evaluated after 48 hrs by measuring the diameter of the inhibition zone of *Pcc* in mm using a ruler. There were three replicates per treatment in a completely randomized design (CRD), and the experiment was repeated three times.

3.2.5 *In vivo* screening of bacterial isolates against bacterial soft rot on 'Sifra' potatoes

Potatoes cv. were 'Sifra' collected at Swayimane (Pietermaritzburg, South Africa). Healthy and undamaged potatoes were surface disinfected with 70% ethanol (v/v) for 1 minute, rinsed with sterile water, and air-dried at room temperature for 1 hr. Four artificial wounds (1.5 mm in depth and 2.5 mm in diameter) were made along the equatorial zone of each potato using a sterile scalpel. Each wound was inoculated with 20 µL of bacterial isolates suspension (1×10^8 cells/mL) and incubated at 25℃ for 24 hr with a relative humidity (RH) of 95%. After 24 hrs, the wounds were inoculated with 20 µL of the pathogen suspension at a concentration of 1×0^4 cells/mL. The inoculated potatoes were stored in carton boxes at 25°C with a 95% RH for 21 days. Each treatment was replicated three times with five tubers per replicate in a completely

randomized design (CRD. The soft rot incidence was recorded after 7, 14 and 21 days post-inoculation and calculated using the formula:

$$\%DI = \frac{\text{The number of infected potato wounds}}{\text{Total number of wounds in a potato}} \times 100$$

3.2.6 Molecular identification of bacterial isolates

The most effective bacterial isolates were identified using the methods described by Altschul *et al*. (1997). Genomic DNA was isolated from bacterial culture using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target areas were amplified using the OneTaq@ Quick load @ 2X Master mix (NEB, Catalogue No M0486) with the primers presented in Table 3.2. The PCR products were analyzed on a gel using the ZymocleanTM Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The isolated fragments were sequenced forward and reverse (Nimagen, BrillianDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM, Catalogue No. D4050). For each reaction and sample, the purified fragments were analyzed on the ABI 3500x1 Genetic Analyser (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was utilized for analysis.

Table 3.2: 16S Primers sequences

Name of the Primer	Target	Sequence (5' to 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

3.2.7 Transmission electron microscopy analysis of the effect of bacterial biocontrol agents against *Pcc*

The best three bacterial isolates that successfully inhibited *Pcc in vitro* and on 'Sifra' potato tubers were viewed under the transmission electron microscope (TEM). The samples were obtained from treated potato wounds and PDA plates around the zone of *Pcc* inhibition using a sterile scalpel. The samples were fixed with 2.5% glutaraldehyde for one hour and then washed three times with 0.05M sodium cacodylate buffer for 30 min. The samples were post-fixed with 2% osmium tetroxide

for 2 hrs and then washed (two times) with sodium cacodylate buffer for 30 min. The potato and cell samples were dehydrated, using graded ethanol concentrations of 10 to 70% for 10 min each and left overnight. The potato and cell samples were dehydrated in 90% ethanol for 15 min, twice in 100% ethanol for 15 min, and two additional immersions in absolute ethanol for 15 min each. The potato and cell samples were immersed (two times) in 100% propylene oxide for 15 min and 50:50 Spurr's resin/propylene oxide for 1 hr and two times in 100% Spurr's resin for 1 hr. The potato and cell samples were placed in an oven at 60℃ for at least 24 hrs and then sectioned with a Reichert Jung Ultracut E Ultra-microtome. The sections were collected in a copper grid 200 mesh and finally stained with Reynolds lead citrate and uranium acetate 1%. The samples were analyzed in a JEOL 1400 TEM.

3.2.8 Statistical analysis

The data obtained was subjected to analysis of variance (ANOVA) using Genstat® 23rd Edition. Fisher's unprotected least significant difference test was performed to test the significance of the mean differences obtained among the treatments at the 5% level of significance.

3.3 Results

3.3.1 Pathogenicity of *Pcc on* 'Sifra' potatoes

The pathogenicity test showed that *Pcc* was pathogenic to 'Sifra' potatoes after 21 days post-inoculation (Figure 3.1). There was a significant difference in bacterial soft rot incidence of potatoes inoculated with concentrations of 1×10^4 and 1×10^3 cells/mL of *Pcc* and the untreated control (Figure 3.2).

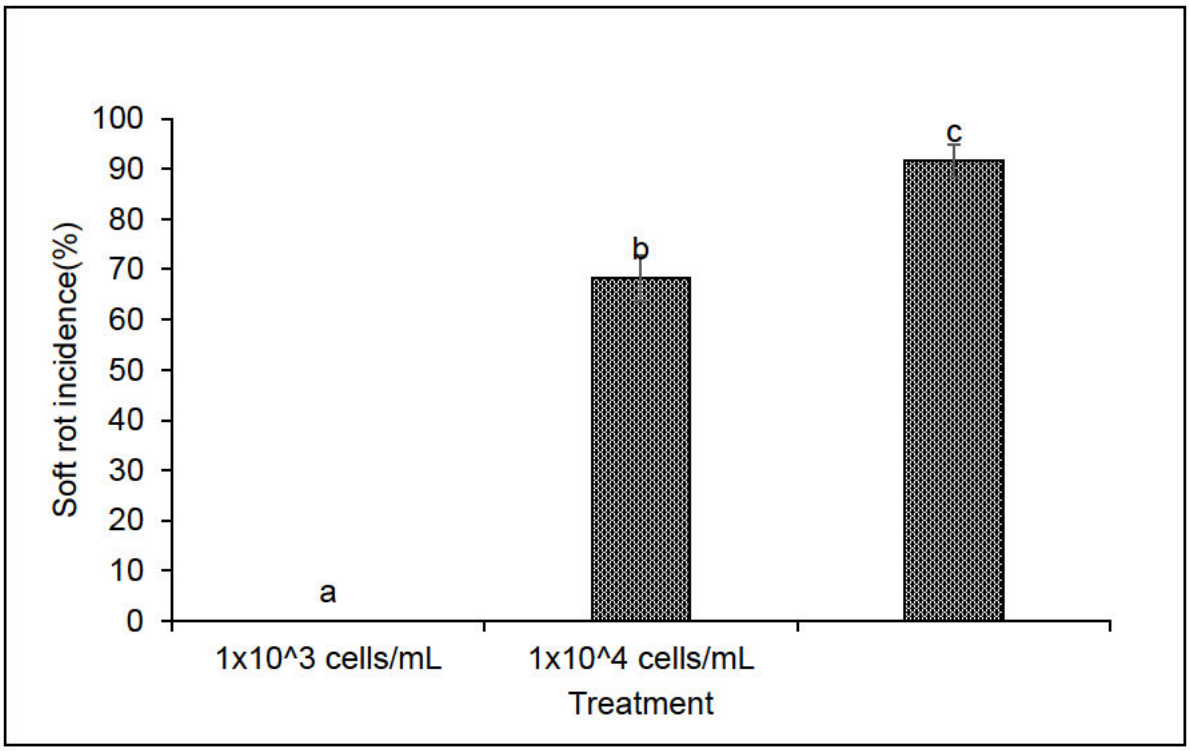

Figure 3.1: Bacterial soft rot incidence on 'Sifra' potatoes after 21 days post-inoculation at 25℃ and 95% relative humidity. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (P = 0 .05).

Figure 3.2: Pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* on 'Sifra' potatoes after 21 days at 25℃ temperature. Control (A), potatoes inoculated with treated with 1×10^3 cells/mL and 1×10^4 cells/mL (C).

3.3.2 *In vitro* screening of bacterial isolates against *Pcc*

A total of 150 bacterial isolates were screened against *Pcc in vitro* (Appendix 1). For the primary screening trial, 82 bacterial isolates inhibited the pathogen with variable results; 68 isolates failed to inhibit the pathogen and were discarded. Bacterial isolates with the largest inhibition zones from 13.00 mm to 22.00 mm were selected for secondary screening. For the secondary screening trial, bacterial isolates BMO3, YMCB and MSAG had the largest inhibition zones (22.83 mm, 20.67 mm and 18.67 mm, respectively) compared to other bacterial isolates and the control with no inhibition zone (Table 3.3; Figure 3.3 B, C and D).

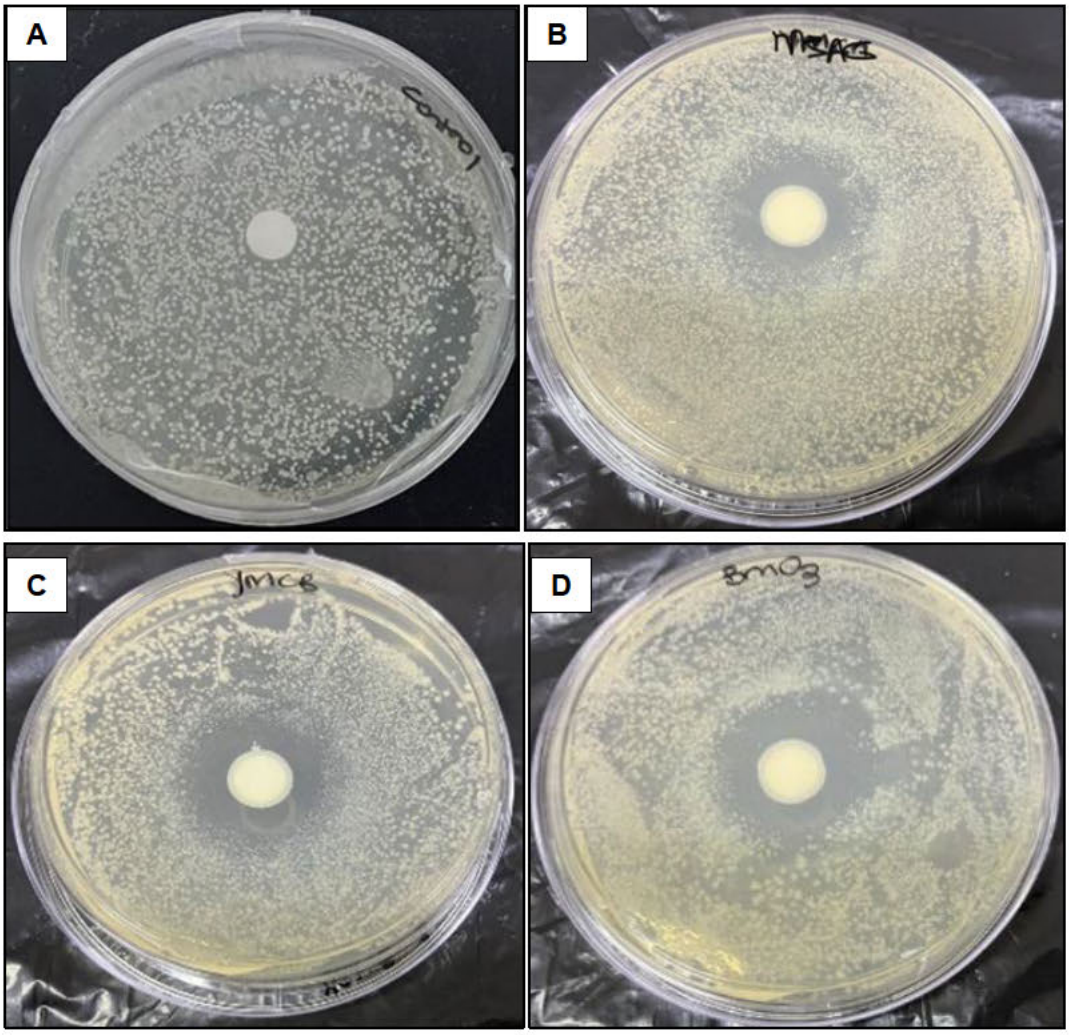

Figure 3.3: *In vitro* screening of the best bacterial isolates against *Pectobacterium carotovorum* subsp. *carotovorum* showing clear inhibition zones on potato dextrose agar after 48 hrs at 25℃. Control(A), MSAG (B), YMCB (C) and BMO3 (D).

Table 3.3: Secondary *in vitro* screening of bacterial isolates against *Pcc* incubated at 25℃ for 48 hrs. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Isolation type	Isolate	Inhibition zone diameter
-	Control	0.00 a
Anacardium occidentale L.	BCA3	13.50 b
Eucalyptus mannifera	GTA2	14.00 bc
Anacardium occidentale L.	YMCP	14.00 bc
Hypoxis hemerocallidea	PTCM	14.33 bc
Eucalyptus mannifera	GTA1	14.50 bc
Aloe vera	AL24	14.67 bc
Artemisia afra	MCN1	14.67 bc
Artemisia afra	MCN2	14.67 bc
Artemisia afra	MNC3	14.83 bcd
Sclerocarya birrea	BMA1	15.33 bcde
Eucalyptus mannifera	GTA4	15.33 bcde
Artemisia afra	BMCN	15.67 cde
Moringa oleifera	BMO1	15.67 cde
Lippia javanica	BGA1	16.67 def
Sclerocarya birrea	CMA1	16.67 def
Lippia javanica	MSGB	16.67 def
Moringa oleifera	BMRG	17.17 efg
Moringa oleifera	BMW1	18.00 fg
Lippia javanica	MSAG	18.67 g
Moringa oleifera	YMCB	20.67 h
Moringa oleifera	BMO3	22.83 i
P-value		0.88
SED		0.978
LSD		1.972
%CV		7.8

3.3.3 Molecular identification of biocontrol agents

All three bacterial isolates (MSAG, BMO3 and YMCB) were identified as *Bacillus amyloliquefaciens* (Table 3.4).

Table 3.4: The primers used to identify the best-performing bacterial agents.

Isolate name	Species name	Primer	Accession no.
MSAG	*Bacillus amyloliquefaciens*	16S rDNA	NR117946.1
BMO3	*Bacillus amyloliquefaciens*	16S rDNA	NR112685.1
YMCB	*Bacillus amyloliquefaciens*	16S rDNA	NR117946.1

3.3.4 *In vivo* screening of bacterial biocontrol agents against bacterial soft rot on 'Sifra' potatoes

Bacterial soft rot incidence on potatoes treated with bacterial isolates MSAG, YMCB and BMO3 was 13.33%, 15% and 16.67%, respectively (Figure 3.4 and Figure 3.5 B, C and D), compared to the control (96.67%) treatment (Figure 3.4 and Figure 3.5 A). There was no significant difference between bacterial soft rot incidence of potatoes treated with MSAG, BGA1, BMO3 and BGA1 means at (P< 0.05) (Figure 3.4).

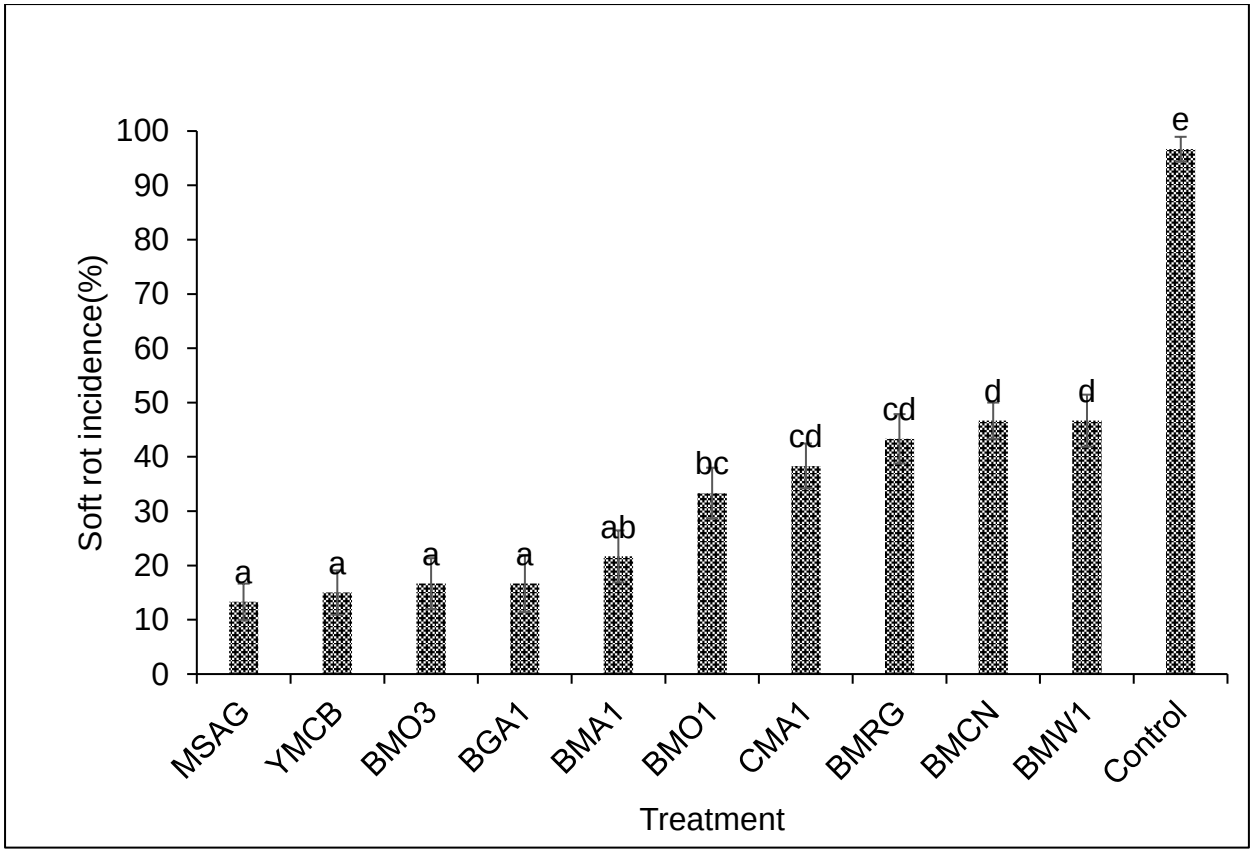

Figure 3.4: Bacterial soft rot incidence of 'Sifra' potatoes inoculated with bacterial isolates after 21 days at 25℃ and 95% relative humidity. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Figure 3.5: Bacterial soft rot incidence on 'Sifra' potatoes treated with bacterial isolates after 21 days post inoculation at 25℃ and 95% relative humidity. Control (A), BMO3 (B), YMCB (C) and MSAG (D).

3.3.3 Transmission electron microscopy analysis of the effect of bacterial biocontrol agents against *Pcc*

The *Pcc* cells were treated with the best performing *B. amyloliquefaciens* isolates (YMCB, BMO3 and MSAG), collected around the inhibition zone, and examined using

TEM. *Pcc* cells treated with YMCB, BMO3 and MSAG isolates showed deformed *Pcc* cells (DPC), leakage of the cell wall (LCW), plasma membrane (LPM) and leakage of the cytoplasm and its constituents (LCP) (Figure 3.6A, B and C; and Figure 3.6B). Untreated *Pcc* cells were intact with their constituents (CCA) and viable *Pcc* cells (VP) (Figure 3. 6A).

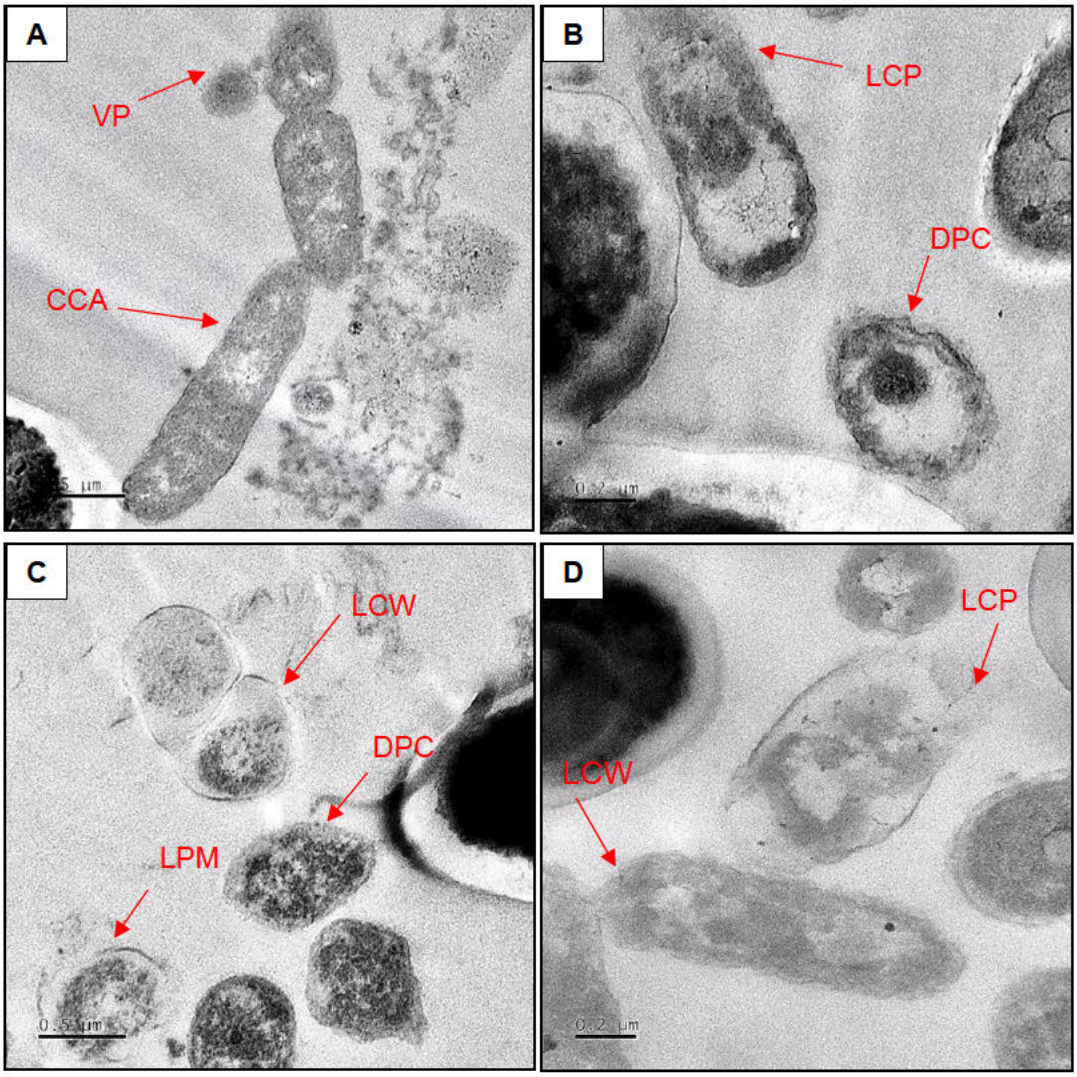

Figure 3.6: TEM micrographs of *Pectobacterium* cells treated with bacterial isolates after 48 hours at 25℃. Control (A), MSAG (B), BMO3 (C), and YMCB (D).

TEM micrographs of potato wounds treated with *B. amyloliquefaciens* (MSAG) and *Pcc* showed damaged *Pcc* cells (DP) (figure 3.7 C, undamaged amyloplast envelope (AME) and amyloplasts (AM) with starch grains inside (Figure 3.7 D). The untreated potato wounds (only treated with *Pcc*) showed viable *Pcc* cells (VP) (Figure 3.7 A), damaged amyloplast (DAM) and damaged amyloplast envelope (DAE) (Figure 3.7B).

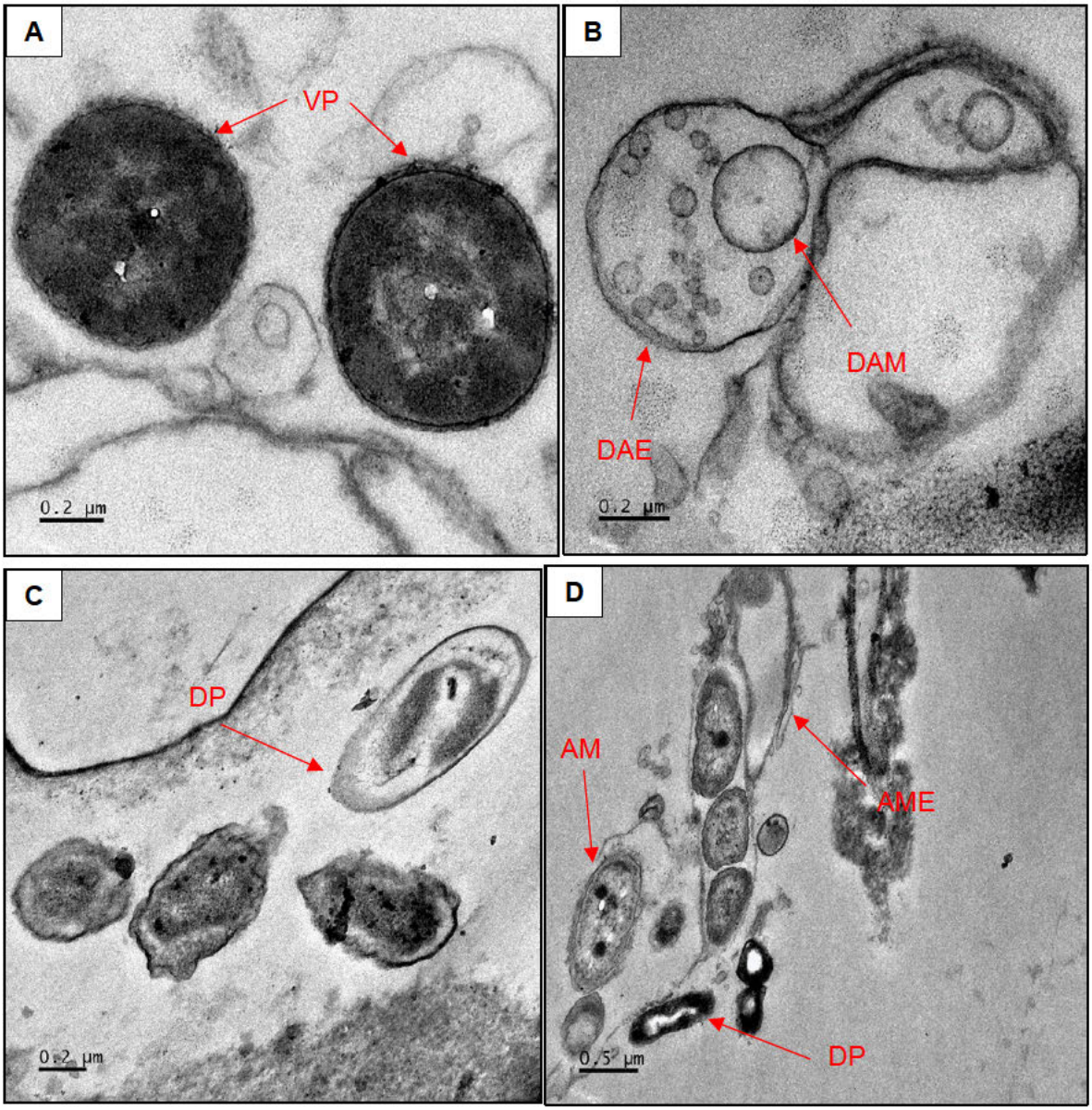

Figure 3.7: TEM micrographs of untreated potato wounds (A and B) and potato wounds treated with *B. amyloliquefaciens* (MSAG) (C and D), 14 days post-inoculation incubated at 25 ˚C at 95% relative humidity.

3.4 Discussion

The research aimed to isolate bacterial biocontrol agents and to evaluate their efficacy in controlling potato soft rot caused by *Pcc*. The experimental data presented in this study demonstrates the occurrence of antagonistic *Bacillus* spp. that can control bacterial soft rot. Using beneficial microorganisms to combat plant diseases is sustainable and environmentally friendly and represents a promising approach to

managing postharvest plant diseases (Dong *et al*., 2023). Biocontrol agents may suppress bacterial and fungal infections by various techniques, including parasitism, antibiosis, competition for resources and space, and enhancing the host plant defenses (Etesami *et al*., 2023).

Bacillus spp. is known for producing a variety of antimicrobial compounds, including peptide and lipopeptide antibiotics (Tran *et al*., 2022). *B. amyloliquefaciens* isolates (MSAG, BMO3 and YMCB) showed the best antibacterial activity against *Pcc in vitro* and reduced bacterial soft rot incidence of 'Sifra' potatoes to 13%, 15%, and 16.67% at $P<0.05$, respectively. The antibacterial activity of *B. amyloliquefaciens* JK6 and *B. velezensis* FJAT-46737 against tomato bacterial wilt was investigated, and it was reported that both bioagents reduced the disease incidence by 52.9% and 66.2% (Chen *et al*., 2000). In another study, Calvo *et al.* (2017) showed a preventive application of *B. amyloliquefaciens* UZ-14 on *Penicillium expansum* (Link) and *Penicillium digitatum* (Pers.) Sacc. and *Penicillium italicum* (Wehmer) reduced disease incidence in apples from 100% to 20%.

The cell wall disruption, plasma membrane and cytoplasm leakage of *Pcc* cells observed on TEM micrographs (Figures 3.6 and 3.7), and protection of the amyloplasts inside the potato cell could be due to the different mechanisms of action by *B. amyloliquefaciens*. The lipopeptides of *Bacillus* spp. are involved in direct antibiosis against plant pathogens (Dimkić *et al*., 2022). The results of this study are consistent with those of Xu *et al*. (2019), who demonstrated that the myriocin generated by *B. amyloliquefaciens* caused morphological alterations in *Fusarium oxysporum* f. sp. *niveum* (Fon) conidia, including vacuole enlargement, conidial shrinkage, variations in cytoplasmic density, and a reduction in liposome membrane fluidity and pore-formation. In addition, Arrebola *et al*. (2010) showed that fengycin, iturin and surfactine are responsible for the antifungal activity of *B. amyloliquefaciens* against citrus postharvest pathogenic fungi.

3.5 Conclusion

The findings of this study showed that *B. amyloliquefaciens* isolated from *Moringa oleifera* and *Lippia javanica* leaves have antibacterial properties and may be used as a natural bactericide that can reduce potato soft rot postharvest

3.6 References

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Chapter 4

In vitro and *in vivo* antibacterial activity of *Cymbopogon citratus L.* essential oil against *Pectobacterium carotovorum* subsp. *carotovorum* causing bacterial soft rot of 'Sifra' potato tubers

Abstract

Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is considered one of the major diseases of potatoes worldwide that cause significant losses of potatoes. Alternative strategies, such as using essential oils to control this disease, are needed. The aim of this study was to investigate the effect of lemongrass (LE) essential oil on *Pcc in vitro* and *in vivo*. LE essential oil was tested *in vitro* using the disc diffusion method and on 'Sifra' potatoes using the dip method. The cellular changes and viability of *Pcc* treated with different concentrations of LE were evaluated under transmission electron microscopy (TEM). The inhibition zones of *Pcc* exposed to LE1.0%, LE2.0%, and LE3.0% were 28.00 mm, 30.00 mm and 34.00 mm, respectively, compared to the control (0.0mm) at $P< 0.05$. Potato tubers treated with LE0.125% and LE0.175% had 94.83% and 89.66% bacterial soft rot incidence reduction. The TEM micrographs of the *Pcc* bacterium treated with LE3.0% showed deformed *Pcc* cells and an accumulation of lemongrass constituents in the plasma membrane. Lemongrass essential oil deposition was also observed inside the potato cell and cell wall; it was also observed on potato wounds treated with LE0.125% and LE0.175%. Due to its high antibacterial activity at low concentrations, lemongrass essential oil has the potential to be used as a bactericide that can control potato soft rot during storage.

Keywords: Lemongrass, *Pectobacterium carotovorum* subsp. *carotovorum*, soft rot incidence, *Solanum tuberosum*

4.1 Introduction

Potatoes (*Solanum tuberosum* L.) are the world's most popular noncereal staple food, renowned mostly for their starchy carbohydrate content. They are also high in vitamins and other phytonutrients that are very beneficial for human health (Zaheer and Akhtar, 2016). Potato is a temperate climate crop grown in conditions where the temperature during the growing seasons is moderately cool (Zaheer and Akhtar, 2016). However,

the production and postharvest quality of potatoes are mainly affected by pests and diseases (Chakrabarti *et al*., 2022). Bacterial soft rot and black leg are significant potato diseases that result in major economic losses of potatoes (Czajkowski *et al*., 2014). Synthetic antimicrobials are commonly used to manage the growth of postharvest pathogens. Using synthetic antimicrobial chemicals as food preservatives has concerned consumers as they pose many toxicological problems and are dangerous for human consumption (Goswami *et al*., 2018). The public's awareness of these concerns caused an interest in safer alternatives to synthetic antimicrobials. One of the alternatives is the use of natural plant protectants with antimicrobial activity, as they are less harmful to humans, have fewer environmental consequences, and are widely accepted by consumers (Youssef *et al*., 2022).

Essential oils are complex, volatile compounds produced in different plant parts, known to have various functions in plants, including conferring pest and disease resistance (Andrade *et al*., 2014). The complexity of essential oils is due to terpene hydrocarbons and their oxygenated derivatives, such as alcohols, aldehydes, ketones, acids, and esters (Elgayyar *et al*., 2001). The ability of essential oil to combat postharvest pathogens has been extensively studied by many researchers (Szczerbanik *et al*., 2007; Du Plooy *et al*., 2009; Abd-Alla and Haggag, 2013; Elshafie *et al*., 2015).

Lemongrass (LE) essential oil has often been used as an antifungal and antibacterial agent in plant and even in human pathogens (Tzortzakis and Economakis, 2007; Naik *et al*., 2010; Mbili *et al*., 2017; Martinazzo *et al*., 2019; Mukarram *et al*., 2021). LE essential oil antibacterial mechanism involves the destruction of bacterial biofilms and hinders bacterial growth and development Moore-Neibel *et al*., 2012). Furthermore, the components of LE can weaken lipid bilayer bonds and destroy bacteria by disintegrating the membrane (Moore-Neibel *et al*., 2012). Popović *et al*. (2018) reported the efficacy of LE antibacterial activity on *Erwinia amylovora* (Burr.) Winsl. *et al*, *Xanthomonas campestris pv. campestris* (Pammel) Dowson and *Pseudomonas syringae pv. syringae* van Hall *in vitro* (Popović *et al*., 2018). Currently, there are no studies published that prove the antibacterial efficacy of LE essential oil in the management of potato soft rot. Therefore, the aim of the study was to investigate the

effect of LE essential oil on *P. carotovorum* subsp. *carotovorum* (*Pcc*) *in vitro* and *in vivo.*

4.2 Materials and methods

4.2.1 Essential oil

LE essential oil was bought from Dis-Chem pharmacy. LE was diluted in 40% ethanol (v/v) to make a concentration of 0.125%, 0.175%, 0.5%, 1.0%, 2.0% and 3.0%. LE concentrations were calculated using a formula:

$$Total\ volume = LE\% \times total\ volume\ + VolumeEthanol$$

4.2.2 Pathogen inoculum preparation

A pure culture of *Pcc* (Accession number: CP003776) used in this study was purchased from the Agricultural Research Council-Plant Health Protection (PHP), Pretoria, South Africa. The bacterial culture was stored in the refrigerator in 70% glycerol (v/v) at -80℃. Fresh cultures of the pathogen were prepared by inoculating PDA Petri plates with 450 µL of the pathogen suspension and incubated for 48 hrs at 25℃, *Pcc* colonies were then streaked in fresh PDA plates and incubated at 25℃ for 48 hrs. PDA plates with *Pcc colonies* were washed with sterile distilled water, and the pathogen suspension was adjusted to a concentration of 1×10^4 cells/mL using a hemocytometer.

4.2.3 *In vitro* screening of lemongrass essential oil against *Pcc*

Potato dextrose agar (PDA) was autoclaved and allowed to cool to 40℃. A volume of 15 mL of PDA was poured into sterile Petri plates and allowed to solidify. The pathogen inoculum of 350 µL was pipetted on the surface of PDA plates and spread using a sterile glass rod. Sterile filter paper discs (9mm diameter) were dipped in different lemongrass concentrations (LE0.125%, LE0.175%, LE0.5%, LE1.0%, LE2.0%, and LE3.0%), 40% ethanol control (v/v), and water were used as a control. The discs were placed at the centre of the PDA plate using sterile forceps. Inoculated PDA plates were sealed with a parafilm and incubated at 25℃ for 48 hrs. The antibacterial activity of LE

essential oil was evaluated by measuring the diameter of the zones of inhibition of *Pcc*. The experiment was repeated three times, with three replicates per treatment.

4.2.4 Investigating the phytotoxic effect of lemongrass essential oil on 'Sifra' potato tubers

Potatoes cv. 'Sifra' used in this trial were collected at Swayimane (Pietermaritzburg, South Africa). Healthy and undamaged potatoes were surface disinfected with 70% ethanol (v/v) for 1 min, rinsed with sterile water, and air-dried at room temperature for 1 hr. The potatoes were dipped for 30 sec in different concentrations of (LE0.125%, LE0.175%, LE0.5%, LE1.0%, LE2.0%, and LE3.0%), and water as the control. Treated potatoes were stored in carton boxes at 25℃ for seven days at 95% relative humidity (RH). Each treatment was replicated three times with five potatoes per replicate in a completely randomized design (CRD). After incubation, the degree of phytotoxicity was measured according to (Elshafie *et al*., 2015).

4.2.5 Efficacy of postharvest dipping of lemongrass essential oil against *Pcc*

Potatoes c.v. 'Sifra' were surface sterilized with 70% ethanol (v/v) for 1 min and allowed to air dry before postharvest dip treatment. Treatments consisted of 0.125%,0.175%,0.5%,1.0%,2.0%, and 3.0% LE essential oil concentration, 40% ethanol (v/v), and distilled water was used as a control. The potatoes were uniformly wounded at (1.5 mm in depth and 2.5 mm in diameter), with a sterile needle at the equator and dipped for 30 sec in LE0.125%, LE0.175%, LE0.5%, LE1.0%, LE2.0%, and LE3.0%), 40% ethanol control (v/v), and water control. After postharvest dip treatments, the treated potatoes were left to air-dry, and each wound was inoculated with 20 µL *Pcc* suspension (1×10^4 cells/mL). Treated potatoes were stored in carton boxes at 25℃ and RH of 95% for 21 days. Each treatment was replicated three times with five tubers per replicate in a completely randomized design (CRD). The experiment was repeated three times. The soft rot incidence on potatoes was measured using the formula:

$$\%DI = \frac{\text{The number of infected potato wounds}}{\text{Total number of wounds in a potato}} \times 100$$

4.2.6 Transmission electron microscopy studies of the effect of lemongrass on *Pcc*

LE essential oil concentrations LE0.125%, LE0.175%, LE2.0%, and LE3.0% that successfully inhibited *Pcc* in dual disc diffusion essay and on potatoes were prepared for TEM analysis. A volume of 350 µL of *Pcc* was spread on PDA plates, and the antibiotic discs were dipped in LE2.0% and LE3.0% essential oil concentrations and placed at the centre of the PDA plate and incubated at 25℃ for 48 hrs. For the *in vivo* trial, potatoes were disinfected with 70% ethanol, wounded and dipped in a suspension of LE0.125% and LE0.175% concentrations, and after 24 hrs, the potatoes were inoculated with 1 x 10^4 cells/mL of *Pcc* and incubated at 25℃, 95% relative humidity for 14 days**.** The treated potato wounds were cut, and for the *in vitro* samples, the bacterial cells around the zone of inhibition were aseptically collected using a sterile scalpel. The potato and cell samples were fixed with 2.5% glutaraldehyde for 1 hr and then washed three times with 0.05M sodium cacodylate buffer for 30 min. The potato and cell samples were post-fixed with 2% osmium tetroxide for 2 hrs and then washed (two times) with sodium cacodylate buffer for 30 min. The potato and cell samples were dehydrated, using graded ethanol concentrations, from 10 to 70% for 10 min each and left overnight. The potato and cell samples were dehydrated in 90% ethanol for 15 min, twice in 100% ethanol for 15 min, and two additional immersions in absolute ethanol for 15 min each. Samples were immersed (two times) in 100% propylene oxide for 15 min and 50:50 Spurr's resin/propylene oxide for 1 hr and two times in 100% Spurr's resin for 1 hr. The potato and cell samples were placed in an oven at 60℃ for at least 24 hrs and then sectioned with a Reichert Jung Ultracut E Ultra-microtome. The sections were collected in a copper grid 200 mesh and finally stained with Reynolds lead citrate and uranium acetate 1%. The samples were analyzed in a JEOL 1400 TEM.

4.2.7 Statistical analysis

The data obtained was subjected to analysis of variance (ANOVA) using Genstat® 23rd Edition. Fisher's unprotected least significant difference test was performed to test

the significance of the mean differences obtained among the treatments at the 5% level of significance.

4.3 Results

4.3.1 *In vitro* effect of LE essential oil against *Pcc*

LE3.0% had the highest inhibition zone of *Pcc* (34.00 mm) (Table 4.1; Figure 4.1E). The lowest inhibition zone of *Pcc* was exhibited by LE0.125% (17.33 mm; Table 4.1) compared to the water control (Table 4.1; Figure 4.1 A) and 40% ethanol control (Table 4.1; Figure 4.1 B), which failed to inhibit *Pcc*.

Table 4.1: *In vitro* inhibition of *Pcc* growth by lemongrass essential oil on potato dextrose agar incubated at 25℃ for 48 hrs. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Treatment	Inhibition zone (mm)
Water control	0.00 a
40% ethanol control	0.00 a
LE0.125 %	17.33 b
LE0.175 %	21.00 c
LE0.5%	23.00 c
LE1.0%	24.33 c
LE2.0%	30.33 d
LE3.0%	34.00 e
P-value	<.001
ESD	1.63
LSD	3.46
%CV	10.7

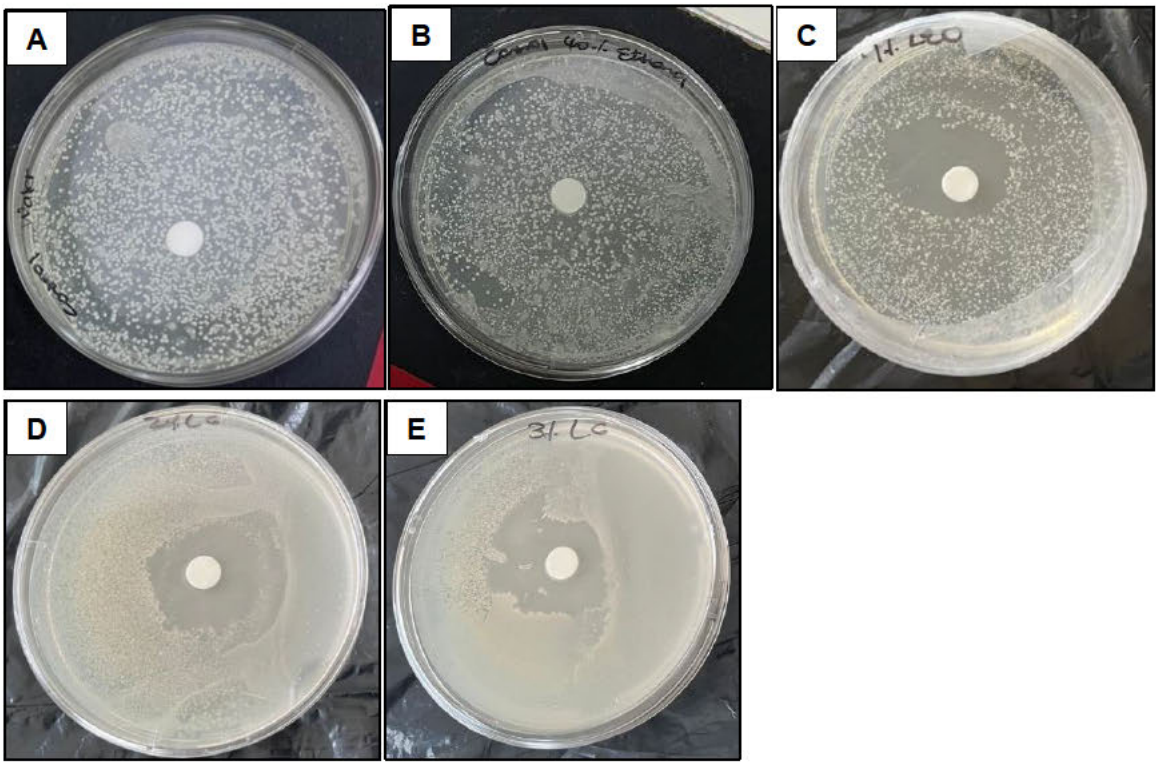


Figure 4.1: *In vitro* inhibition of *Pectobacterium carotovorum* subsp. *carotovorum* by lemongrass essential oil on potato dextrose agar incubated at 25℃ for 48 hrs. Water control (A); 40% ethanol control (B); LE1.0% (C); LE2.0% (D) and LE3.0% (E).

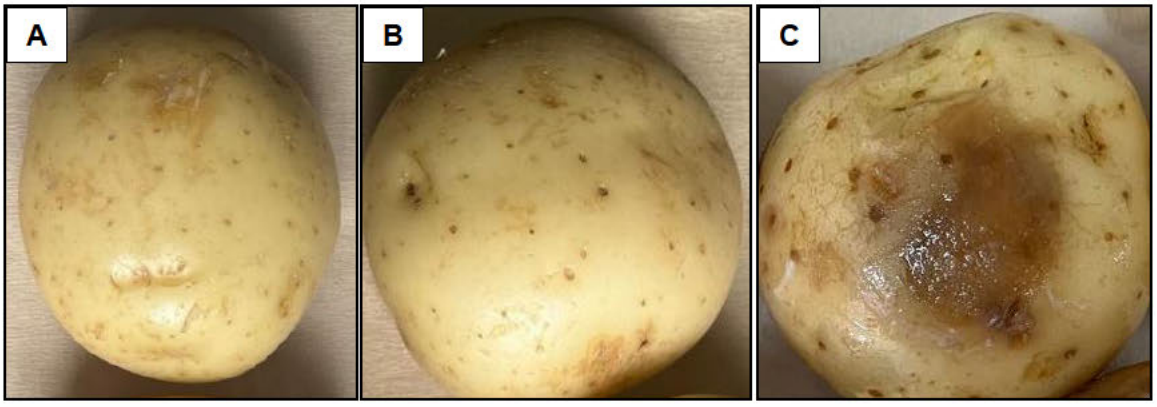
4.3.3 Phytotoxic effect of lemongrass essential oil on 'Sifra' potato tubers

It was observed that dipping potatoes in different concentrations of LE essential oil for 30 sec showed different levels of phytotoxicity (Table 4.2). Potato tubers treated with LE0.125%, LE0.175%, LE0.5%, and LE1.0% showed no phytotoxicity (Table 4.2; Figure A and Figure B) compared to LE2.0%, and LE3.0% which showed phytotoxicity as a result of black discolouration on potato skin (Table 4.2; Figure 4.2 C)

Table 4.2: Phytotoxicity of lemongrass essential oil on 'Sifra' potato tubers after seven days at 25℃ and 95% relative humidity.

Treatments	Phytotoxicity (+ or -)
Control	–
LE0.125 %	–
LE0.175 %	–
LE0.5%	–
LE1.0%	–
LE2.0%	+
LE3.0%	+

+ (phytotoxicity); - (no phytotoxicity)



4.3: Phytotoxic effect of LE essential oil on 'Sifra' potato tubers after seven days at 25℃ and 95% relative humidity. Control (A), LE 0.125% (B) and LE3.0% (C).

4.3.2 Efficacy of lemongrass essential oil against postharvest bacterial soft rot

LE essential oil postharvest treatment significantly reduced soft rot incidence ($P < 0.05$) on 'Sifra' potato tubers compared to the control (Table 4.3; Figure 4.4) at ($P<0.05$). LE essential oil concentrations (0.125% and 0.175%) provided the best control of bacterial soft rot infection on 7 and 14 days post inoculation with 100% soft rot incidence reduction (Appendix 2 and Appendix 3) and reduced bacterial soft rot incidence by 94.83% and 89.66%,21 dpi (Table 4.3; Figure 4.5 B and C), respectively, compared to the other treatment. LE2.0% and LE 3.0% concentrations were less

effective, with a disease incidence reduction of 1.73% and 25.86% respectively (Table 4.3).

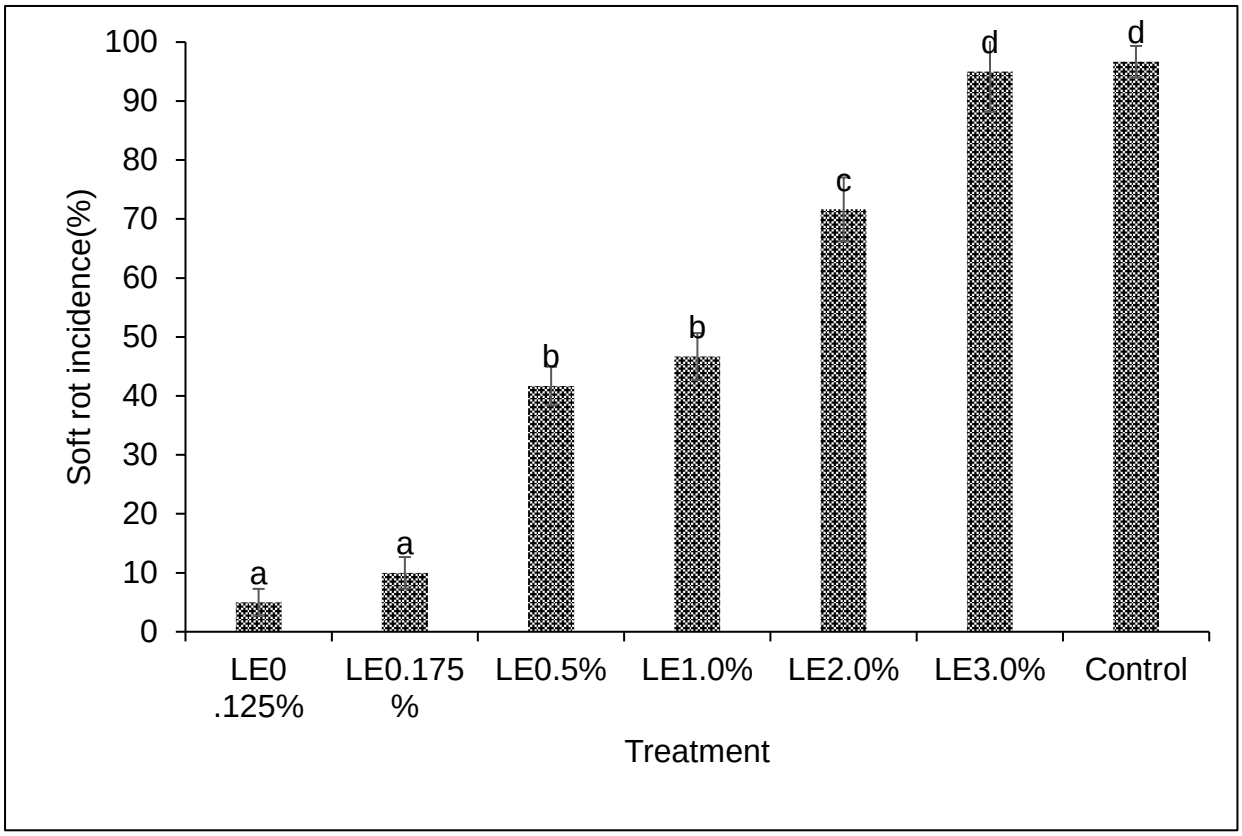


Figure 4.4: Soft rot incidence on 'Sifra' potatoes treated with lemongrass essential oil 21 days post-inoculation at 25℃ and 95% relative humidity. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Figure 4.5: Bacterial soft rot incidence on 'Sifra' potatoes dipped in lemongrass concentrations and stored a 25℃ and 95% relative humidity. Control (A); LE0.175% (B) and LE 0.125%(C).

Table 4.3: Inhibition of bacterial soft rot incidence on 'Sifra' potatoes after 21 days post inoculation at 25℃ and 95% relative humidity.

Treatments	Inhibition of bacterial soft rot incidence (%)
Control	0.00
LE3.0%	1.73
LE2.0%	25.86
LE1.0%	51.72
LE0.5%	56.89
LE0.175%	89.66
LE0.125%	94.83

4.3.3 Transmission electron microscopy studies of the effect of lemongrass on *Pcc*

The *Pcc* cells treated with different LE essential oil concentrations were collected around the zone of inhibition and examined using TEM. *Pcc* cells treated with LE3.0%, and LE2.0% showed LE constituents embedded around the plasma membrane (LDC), deformed *Pcc* cells (DPC), leakage of the cell wall (CW), leakage of the plasma

membrane (LPM) and eroded outer membrane (Figure 4.6 B, C and D). Untreated *Pcc* cells showed an intact outer membrane (OM), cell wall (CW), and viable *Pcc* cells(Figure 4.6 A).

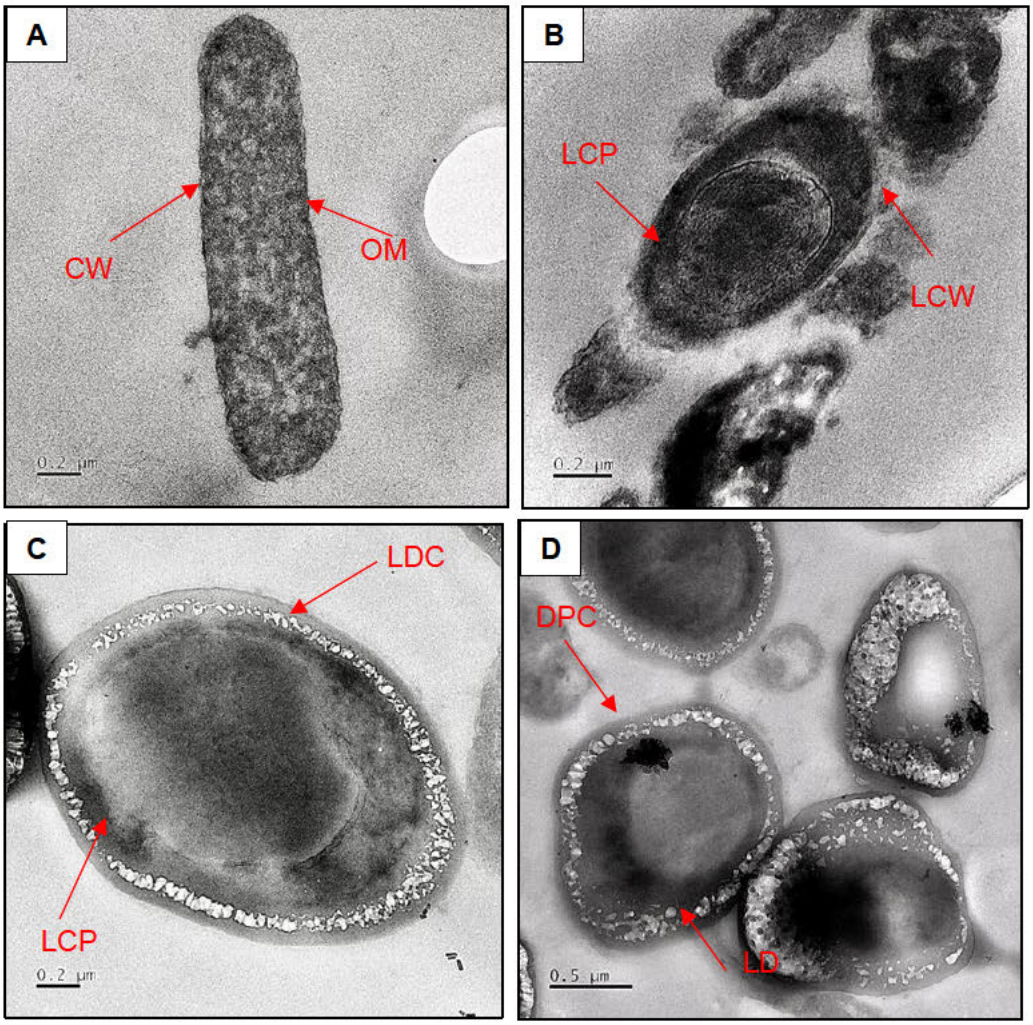


Figure 4.6: TEM micrographs of *Pcc* cells treated with LE essential oil, Control(A), LE2.0% (B and C) and LE3.0% (D).

Potato wounds treated with LE0.125% and LE0.175% were observed using TEM. The TEM micrograph for the control treatment showed a cross-section of viable *Pcc* cells inside the potato cell (Figure 4.7 A), and LE essential oil deposition was observed in the cell wall of the potato cell (LEDC) and on the amyloplast organelle (LEDA) (Figure 4.6B). Antibacterial activity of LE essential oil (LEAA) was observed in amyloplast (Figure 4.6 C).

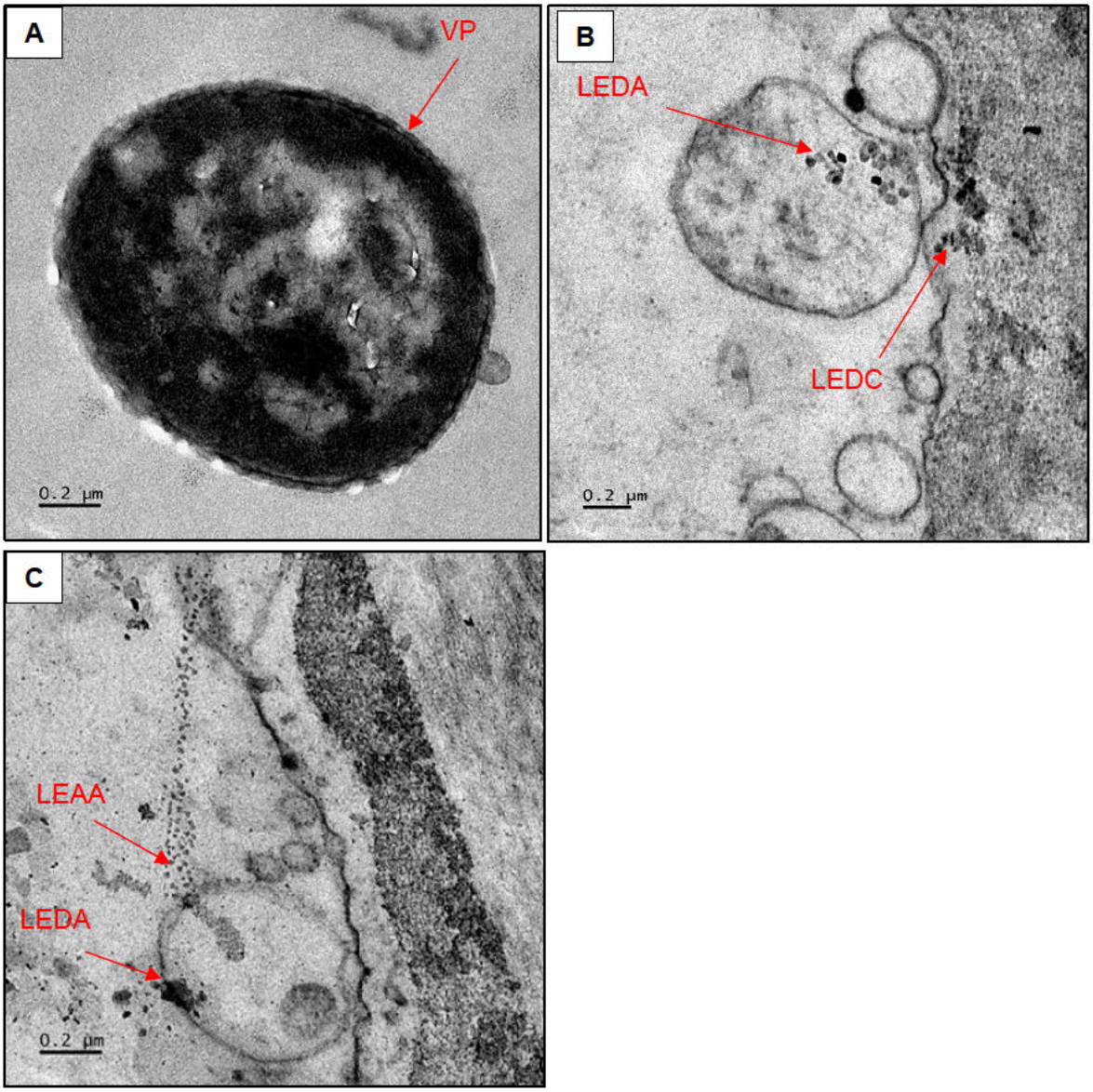


Figure 4.7: TEM micrograph of potato wounds treated with LE essential oil and *Pcc* at 25℃ and 95% relative humidity for 14 days. Control (A), LE0.175% (B) and LE0.125% (C).

4.4 Discussion

This study aimed to investigate the antibacterial effect of LE essential oil on *Pcc in vitro* and storage. The *in vitro* studies showed that all LE essential oil concentrations tested significantly inhibited *Pcc* ($P < 0.05$). The disease inhibition was directly proportional to the LE essential oil concentration. According to Mbili *et al.* (2017), increasing the concentration of essential oils increased fungal growth inhibition and spore germination inhibition of *B. cinerea*. LE essential oil showed the highest antibacterial activity against *Pcc* at LE3.0%. The *in vitro* antibacterial activity of LE essential oil has been proven by Popovic *et al.* (2018), who reported LE essential oil

antibacterial activity on *Erwinia amylovora* (fire blight), *Xanthomonas campestris pv*. *campestris* (black rot) and *Pseudomonas syringae* pv. *syringae* (bacterial canker and leaf spot) *in vitro*. The *in vivo* studies showed that LE essential oil reduced the bacterial soft rot incidence in 'Sifra' potatoes compared to the control treatment. LE essential oil significantly reduced bacterial soft rot incidence of *Pcc* on 'Sifra' potatoes. LE0.125% and LE0.175% were the most effective concentrations that showed significant disease control compared to the other concentrations and the control at ($P < 0.05$). Mbili *et al*. (2017) found that LE essential oil was fungicidal against *B. cinerea* in direct contact and volatile phase at 1.5 and 0.125% LE concentrations.

The TEM images of the treated bacterium cells with 3.0% and 2.0% LE essential oil concentration showed LE deposition in the plasma membrane of *Pcc* cells and deformation of *Pcc* cells. Disappearance of the cell wall and the plasmatic membrane were also observed. LE essential oil deposition was also observed in the potato cell wall and inside the amyloplast of potatoes treated with LE 0.175% and 0.125%. The mechanisms of action of LE include the degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm coagulation, damaging the membrane proteins, and increased permeability leading to leakage of the cell contents (Mukarram *et al*., 2021). The results align with those of Sotelo-Boyás *et al*. (2019), who demonstrated that *Pcc* cells treated with formulations of thyme (*Thymus vulgaris*) essential oil-loaded chitosan nanoparticles for a maximum period of 48 hrs showed the treated bacterium cell surface alterations such as deformation and disappearance of the cell wall and the plasmatic membrane.

The antibacterial activity of LE essential oil could be due to the major component citral, a significant constituent of lemongrass oil, which showed very high bactericidal activity in disc diffusion essays on different bacterial pathogens (Mangalagiri *et al*., 2021). The current study showed that LE essential oil has antibacterial properties and can be used as a bactericide to control soft rot pathogens.

4.5 Conclusion

The activity of essential oil and their components on bacteria remains a focal area for future research. Studying the synergistic effects among essential oils and their components could help enhance the antibacterial efficacy while reducing the concentration of essential oils used to achieve a particular antibacterial effect for food safety and health purposes. LE essential oil has the potential to be used as a bactericide that can control *Pectobacterium carotovorum* subsp. *carotovorum in vitro* and storage due to its high antibacterial activity.

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Chapter 5

The antibacterial effect of *Cympopogon citratus* L. and *B. amyloliquefaciens* on postharvest potato soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*

Abstract

The importance of plant protection has evolved from using chemical methods to implementing integrated disease management strategies, with emphasis on using biological control methods and other natural antimicrobials. Using biological control agents and essential oils is a promising alternative to the dependence on using chemicals. The aim of this study was to investigate the effect of integrated control of *B. amyloliquefaciens* with lemongrass (LE) essential oil on potato soft rot *in vitro* and *in vivo*. The antibacterial activity of the treatment combination was evaluated by measuring the diameter of the inhibition zones of *Pcc*. The antibacterial activity of LE essential oil combined with *B. amyloliquefaciens* was evaluated *in vivo* by dipping 'Sifra' potatoes in different treatment combinations. The cellular changes and viability of *Pcc* were evaluated by transmission electron microscopy (TEM). A combination of the best biocontrol and best LE concentration was tested. LE3.0% + YMCB had the highest pathogen inhibition zone with a mean of (30mm) compared to the control treatment, where no *Pcc* inhibition was observed. A treatment combination of LE0.125% + *B. amyloliquefaciens* (MSAG) and LE0.125% + B. *amyloliquefaciens (*BMO3) provided the best control of bacterial soft rot incidence by reducing bacterial soft rot incidence to 3.33% and 5%, although not significantly different at ($P<0.05$).

The TEM micrographs of the bacterium treated with LE3.0% + *B. amyloliquefaciens* YMCB showed cell surface disruption and leakage of the cytoplasm, plasma membrane and cell wall. In the potato wounds treated with LE0.125% + B. amyloliquefaciens MSAG, an interaction of different antimicrobials was observed on the cell wall and also inside the potato cell. In storage, LE essential oil at lower concentrations can be combined with *B. amyloliquefaciens* to control potato soft rot.

Keywords: Lemongrass, *B. amyloliquefaciens*, integration; potato soft rot

5.1 Introduction

Potato (*Solanum tuberosum* L.) is an economically significant staple crop widely grown, consumed, and accessible in the open market (Ortiz and Mares, 2017). Potatoes provide essential nutrients such as carbohydrates, nutritional fibre, vitamins, and minerals (Devaux *et al*., 2021). The potato industry is one of the essential food providers in South Africa, with a gross value of 43 % of significant vegetables, 15 % of horticultural products and 4 % of total agricultural production (DAFF, 2016). Potatoes were reported to be lost yearly to bacterial, fungal, and viral diseases (Chakrabarti *et al*., 2022). Potato soft rot caused by *P. carotovorum* subsp. *carotovorum* is a damaging pathogen known for breaking down of plant cell walls, resulting in soft rot symptoms in potato tubers (Czajkowski *et al*., 2011). Because of its broad host range, ability to thrive in soil and on plant debris, and resilience, this bacterium presents a challenge for disease control measures (Pérombelon, 2002).

The importance of plant protection has evolved from using chemical methods to integrated disease management strategies, and the emphasis is on using biological control methods and other natural antimicrobials (Hong *et al*., 2013). Using biological control agents and essential oils is a promising alternative for reducing chemical dependence (Ab-Rahman *et al*., 2018). Several *Bacillus* species are essential microbiome members that assist in protecting plants from diseases. *Bacillus amyloliquefaciens* can successfully colonise the epidermis of fruits and vegetables, compete with pathogens for nutrients, secrete antimicrobial compounds to inhibit pathogen growth, and induce successful plant biological control (Hong *et al*., 2013). Increasing studies have indicated that *B. amyloliquefaciens* could be used in the biological control of postharvest plant diseases (Zhao *et al*., 2013; Li *et al*., 2013; Calvo *et al*., 2017; Chen *et al*., 2018; Zhou *et al*., 2020). Zhao *et al*. (2013) reported the antibacterial activity of *B. amyloliquefaciens* subsp. *plantarum* strain BGP20 on bacterial soft rot of potatoes, green pepper and Chinese cabbage.

The bioactive, nontoxic, biodegradable, ecologically and economically viable qualities of essential oils make them a potential intriguing approach for biocontrol plant diseases (Mangalagiri *et al*., 2021). Several studies reported antimicrobial activities of LE essential oil on postharvest pathogens (Tzortzakis and Economakis, 2007; Duamkhanmanee *et al*., 2008; Mpho *et al*., 2023; Mbili *et al*., 2017; Lopes *et al*., 2023).

LE essential oil may have antimicrobial effects through several mechanisms, including producing inhibiting enzymes that damage bacterial cell walls and target metabolic processes. Arrebola *et al*. (2010) demonstrated the potential of using *B. amyloliquefaciens* PPCB004 in combination with LE essential oil in a pad delivery system. According to the current information, no previous studies have been reported on the effect of *B. amyloliquefaciens* and LE essential oil combination to control bacterial soft rot of potatoes. Therefore, this study aims to investigate the effect of *B. amyloliquefaciens* integrated with LE essential oil on bacterial soft rot of potatoes *in vitro* and *in vivo*.

5.2 Materials and Methods

5.2.1 Essential oil

LE essential oil was bought from Dis-chem pharmacy. LE essential oil was diluted in 40% ethanol(v/v) to make the required concentrations: 0.125%,0.175%,0.5%, 1.0%, 2.0% and 3.0% LE. The LE essential oil concentrations were calculated by using a formula:

$$Total\ volume = LE\% \times total\ volume\ + VolumeEthanol$$

5.2.2 Pathogen inoculum preparation

A pure culture of *Pcc* (Accession number: CP003776) used in this study was purchased from the Agricultural Research Council-Plant Health Protection (PHP), Pretoria, South Africa. The bacterial culture was stored in the refrigerator in 70% glycerol (v/v) at -80℃. Fresh cultures of the pathogen were prepared by inoculating Potato dextrose agar (PDA) plates with 350 µL of the pathogen suspension and incubated for 48 hrs at 25℃, *Pcc* colonies were then streaked in fresh PDA plates and incubated at 25℃ for 48 hrs. PDA plates with *Pcc colonies* were washed with sterile distilled water, and the pathogen suspension was adjusted to a concentration of 1×10^4 cells/mL using a hemocytometer.

5.2.3 Compatibility of *B. amyloliquefaciens* with lemongrass essential oil

PDA was prepared, and 15mL of PDA was poured into sterile petri plates and allowed to solidify. Sterile filter paper discs (9mm diameter) were dipped in different

lemongrass concentrations of (0.125%, 0.175%, 0.5%,1.0%,2.0%, and 3.0%), 40% ethanol (v/v), and water was used as a control. A 4 day old *B. amyloliquefaciens* isolate was streaked on different sides of the PDA plates. The PDA plates were sealed with a parafilm and incubated at 25°C for seven days.

5.2.4 *In vitro* screening of lemongrass essential oil and *B. amyloliquefaciens* against *Pcc*

PDA was prepared, and 15mL of PDA was poured into sterile petri plates and allowed to solidify. A 48-hour-old *Pcc* culture was washed with sterile distilled water to make a $1x10^4$ cells/mL bacterial suspension using a haemocytometer. The pathogen inoculum of 350 µL containing $1x10^4$ cfu/mL was pipetted on the surface of PDA plates and spread using a glass rod. Sterile filter paper discs (9mm diameter) were dipped in different LE essential oils and *B. amyloliquefaciens* mixtures. The mixture of the two treatments was created by combining (1:1) from each treatment. The antibiotic discs were placed at the centre of the PDA plate using sterile forceps. PDA plates were sealed with parafilm and incubated at 25℃ for 48 hrs. The antibacterial activity of the treatment combination was evaluated by measuring the diameter of the inhibition zones. The experiment was repeated thrice, with three replicates per treatment.

5.2.5 *In vivo* screening of LE essential oil + *B. amyloliquefaciens* against *Pcc* on 'Sifra' potatoes

Treatments consisted of 0.175% and 0.125 % concentrations of lemongrass integrated with *B. amyloliquefaciens* isolates: BMO3, MSAG and YMCB. The treatment combination was accomplished by combining (1:1) LE essential oil concentration and *B. amyloliquefaciens*. Potatoes cv. 'Sifra' were collected at Swayimane (Pietermaritzburg, South Africa). Healthy and undamaged potatoes were surface sterilized with 70% ethanol (v/v) for 1 min, washed with distilled water and allowed to air dry before a dip. The potatoes were uniformly wounded (1.5 mm in depth and 2.5 mm in diameter wide), with a sterile needle at the equator. Wounded potatoes were dipped for 30 sec at different test treatments and allowed to air-dry after 24 hrs; the potatoes were inoculated with *Pcc* ($1×10^4$ cells/mL) suspension. Inoculated potatoes were stored in carton boxes and enclosed in black plastic bags with a piece of wet paper towel to ensure high humidity of approximately 95% at 25℃ for 21 days. Each

treatment was replicated three times with five tubers per replicate in a completely randomized design (CRD). The experiment was conducted three times. After incubation, the soft rot incidence was recorded after 7, 14, and 21 days post-inoculation. The percentage of disease incidence (DI) was determined as follows:

$$\%DI = \frac{\text{The number of infected potato wounds}}{\text{Total number of wounds in a potato}} \times 100.$$

Limpel's formula, as described by Richer (1987), was used to determine synergistic interactions between LE essential oil and *B. amyloliquefaciens*. Limpel's formula is Ee= X + Y - (XY/100), where X and Y are the percentages of disease reduction in relation to each antimicrobial used alone, and Ee is the expected effect from the additive response of two treatments. Consequently, synergism exists if the two agents work together to reduce disease to a value higher than Ee.

5.2.6 Transmission electron microscopy studies of the antibacterial effect of lemongrass essential oil + *B. amyloliquefaciens* on *Pcc*

LE essential oil and *B. amyloliquefaciens* treatment combinations that successfully inhibited *Pcc* in dual disc diffusion essay and on potatoes were prepared for TEM analysis. The 350 µL of *Pcc* was spread on PDA plates, and the antibiotic discs were dipped in different LE essential oils and *B. amyloliquefaciens* concentration combinations placed at the center of the PDA plate and incubated at 25℃ for 48 hrs. For the *in vivo* trial, potatoes were disinfected with 70% ethanol, wounded and dipped in suspension LE and *B. amyloliquefaciens* combination, and after 24 hrs, the potatoes were inoculated with 1 ×10^4 cells/mL of *Pcc* and incubated at 25°C, 95% RH for 14 days. The treated potato wounds were cut, and for the *in vitro* samples, the bacterial cells around the zone of inhibition were aseptically collected using a sterile scalpel and all the samples were fixed with 2.5% glutaraldehyde for 1 hr and then washed three times with 0.05M sodium cacodylate buffer for 30 min. The potato and cell samples were post-fixed with 2% osmium tetroxide for 2 hrs and then washed (two times) with sodium cacodylate buffer for 30 min. The samples were dehydrated, using graded ethanol concentrations, from 10 to 70% for 10 min each and left overnight. The potato and cell samples were dehydrated in 90% ethanol for 15 min, twice in 100% ethanol for 15 min, and two additional immersions in absolute ethanol for 15 min each. Potato

and cell samples were immersed (two times) in 100% propylene oxide for 15 min and 50:50 Spurr's resin/propylene oxide for 1 hr and two times in 100% Spurr's resin for 1 hr. The potato and cell samples were placed in an oven at 60 ℃ for at least 24 hrs and then sectioned with a Reichert Jung Ultracut E Ultra-microtome. The sections were collected in a copper grid 200 mesh and finally stained with Reynolds lead citrate and uranium acetate 1%. The samples were analyzed in a JEOL 1400 TEM.

5.2.7 Statistical analysis

The data obtained was subjected to analysis of variance (ANOVA) using Genstat® 23rd Edition. Fisher's unprotected least significant difference test was performed to test the significance of the mean differences obtained among the treatments at the 5% level of significance.

5.3. Results

5.3.1 Compatibility test between *B. amyloliquefaciens* and lemongrass essential oil

B. amyloliquefaciens isolates grew when they were exposed to PDA with antibiotic discs impregnated with 0.125% to 3.0% LE concentrations (Figure 5.1 A and B). The two antimicrobials were compatible since they could grow together in the same medium without inhibiting each other.

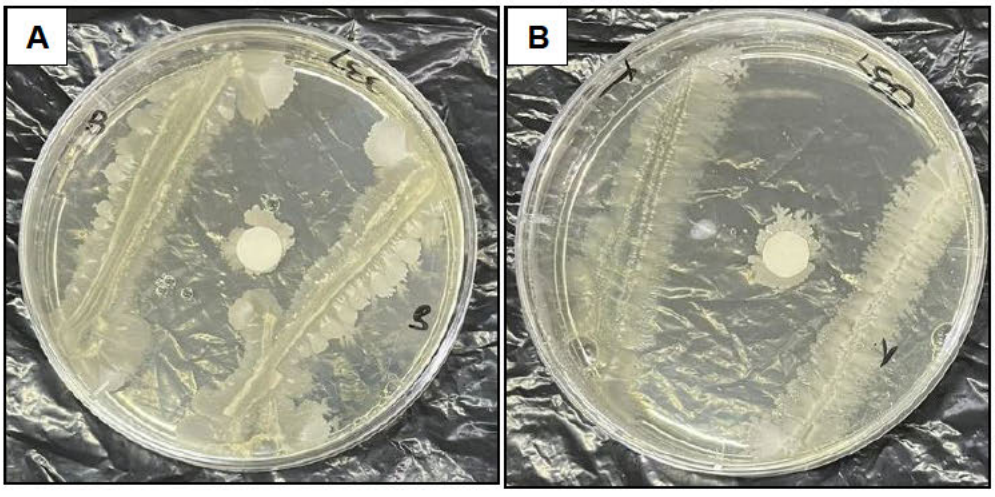

Figure 5.1: Compatibility test between lemongrass essential oil and *B. amyloliquefaciens* isolates after 7 days at 25℃ and 95% relative humidity. ; A- LE3.0% and BMO3; B- LE3.0% and YMCB.

5.3.2 *In vitro* screening of *B. amyloliquefaciens* + lemongrass essential oil against *Pcc*

A combination of the best biocontrol and best LE essential oil concentration was tested. LE3.0%+ *B. amyloliquefaciens* (YMCB) had the highest pathogen inhibition zone of (30 mm) compared to the control treatment, where no bacterium inhibition was observed (Table 5.1; Figure 5.3 A). There was a significant difference between LE3.0% + *B. amyloliquefaciens* (YMCB) and LE3.0% + *B. amyloliquefaciens* (BMO3) means, respectively, at P< 0.05 (Table 5.1). There was no significant difference between the treatment means of 2.0% LEO + *B. amyloliquefaciens* (YMCB), 2.0% LEO + MSAG, 2.0% LEO + *B. amyloliquefaciens* (BMO3), and 3.0% LEO + *B. amyloliquefaciens* (MSAG), respectively (Table 5.1).

Table 5.1: The antibacterial effect of lemongrass essential oil and *B. amyloliquefaciens* combination on *Pcc in vitro*. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Treatment	Inhibition zone (mm)
Control	0.00 a
LE2.0% + *B. amyloliquefaciens* (BMO3)	21.00 b
LE3.0% + *B. amyloliquefaciens* (MSAG)	21.00 b
LE2.0% + *B. amyloliquefaciens* (MSAG)	21.67 b
LE 2.0% + *B. amyloliquefaciens* (YMCB)	21.67 b
LE3.0% + *B. amyloliquefaciens* (BMO3)	24.67 c
LE 3.0%+ *B. amyloliquefaciens* (YMCB)	30.00 d
P-value	<.001
LSD	2.195
SED	1.024
%CV	6.3

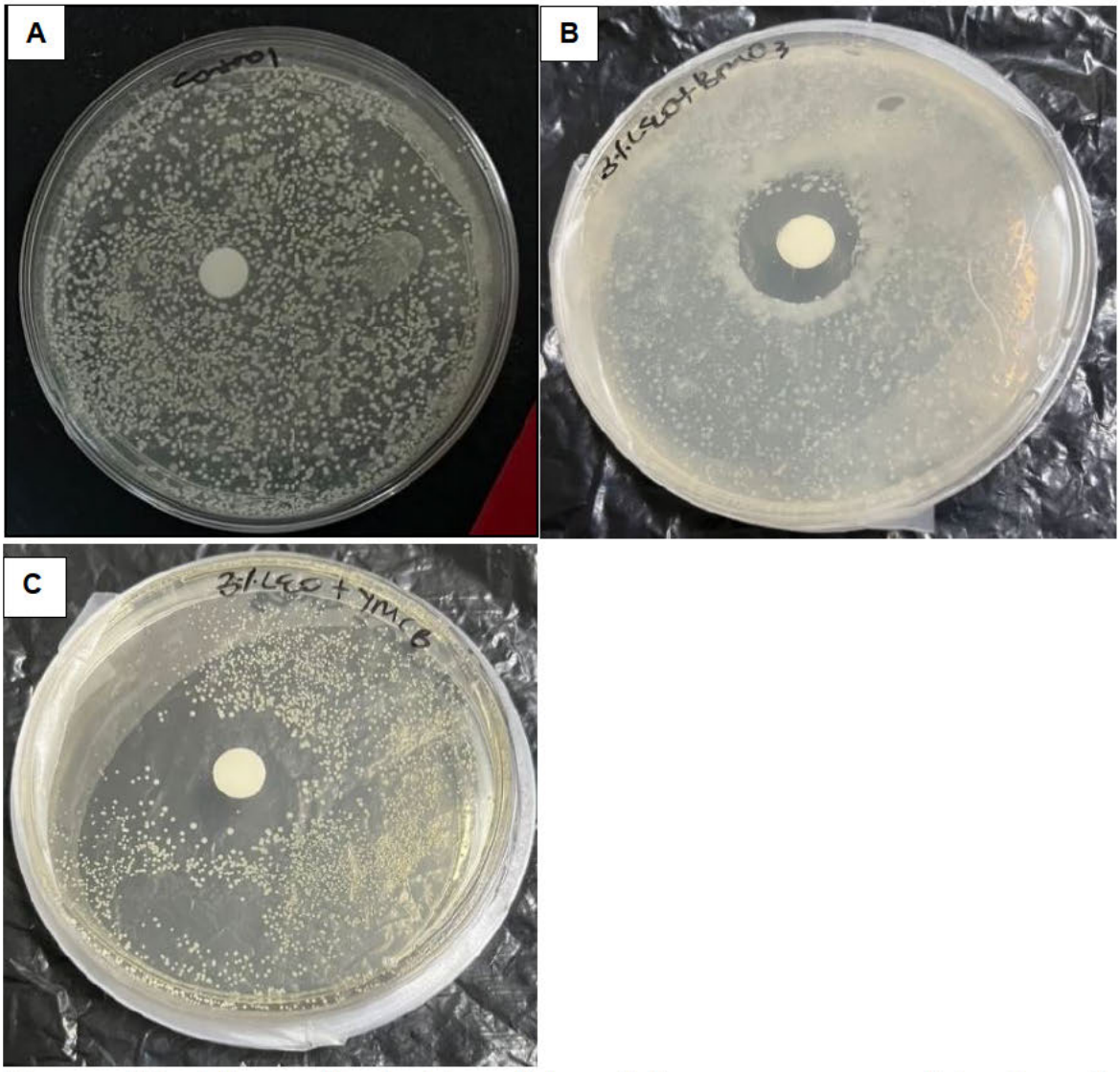

Figure 5.2: The antibacterial activity of lemongrass essential oil and *B. amyloliquefaciens* against *Pcc* after 2 days at 25℃ temperature and 95 % relative humidity. Control (A); LE3.0% + *B. amyloliquefaciens* (YMCB) (B) and LE3.0% + *B. amyloliquefaciens* (BMO3) (C).

5.3.4 The effect of integrating lemongrass essential oil and *B. amyloliquefaciens* against *Pcc* in 'Sifra' potatoes

On 'Sifra' potatoes, all treatment combinations significantly reduced bacterial soft rot (Table 5.2) at ($P<0.05$) compared to the control treatment with a soft rot incidence of

96.67%. A treatment combination of LE0.125%+ *B. amyloliquefaciens* (MSAG) and LE0.125%+ *B. amyloliquefaciens* (BMO3) provided the best control of bacterial soft rot incidence by reducing bacterial soft rot incidence by 96.55% and 94.83% at ($P<0.05$) (Table 5.2 and Figure 5.3 C and D).

Table 5.2: The antibacterial effect of LE essential oil + *B. amyloliquefaciens* on potato soft rot 21 days post-inoculation, at 25℃, 95% relative humidity. Treatment means with the same letters are not significantly different according to Fisher's least significant different test (*P* = 0 .05).

Treatment	Soft rot incidence (%)	DI reduction (%)	Ee (%)
LE0.125% + *B. amyloliquefaciens* (MSAG)	3.33a	96.56	99.29
LE0.125%+ *B. amyloliquefaciens* (BMO3)	5.00ab	94.83	99.11
LE0.175% + *B. amyloliquefaciens* (BMO3)	8.33abc	91.38	98.21
LE0.125% + *B. amyloliquefaciens* (YMCB)	10.00abc	89.66	99.20
LE0.175%+ *B. amyloliquefaciens* (MSAG)	11.67bc	87.93	98.57
LE0.175%+ *B. amyloliquefaciens* (YMCB)	15.00c	84.48	98.40
Control	96.67d	0.00	-
P-value	<.001		
LSD	8.373		
SED	4.231		
%CV	50.5		

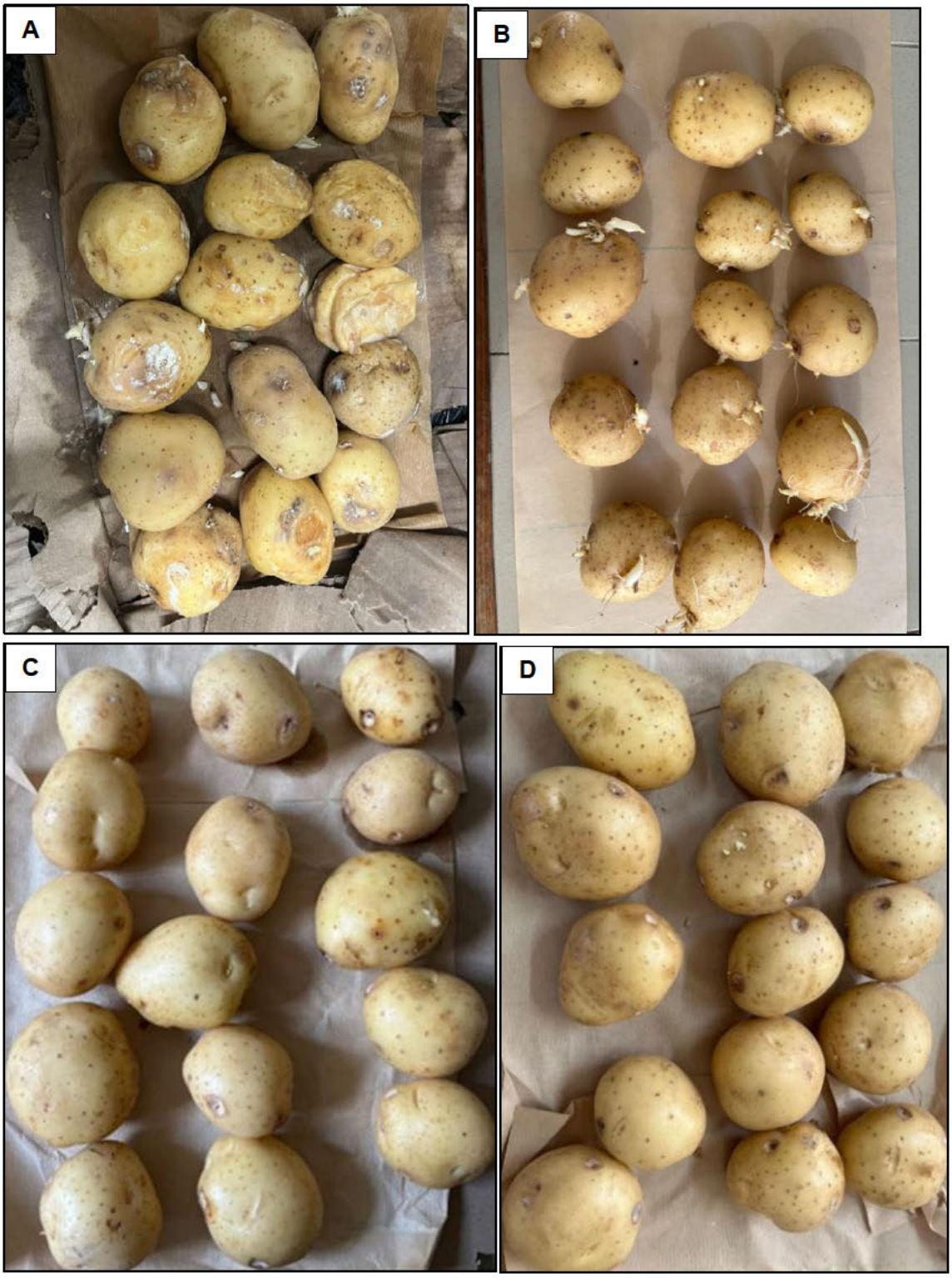

Figure 5.3: The effect of LE essential oil + *B. amyloliquefaciens* on soft rot incidence of potatoes after 21 days at 25℃, 95% relative humidity. Control (A), LE0.175% + *B. amyloliquefaciens* (BMO3) (B), LE0.125% + *B. amyloliquefaciens* (BMO3) (C) and LE0.125% + *B. amyloliquefaciens* (MSAG)(D).

5.3.5 Transmission electron microscopy studies of the antibacterial effect of LE essential oil + *B. amyloliquefaciens* on *Pcc*

The *Pcc* cells around the inhibition zone were collected and observed using the TEM to see the effect of the treatment combination. *Pcc* cells treated with LE3.0% + *B. amyloliquefaciens* (YMCB) and LE3.0% + *B. amyloliquefaciens* (BMO3) showed deformed *Pcc* cells (DP), leakage of the cell wall (CLW) and plasma membrane (LPM) (Figure 5.4B and Figure 5.4C) Untreated *Pcc* cells showed viable *Pcc* cells (VP), an intact cell wall (CW), plasma membrane (PM) and cytoplasm(CP) with cell constituents inside(Figure 5.4A).

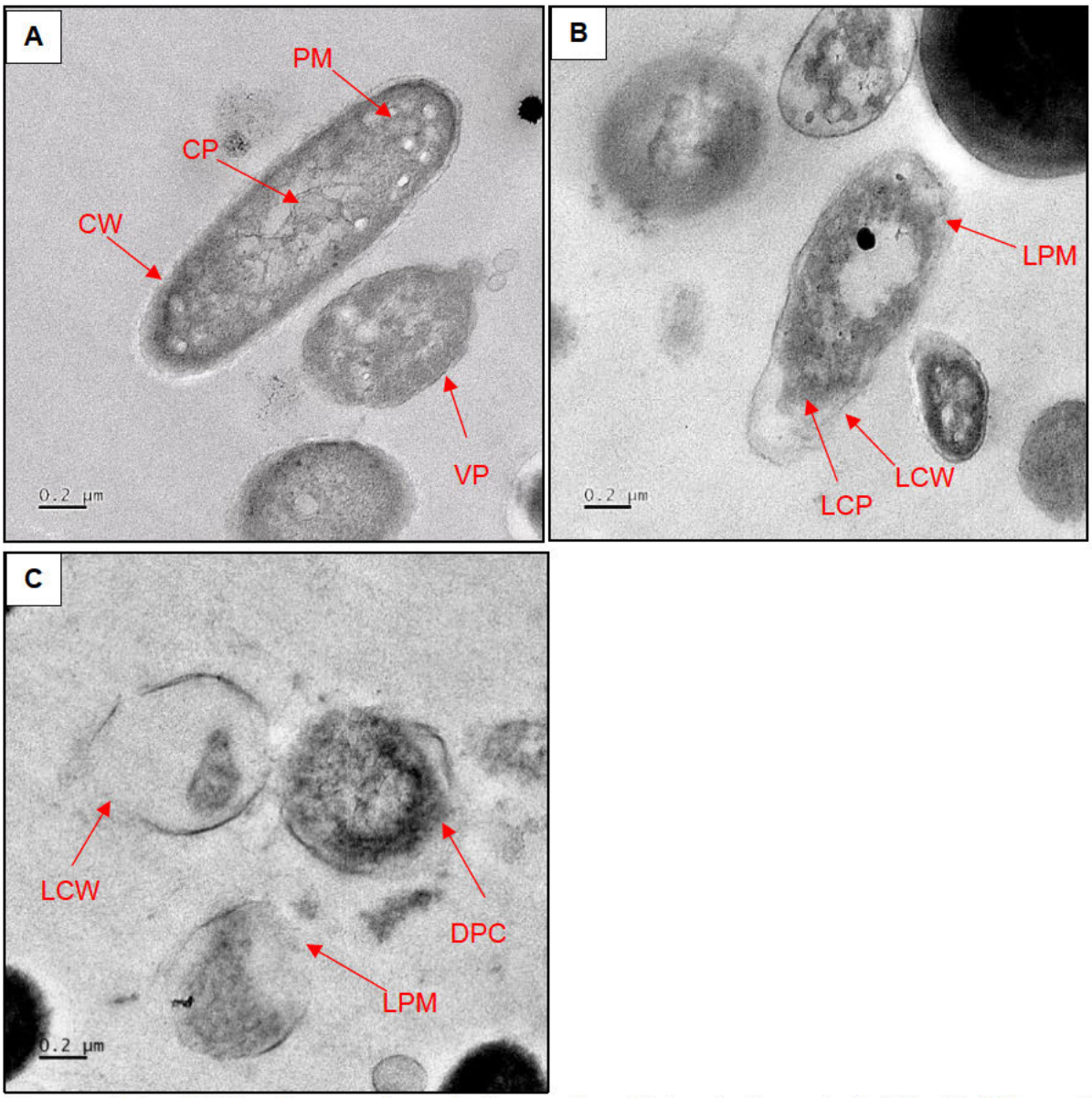

Figure 5.4: TEM micrographs of *Pcc* cells. Untreated control (A), LE3% + *B. amyloliquefaciens* (BMO3) (B) and LE3% + *B. amyloliquefaciens* (YMCB) (C).

Artificial potato wounds treated with LE0.125% + *B. amyloliquefaciens* (MSAG) and untreated potatoes (*Pcc* only) were observed using TEM; the control treatment showed a longitudinal section of viable *Pcc* cells (VP) with intact cell constituents (figure 5.5A). Antimicrobial interactions were observed on the potato cell wall (ACW) and were also observed inside the potato cell (IAC) (Figure 5.5B, C and D).

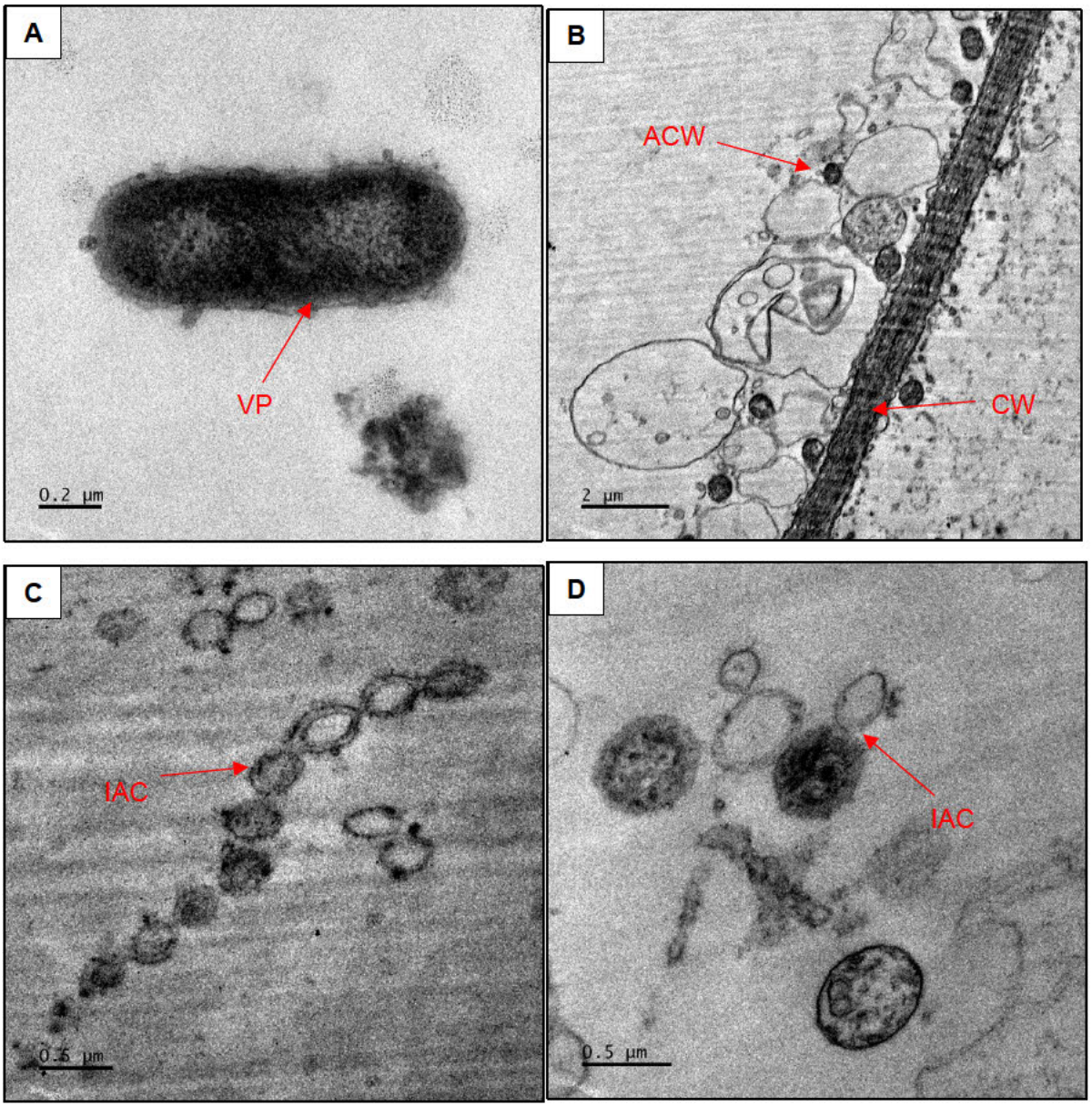

Figure 5.5: TEM micrograph of potato treated with Untreated control(A) and LE0.125% + *B. amyloliquefaciens* (MSAG) (B),21 days post-inoculation at 25℃,95% relative humidity.

5.4 Discussion

The present study aimed to investigate the effect of combining *B. amyloliquefaciens* with LE essential oil to test their efficiency in managing potato soft rot. Natural compounds such as biological control agents, essential oils, essential oil-based products, and plant growth-promoting rhizobacteria (PGPRs) are promising alternatives for reducing chemical control dependence in postharvest disease management (Jobling, 2000; Wilson *et al*., 2000; Fira *et al*., 2018). Researchers are increasingly interested in using combinations of antagonistic microorganisms to exploit the possible synergistic effects and improve control strategies.

The current findings showed the antibacterial activity of integrating LE essential oil and *B. amyloliquefaciens* to control potato soft rot *in vitro* and storage. *In vitro,* disc diffusion assay (Table 5.1 and Figure 5.2) showed that all treatment combinations inhibited *Pcc* and LE3.0% + *B. amyloliquefaciens* (YMCB) treatment significantly inhibited *Pcc* with the largest zone of inhibition (30 mm). In 'Sifra' potatoes, all treatment combinations significantly reduced bacterial soft rot incidence compared to the control treatment. LE0.125%+ *B. amyloliquefaciens* (MSAG) and LE0.125%+ *B. amyloliquefaciens* (BMO3) reduced bacterial soft rot incidence by 96.56% and 94.83% (Table 5.2). Although the treatment combinations significantly reduced soft rot incidence by up to 96.55%, the expected additive response (Ee), determined according to Limpel's formula (Table 5.2), showed that the treatment combinations were not synergetic. The results are similar to those of Arrebola *et al*. (2010), who showed that a combined treatment of *B*. amyloliquefaciens PPCB004+ LE essential oil at 25℃ for five days showed a higher percentage of disease inhibition and severity reduction on peaches artificially inoculated with *Botrytis cinerea (Pers)*, *P. expansum (Link)* and *Rhizopus stolonifera* (Vuillemin).

Chen *et al*. (2007) revealed the capacity of *B. amyloliquefaciens* FZB42 to produce several lipopeptides such as surfactin, bacillomycin, fengycin and bacillibactin with antifungal, antibacterial and even nematocidal activity. A high concentration of citral, made up of neral and geranial isomers, characterises LE essential oil and is responsible for its antimicrobial effect (Mukarram *et al*., 2021). The hydrophobic feature of LE essential oil is the leading cause of cellular death, and it can target cell wall components. It also increases the permeability of the cell membrane after it accumulates within the cell membrane, ultimately resulting in membrane breakdown

and the release of cellular components (Mukarram *et al*., 2021). The TEM micrographs (Figure 5.4) of *Pcc* cells treated with a combination of LE3.0% + *B. amyloliquefaciens* (YMCB) and LE3.0% + *B. amyloliquefaciens* (BMO3) showed deformed *Pcc* cells with leakage of the cell wall, plasma membrane and the cytoplasm with its constituents. Sotelo-Boyás *et al*. (2019) showed that *Pcc* cells were damaged, other cells were broken, and cytoplasm linkage on a TEM micrograph when *Pcc* was treated with chitosan loaded with thyme essential oil. The TEM micrographs of treatment combinations LE0.125% + *B. amyloliquefaciens* (MSAG) and LE0.125% + *B. amyloliquefaciens* (BMO3) on 'Sifra' potatoes(Figure 5.5) showed the interaction of different antimicrobials in the potato cell wall and inside the potato cell.

The interaction of different antimicrobials could be due to the mode of action of *B. amyloliquefaciens* and LE essential oil. Plant defence mechanisms activated by *Bacillus* include lignin accumulation and structural changes in the cell wall (Singh *et al*., 2016) as well as the synthesis of secondary metabolites such as flavonoids, phytoalexins, auxins, or glucosinolates (Dimkić *et al*., 2022) Essential oils' antimicrobial activity, which is effective for controlling fungal diseases, is mostly because of the synergism of the different chemical components (Spadaro *et al.*, 2017). Spadaro *et al.* (2017) observed that apples treated with thyme (*thymus vulgaris*) essential oil at 1% concentration had a lower disease incidence of grey mould caused by *B. cinerea* and induced resistance in the apples by increasing the expression of the pathogenesis-related gene PR-8.

5.5 Conclusion

The present study demonstrated the potential of using *B. amyloliquefaciens* isolates combined with LE essential oil in *vitro* and storage. The current findings showed that LE essential oil at lower concentrations can be combined with *B. amyloliquefaciens* strains to control potato soft rot in storage. Future studies could involve studying the effect of the combination of LE essential oil and *B. amyloliquefaciens* on potato quality parameters such as antioxidant activity and protein content. Future studies can also focus on applying the treatment combination in potato fields during the preharvest stage.

5.6 References

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Chapter 6

Thesis overview

6.1 Introduction

Potato is the world's most popular non-cereal staple food, recognized mostly for its starchy carbohydrate content. Potatoes are also high in vitamins and other phytonutrients that are quite beneficial (Zaheer and Akhtar, 2016). Pests, diseases, and climatic changes are the most important factors that affect potato yield and quality (Chakrabarti *et al*., 2022). One of the most prevalent diseases that damage potatoes in the field and during storage is bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*.

Researchers are increasingly interested in using environmentally friendly antimicrobials such as essential oil and biocontrol agents to improve postharvest disease control strategies. Lemongrass (LE) essential oil has often been used as an antimicrobial agent to combat postharvest pathogens (Mbili *et al*., 2017; Mukarram *et al*., 2021). LE essential oil antibacterial mechanism involves the destruction of bacterial biofilms and hinders bacterial growth and development; also, LE essential oil components can weaken lipid bilayer bonds and destroy bacteria by disintegrating the membrane (Moore-Neibel *et al*., 2012). The use of *Bacillus* spp. for biocontrol of various postharvest diseases in a wide range of fruits and vegetables has been reported (Calvo *et al*., 2017; Zhang *et al*., 2019; Taha *et al*., 2023). *Bacillus spp.* efficacy depends on antibiosis, siderophores production, and hydrolytic enzyme secretion (Rani *et al*., 2019).

According to our knowledge, no research has focused on the management of potato soft rot using a combination of LE essential oil and *B. amyloliquefaciens.* This study's significance was integrating generally recognized as safe (GRAS) control measures to reduce the soft rot incidence of potatoes *in vitro* and in storage with an interest in minimizing the use of synthetic antimicrobials currently utilized in the postharvest management of potatoes.

6.2 Research objectives and major findings

This study investigated the effect of bacterial biological control agents integrated with LE essential oil on bacterial soft rot of potatoes. The specific research objectives were as follows:

- To investigate the effect of biological control agents against *P. carotovorum* subsp. *carotovorum in vitro* and *in vivo*
- To investigate the effect of lemongrass essential oil on *P. carotovorum* subsp. *carotovorum in vitro* and *in vivo*
- To investigate the integration of *B. amyloliquefaciens* and lemongrass essential oil on potato soft rot *in vitro* and *in vivo.*

Chapter 3: *In vitro* and *in vivo* effect of bacterial biocontrol agents against *Pectobacterium carotovorum* subsp. *carotovorum* of potato

- YMCB, BMO3 and MSAG isolates successfully suppressed the pathogen and showed high antibacterial activity against *Pcc* both *in vitro* and *in vivo*.
- BMO3 showed the most excellent antibacterial activity against *Pcc.*
- MSAG had the highest soft rot incidence reduction on Sifra potatoes treated with *Pcc.*
- The TEM micrograph revealed that the *B. amyloliquefaciens* isolates caused the disappearance of the outer membrane, leakage of the cell wall and plasma membrane and leakage of the cytoplasm and its constituents.
- The TEM micrograph of potato wounds treated with *B. amyloliquefaciens* showed healthy potato cells with deformed *Pcc* cells.

Chapter 4: *In vitro* and *in vivo* antibacterial activity of *Cymbopogon citratus L.* essential oil against *Pectobacterium carotovorum* subsp. *carotovorum* causing bacterial soft rot of 'Sifra' potato tubers

- LE3.0% and LE2.0% concentrations successfully suppressed the pathogen and showed high antibacterial activity against *Pcc* both *in vitro* and *in vivo*.

- LE0.125 % and 0.175% had the highest soft rot incidence reduction on Sifra potatoes treated with *Pcc.*
- The TEM micrograph revealed that the LE3.0%, 2.0% and 1.0% caused the disappearance of the outer membrane and deformed *Pcc* cells and showed the LE essential oil attached to the plasma membrane and cell wall.
- Lemongrass EO causes permeability of the cell wall and plasma membranes, which results in the leakage of bacterial proteins and other cytoplasm constituents.
- The TEM micrograph of potato wounds treated with 0.125 % and 0.175% lemongrass EO showed LE deposition in the potato cell wall and inside the cell.

Chapter 5: The antibacterial effect of *Cympopogon citratus L.* and *B. amyloliquefaciens* on postharvest potato soft rot caused by *Pectobacterium carotovorum* subsp*. carotovorum*

- A LE3.0% + *B. amyloliquefaciens* YMCB treatment combination had the highest pathogen inhibition zone.
- The *in vitro* treatment combinations were not synergistic but had additive effects on each other.
- A treatment combination of LE0.125% + *B. amyloliquefaciens* MSAG and LE 125%+ *B. amyloliquefaciens* BMO3 provided the best control of bacterial soft rot incidence.
- The TEM micrographs of the bacterium treated with LE3.0% + *B. amyloliquefaciens* YMCB showed cell surface disruption, such as deforming and disappearance of the cell wall and the plasmatic membrane.
- TEM micrograph of potato cells treated with LE0.125% + *B. amyloliquefaciens* MSAG showed an interaction of different antimicrobials inside the potato cell and the cell wall.
- In storage, lemongrass essential oil at lower concentrations can be combined with *B. amyloliquefaciens* to control potato soft rot.

6.3 Recommendations and future research

This study demonstrated the efficacy of LE essential oil and *B. amyloliquefaciens* as antibacterial agents that can be used to manage bacterial soft rot of potatoes caused by *Pcc*. Based on the findings of this study, future research may focus on the following recommendations:

- Explore the synergism of LE essential oil components and mode of action for postharvest disease management in potatoes and other crops.
- Evaluation of the effects of *B. amyloliquefaciens* and LE essential oil on potato quality parameters such as phenolic content, ascorbic acid content, antioxidant activity, and protein content.
- Exploring the effects of LE essential oil and *B. amyloliquefaciens* mode of action by studying the expression of defence-related enzymes and secondary metabolites.
- The efficacy of using LE essential oil and *B. amyloliquefaciens* and bactericides sprays or fumigants during the preharvest growing stage of potatoes.
- Studying the effect of LE essential oil and *B. amyloliquefaciens* in controlled environments such as cold temperatures to control postharvest potato diseases.

6.4 References

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Appendices

Appendix 1: Primary screening of bacterial isolates against *Pectobacterium carotovorum* subsp. *carotovorum* on potato dextrose agar after 7 days at 25°C and 95% relative humidity.

Isolate source	Treatment	Inhibition zone(mm)±SE
Control	-	0.00±0.00
Lippia javanica	YUM4	11.33±0.66
L. javanica	BMJ	14.00±0.33
L. javanica	UMB	12.33±0.66
L. javanica	YUM4	11.33±0.66
L. javanica	MSA	12.66±0.33
L. javanica	UMB	11.33±0.66
L. javanica	MSE	12.00±0.66
L. javanica	YMCU	13.67±0.33
L. javanica	YUM2	11.66±0.33
L. javanica	YUM1	12.66±0.66
L. javanica	BMW1	18.00±1.15
L. javanica	MSAP	13.66±0.33
L. javanica	MSGB	13.50±0.29
L. javanica	MSXTR	11.66±0.33
Anacardium occidentale L.	BCA4	10.33±0.17
A. occidentale L	BCA1	12.00±0.58
A. occidentale L	BCA3	11.83±0.17
A. occidentale L	BCA24	11.33±0.33
A. occidentale L	BCA3	15.33±0.33
A. occidentale L	YMCP	14.00±0.58
Moringa oleifera	BMW1	18.00±0.17
M. oleifera	BMO1	10.83±0.17
M. oleifera	BMO2	14.67±0.33
M. oleifera	YMCB	20.67±0.33
M. oleifera	BMRG	18.67±0.88
M. oleifera	BMCN	18.66±0.33

M. oleifera	BCMB	13.33±0.88
M. oleifera	UME	11.66±0.33
M. oleifera	BMO3	22.00±0.88
M. oleifera	MSAG	18.66±1.20
Hypoxis hemerocallidea	YAP1	11.66±0.33
H. hemerocallidea	YAP2	12.33±0.33
H. hemerocallidea	YAP3	12.33±0.33
H. hemerocallidea	PTCM	14.33±0.66
H. hemerocallidea	PTA10	12.00±0.58
Sclerocarya birrea	BMA3	11.16±0.44
S. birrea	CMA1	14.17±0.17
S. birrea	BMA1	16.17±0.44
S. birrea	YMA2	12.66±0.33
S. birrea	BMA4	12.33±0.33
S. birrea	BMA6	10.66±0.33
Artemisia afra	MNC3	14.83±0.44
A. afra	MNC1	13.00±0.58
A. afra	MNC2	14.17±0.60
A. afra	MNC5	11.00±0.58
A. afra	MNC4	12.33±0.33
Apodytes dimidiate	CLVB	11.66±0.33
A. dimidiate	CL20	10.50±0.33
A. dimidiate	CLCA	12.17±0.17
A. dimidiate	CLMV	10.17±0.17
Persea americana	LVEV	10.83±0.17
P. americana	YAV3	13.33±0.33
P. americana	YAY	13.66±0.66
Tetradenia riparia	IBC	10.33±0.33
T. riparia	IBB	13.66±0.66
T. riparia	IB2	12.66±0.33
T. riparia	IBD	12.66±0.33
Nuxia floribunda	TSD	13.66±0.33
N. floribunda	TSA	12.00±0.17

N. floribunda	TSE	11.00±0.58
Psidium guajava	YOG1	10.33±0.33
P. guajava	YGA3	13.33±0.33
P. guajava	BGA3	12.33±0.33
P guajava	BGA1	16.66±1.20
Eucalyptus mannifera	GTA2	14.00±0.58
E. mannifera	GTA4	16.66±1.20
E. mannifera	GTA1	14.50±0.29
Trichilia dregeana	NPM2	15.66±0.88
T. dregeana	NVM	10.66±0.33
T. dregeana	NCF	12.33±0.17
T. dregeana	NMF	10.66±0.33

Appendix 2: Soft rot incidence of ‘Sifra’ potatoes 7 days and 14 days post-inoculation at 25℃ ,95% relative humidity.

	Disease Incidence (%)	
Treatment	7 dpi	14 dpi
LE0.125 %	0.00	0.00
LE0.175 %	0.00	0.00
LE0.5%	25.00	28.33
LE1%	25.00	41.67
LE2%	43.33	68.33
LE3%	48.33	75.00
Control	33.33	76.67

P-value	0.001	0.001
SED	2.47	3.563
LSD	4.910	7.072
%CV	27.1	23.6

Appendix 3: Disease incidence reduction of potato soft rot 7 days and 14 days post inoculation at 25℃ ,95% relative humidity.

Treatment	7 dpi	14 dpi
LE0.125 %	100.00	100.00
LE0.175 %	100.00	100.00
LE0.5%	24.99	67.39
LE1%	24.99	67.39
LE2%	- 23.08	43.49
LE3%	- 23.08	36.96
Control	0.00	0.00